THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 261, No. 7, Issue of March 5, pp. 3156–3163, 1986
Printed in U.S.A.

Determination of *in Vivo* Protein Binding of Homocysteine and Its Relation to Free Homocysteine in the Liver and Other Tissues of the Rat*

(Received for publication, May 13, 1985)

Asbjørn Svardal‡, Helga Refsum§, and Per Magne Ueland

From the Clinical Pharmacology Unit, Department of Pharmacology, University of Bergen, 5016 Haukeland Sykehus, Bergen, Norway

Low concentrations (0.5–6 nmol/g) of homocysteine (Hcy) have recently been demonstrated in acid extracts of various tissues of the mouse and rat (Ueland, P. M., Helland, S., Broch, O.-J., and Schanche, J.-S. (1984) *J. Biol. Chem.* 259, 2360–2364). This is referred to as free Hcy in tissues. This paper describes a method for the determination of protein-bound Hcy, which involves precipitation and washing of tissue protein with ammonium sulfate, release of Hcy from native proteins in the presence of dithioerythritol, and determination of free Hcy by a sensitive radioenzymic assay. Both free and bound Hcy decreased markedly in rat tissues within a few seconds following death of the animal. The amount of protein-bound Hcy was highest in liver, somewhat lower in kidney, brain, heart, lung, and spleen. The ratio between free and bound Hcy was between 1 and 2 in most tissues, except in cerebellum, containing a large excess of free Hcy (free/bound ratio of 18). Free Hcy was almost exclusively localized to the soluble fraction of rat liver, whereas protein-bound Hcy was about equally distributed between this fraction and the microsomes. Isolated rat hepatocytes contained free and protein-bound Hcy in proportions observed in whole liver, but a large amount of Hcy was exported into the extracellular medium. The half-lives, as determined from pulse-chase experiments with [^{35}S] methionine, were 53 s for *S*-adenosylmethionine, 2 s for *S*-adenosylhomocysteine and 3 s for Hcy (free and bound regarded as a single pool). Furthermore, isotope equilibrium between these metabolites and between free and bound Hcy throughout the rapid chase period suggests the turnover rates of *S*-adenosylhomocysteine and Hcy to be production rate limited, and the dissociation rate of the Hcy-protein complex may greatly exceed the turnover rate of Hcy. Thus, the half-lives of Hcy are such that participation of both free and bound Hcy in metabolic regulation is feasible.

There has been a growing interest in the sulfur amino acid, Hcy,[1] especially because this compound seems to be involved in various pathological processes. Inborn error of Hcy metabolism is associated with the inherited disease, homocystinuria (1). Patients afflicted with this disease suffer from thromboembolism, and Hcy has been suggested as an atherogenic agent (1, 2). Reduced remethylation of Hcy to methionine in post-menopausal women may be responsible for the increased tendency of coronary artery disease in women at old age (3). Malignant transformation seems to be associated with altered cellular disposition of Hcy, because normal cells, but not cancer cells, thrive in a medium where methionine is replaced by Hcy (4). Perturbation of Hcy metabolism by methotrexate is probably responsible for the hepatotoxic effect of this drug (5). Furthermore, we have recently demonstrated that methotrexate enhances Hcy secretion in chemically transformed fibroblasts (6).

Knowledge of the metabolic origin and fate of Hcy in mammalian cells is accumulating. Degradation of the endogenous transmethylase inhibitor, AdoHcy, to adenosine and Hcy, which is catalyzed by the enzyme AdoHcy hydrolase (EC 3.3.1.1), seems to be the only source of Hcy in vertebrates (7). Once formed, Hcy is converted either to cystathionine or is remethylated to methionine. The latter reaction is catalyzed by two enzymes, one of which is widely distributed and requires 5-methyltetrahydrofolate as methyl donor (1). The distribution of Hcy between these competing pathways in the rat liver has recently been evaluated (8).

The advance in our knowledge of the metabolism and function of Hcy has been hampered by lack of sensitive methods for the determination of Hcy in biological material. The view has long been held that Hcy is not present in plasma under normal conditions (9), but this has been refuted by demonstrating Hcy in human plasma, using either an amino acid analyzer (10) or a sensitive radioenzymic method (11–14). We have recently demonstrated Hcy in acid extract of tissues from rat and mouse (14). This finding is not in accordance with the current belief that intracellular Hcy does not exist as free Hcy (15) but is associated with proteins (8).

A sensitive assay for Hcy has recently been developed, which involves the enzymic conversion of Hcy to AdoHcy, and the latter compound is isolated by HPLC (14). With this method it is possible to quantify Hcy in small amounts of tissue and in isolated cells (14). It has been modified in the present work, to determine the amount of Hcy associated with intracellular proteins. This fraction of Hcy and its relation to

* This work was supported by grants from the Norwegian Cancer Society and the Norwegian Research Council for Science and the Humanities. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Research fellow of the Norwegian Cancer Society.

§ Research fellow of the Norwegian Research Council for Science and the Humanities.

[1] The abbreviations used are: Hcy, L-homocysteine; AdoHcy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosylmethionine; DTE, dithioerythritol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography.

free Hcy were determined in various tissues of the rat and in isolated rat hepatocytes.

EXPERIMENTAL PROCEDURES

Materials—L-Homocysteine, DL-homocysteine, adenosine, AdoHcy, DL-dithioerythritol were purchased from Sigma. Ammonium sulfate, proanalysis, was purchased from Merck, Darmstadt, Federal Republic of Germany. D-Eritadenine was a gift from Dr. A. Holy, Institute of Organic Chemistry and Biochemistry, Praha, Czechoslovakia. Other reagents were obtained from sources given previously (14).

Isolation of Organs—The organs were either removed and frozen after the animal was killed, or the organs were frozen *in vivo*, using liquid nitrogen. Details have been given previously (14).

Preparation of Subcellular Fractions of Rat Liver—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) and 1 mM EDTA. All further procedures were carried out at 0–5 °C. Livers were chopped into small pieces and rinsed with the same medium. The tissue was then homogenized in 4 volumes of medium with two strokes at 720 rpm, using a Potter-Elvehjem homogenizer equipped with a loose-fitting Teflon pestle. Fractionation of the homogenate was carried out by a modification of the method of de Duve *et al.* (16).

The homogenate was centrifuged at 1,000 × *g* for 10 min to remove nuclei and unbroken cells. The pellet obtained was called the nuclear fraction (N). The supernatant was centrifuged at 10,500 × *g* for 10 min, the pellet giving the mitochondrial fraction (M). Centrifugation of the resulting supernatant at 23,500 × *g* for 30 min yielded a pellet which was called the lysosomal fraction (L). The 23,500 × *g* supernatant was then centrifuged at 105,000 × *g* for 60 min to give the microsomal pellet (P) and the soluble fraction (S). To obtain as pure subcellular fractions as possible, all of the fractions N, M, L, and P were washed twice and the supernatant combined with those from the previous step. The M, L, and P fractions were usually resuspended in approximately 1 ml of medium/g of original tissue.

Assay for Marker Enzymes—Glutamate dehydrogenase (EC 1.4.1.3) (17), succinate phenazine methosulfate oxidoreductase (EC 1.3.99.1) (18), acid phosphatase (EC 3.1.3.2) (19), rotenone-insensitive NADPH cytochrome *c* oxidoreductase (EC 1.6.2.4) (20), and lactate dehydrogenase (EC 1.1.1.27) (21) 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 (22) and were incubated in an isotonic salt solution containing Hepes buffer (pH 7.4), bovine serum albumin, glucose, salts, and antibiotics, as described elsewhere (23). The temperature was 37 °C.

Pulse-chase Experiments with [^{35}S]Methionine—Hepatocytes (9.6×10^6 cells/ml) were incubated for 30 min with 1 μM [^{35}S]methionine (20 Ci/mmol). The radioactive methionine was chased by addition of 2 mM cold methionine. Immediately before the chase and during the chase period, samples of 500 μl 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 s 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.

Preparation of Perchloric Acid Extract of Tissues and Cells—Tissues or cells were routinely homogenized in 0.6 N perchloric acid (1/10–1/16, w/v). The precipitated proteins were removed by centrifugation. The perchloric acid extract was neutralized by alkali, and the insoluble potassium perchlorate removed by centrifugation, as described previously (14).

Treatment of Tissues and Cells with Ammonium Sulfate—Tissues or cells were routinely homogenized (1/12, w/v) in ice-cold, saturated ammonium sulfate, using a Ultra Turrax tissue homogenizer. The precipitated protein in samples of 500 μl from the tissue homogenate was immediately 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.

Determination of Free Hcy—This fraction of Hcy was assayed in the neutralized perchloric acid extract of tissues and cells, by a radioenzymic assay described in detail elsewhere (14).

Determination of Protein-bound Hcy—Proteins from tissues or cells, precipitated with ammonium sulfate and collected on filters, were dissolved in 500 μl of ice-cold 50 mM potassium phosphate buffer (pH 7.4), containing 100 μM D-eritadenine. The solution was then frozen and stored at −80 °C until assay. The amount of Hcy associated with the proteins was routinely determined by the following modification of the radioenzymic assay developed for the determination of free Hcy. Samples of 100 μl were supplemented with dextran-coated charcoal containing DTE and incubated for 10 min at 37 °C. The final concentrations were 0.5 mg/ml of dextran, 5 mg/ml of Norit, and 10 mM DTE. Then the charcoal was removed by centrifugation, and the treatment with charcoal plus DTE was repeated to remove trace amounts of free D-eritadenine; this second treatment was performed at 0 °C for 10 min. The extract was then supplemented with [^{14}C]adenosine (20 μM), 2′-deoxycoformycin (1 μM), and AdoHcy hydrolase (9×10^{-3} units/ml), and incubated for 40 min at 37 °C. The reaction was stopped and the mixture deproteinized by adding perchloric acid (final concentration of 0.6 N), which was neutralized to pH 7.4 prior to quantitation of [^{14}C]AdoHcy by HPLC (14).

Determination of Specific Radioactivity of Hcy in Hepatocytes—The radioactivity in both free and protein-bound Hcy in isolated rat hepatocytes exposed to [^{35}S]methionine was determined by the following modification of the procedure described for the determination of free and bound Hcy. [^{14}C]Adenosine was replaced by unlabeled adenosine in the assay mixture, and unlabeled AdoHcy was not added as carrier before the HPLC step. Hcy was quantified by integration of the AdoHcy peak, using a reporting integrator model HP 3390 A from Hewlett-Packard. The peak was collected, using a programmable fraction collector, model Foxy, from ISCO, and the radioactivity determined by liquid scintillation counting.

Determination of Cellular Content and Specific Radioactivity of AdoHcy and AdoMet—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, 0.46 × 25 cm), which was equilibrated at ambient temperature with 60 mM ammonium formate (pH 3.5). The flow rate was 1.5 ml/min. AdoHcy eluted after 6.5 min. At this time point, the ammonium formate concentration was increased to 330 mM. The detector (Spectroflow 773 from Kratos) received an auto zero signal from the HPLC autosampler (model ISS-100 from Perkin-Elmer) at time 10 min, and AdoMet eluted at 11.6 min. The absorbance was recorded at 260 nm.

Statistical Methods—The values (for free and protein-bound Hcy and the free/bound ratio, Table I) were tested for normal distribution by Q-Q plots and for identical variance using the Bartlett test for homogeneity before and after appropriate transformation of data (24, 25). The former test requires more than 10 observations, and values with the same mean from two or three organs within the same group were pooled for analysis. The difference between various organs within a group was investigated using the Newman-Keul test for multiple comparisons (25). Difference in Hcy content in tissues between the two procedures for isolation of organs (freezing *in vivo versus* freezing after death of the animal) was tested for using analysis of variance (two-way).

Determination of Protein—Protein was determined with a Bio-Rad Protein Assay Kit (Bio-Rad) which is based on the absorbance at 595 nm of the complex between Coomassie Brilliant Blue G-250 and protein. Bovine γ-globulin was used as standard.

RESULTS

Principles—A portion of intracellular Hcy is assumed to be associated with proteins, probably via disulfide bonds. To quantify this fraction, the proteins must be separated from the medium containing free Hcy. This could be obtained by precipitation of proteins followed by washing. Ideally, neither release of protein-bound Hcy nor binding of free Hcy to proteins should take place during these steps. Finally, protein-bound Hcy must be quantitatively released (in the presence of a reducing agent), and free Hcy is determined after being condensed with [^{14}C]adenosine to form [^{14}C]AdoHcy, as described (14).

Precipitation of Proteins and Release of Hcy—Proteins in high-speed liver supernatant (subjected to gel filtration to remove free Hcy) and in whole liver were precipitated with either saturated ammonium sulfate or perchloric acid. The

ammonium sulfate precipitate was collected and washed on filters (as described under "Experimental Procedures") while the acid precipitate was collected by centrifugation, washed, and then resuspended and neutralized to pH 7.4.

Hcy was rapidly released from native cytosol proteins isolated by ammonium sulfate precipitation. The recovery of Hcy from protein obtained by ammonium sulfate treatment of whole liver was characterized by a burst phase followed by a slow progressive release (Fig. 1). Notably, the amount of Hcy recovered from the high-speed liver supernatant, was the same whether determined before or after the ammonium sulfate precipitation/filtration step (Fig. 1). This suggests that soluble Hcy binding proteins in liver were quantitatively recovered by the ammonium sulfate precipitation technique.

There was only a slow and minimal liberation of Hcy from proteins exposed to acid (Fig. 1). No free Hcy (extracted by perchloric acid) was found in the cytosol subjected to gel filtration (data not shown) indicating that treatment with acid (for the determination of free Hcy) does not liberate Hcy bound to soluble proteins.

Whole liver homogenate was incubated with DTE (10 mM) for 20 min at 37 °C, and the proteins were precipitated with ammonium sulfate. The proteins were washed and resuspended in buffer, and the DTE treatment was repeated. Protein-bound Hcy in liver homogenate so treated was reduced by about 70%, but was not reduced when DTE was omitted from the incubations (data not shown). This finding suggests the involvement of disulfide linkage in the association of Hcy with liver proteins.

Optimization of the Assay Procedure—We observed that some endogenous AdoHcy was bound to proteins (mainly associated with plasma membranes and microsomes) and precipitated with ammonium sulfate (data not shown). Accordingly, enzymic conversion of AdoHcy to Hcy during processing of tissue and extract may be a source to erratic results. Therefore, the precipitate was dissolved in a buffer containing D-eritadenine, a potent, metabolically stable inhibitor of AdoHcy hydrolase (26). The data presented in Fig. 2 show that a high concentration of D-eritadenine (about 100 μM) was required to prevent interference from AdoHcy added to the tissue extract or from endogenous AdoHcy.

The next step was the removal of interfering compounds (AdoHcy, adenosine, and free D-eritadenine) from the tissue extract by treatment with dextran-coated charcoal. We observed that charcoal treatment at 37 °C, but not at 0 °C, removed endogenous AdoHcy. Then a second charcoal treatment was performed at 0 °C to remove trace amounts of free D-eritadenine, which may block the enzymic conversion of Hcy to [^{14}C]AdoHcy. DTE was included in the charcoal suspensions, to release Hcy from Hcy-protein mixed disulfides.

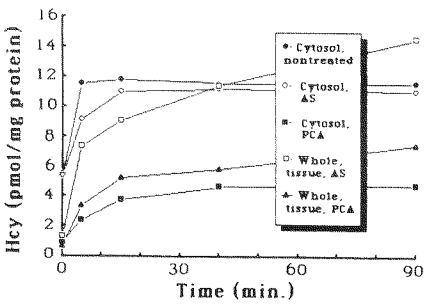

FIG. 1. **Time course for the release of Hcy from proteins.** High-speed supernatant of liver homogenate (cytosol) was prepared as described under "Experimental Procedures." The cytosol was subjected to gel filtration on a Sephadex G-25 column to remove free homocysteine from the extract. Then the protein were precipitated in either saturated ammonium sulfate or perchloric acid. Whole liver was homogenized (1/12, w/v) in either saturated ammonium sulfate or perchloric acid. The proteins precipitated in acid were washed in acid and resuspended in 0.1 N NaOH. After the protein pellet was dissolved, the alkaline solution was neutralized to pH 7.4. The ammonium sulfate precipitate was dissolved in phosphate buffer, (pH 7.4). Details have been given under "Experimental Procedures." The extract obtained by homogenization of whole liver in ammonium sulfate was treated with charcoal and DTE at 37 °C for 10 min, to remove endogenous AdoHcy and adenosine. This step was not required with the other extracts. The extracts were supplemented with AdoHcy hydrolase, DTE, and adenosine and incubated at 37 °C. The time course of the release of Hcy from proteins, assayed as the formation of AdoHcy from Hcy, was determined for the cytosol before (*Cytosol, nontreated*) and after ammonium sulfate treatment (*Cytosol, AS*), cytsol exposed to acid (*Cytosol, PCA*), and whole liver treated with either perchloric acid (*Whole tissue, PCA*) or ammonium sulfate (*Whole tissue, AS*).

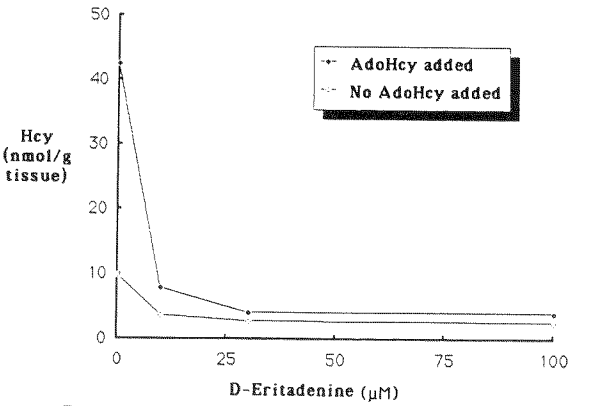

FIG. 2. **Interference with the assay for protein-bound Hcy by AdoHcy in the presence of D-eritadenine.** Whole liver was homogenized in ammonium sulfate, the precipitate washed, resuspended in buffer, treated with DTE and charcoal, and protein-bound Hcy determined as described in "Experimental Procedures," except for the following modifications. The buffer in which the ammonium sulfate pellet was resuspended, was supplemented with increasing concentrations of D-eritadenine (0–100 μM). In addition, in one series the buffer contained 20 μM AdoHcy. The amount of Hcy associated with the proteins was determined with and without addition of AdoHcy and in the presence of increasing concentrations of D-eritadenine.

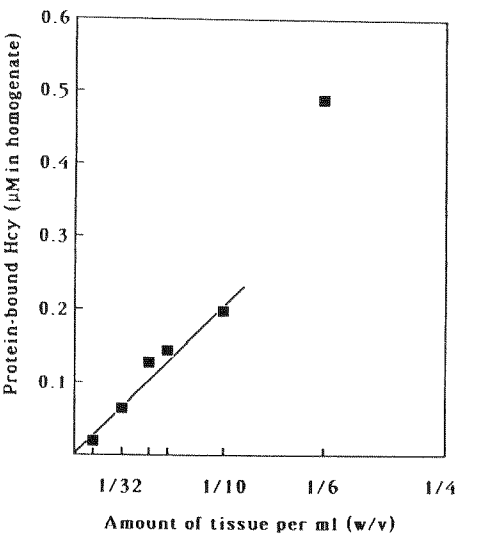

FIG. 3. **Determination of protein-bound Hcy in liver homogenized at various dilutions.** Whole liver was homogenized in ammonium sulfate at the dilutions indicated on the *abscissa*. Protein-bound Hcy was otherwise determined as described in the text.

The amount of protein-bound Hcy in liver was determined after homogenization of tissue at various dilutions (1/4–1/80, w/v) (Fig. 3). Linearity of Hcy *versus* dilution was obtained at dilutions higher than and equal to 1/10. More concentrated extracts gave variable and artificially high values for Hcy. This may be related to filter clogging preventing the removal of free Hcy, overloading of the inhibitory capacity of 100 μM D-eritadenine or overloading of the adsorptive capacity of active charcoal. The latter possibility was supported by the finding of residual endogenous AdoHcy after charcoal treatment of concentrated extracts (data not shown). Tissue was routinely homogenized at 1/12, w/v.

Interference and Recovery—The possibility existed that free Hcy was rapidly bound to proteins during extraction in ammonium sulfate. Therefore, interference from free Hcy was tested for as follows:

Hcy (20 μM) was added to the homogenization medium (ice-cold ammonium sulfate). There was no oxidation of Hcy to homocystine within 30 min, as judged by reaction of the former compound with 5,5′-dithiobis(2-nitrobenzoic acid). Liver was thus homogenized in the presence of 20 μM Hcy, which greatly exceeds the concentration derived from tissue subjected to the same dilution. The precipitate was washed with ammonium sulfate containing no Hcy, as described under "Experimental Procedures." Hcy in the homogenization medium did not increase the amount of protein-bound (data not shown).

In other experiments, liver was homogenized (1/12) in ammonium sulfate containing *N*-ethylmaleimide (0.1–1 mM), which would block thiol oxidation and thiol-disulfide interchange, or in ammonium sulfate containing DTE (50 mM). The precipitate was washed with ammonium sulfate containing neither compound. The presence of *N*-ethylmaleimide or DTE did not affect the amount of Hcy associated with proteins (data not shown).

The observation that addition of Hcy, *N*-ethylmaleimide, or DTE to the extraction medium was without effect suggests that protein binding of Hcy is not an artifact related to association of free Hcy with proteins or to the occurrence of thiol oxidation and disulfide exchange during extraction of tissue in ammonium sulfate.

Hcy was added to liver extract, which had been treated with DTE to remove endogenous Hcy, and incubated to allow a fraction of Hcy to form a complex with proteins. The recovery of Hcy (free plus bound) was almost 100%. The bound fraction increased whereas the free fraction decreased in proportion to the concentration of tissue extract (Fig. 4).

Relation between Free and Bound Hcy in Various Tissues of the Rat—The amount of free (extracted with perchloric acid) and protein-bound Hcy was determined in various tissues (liver, kidney, heart, lung, spleen, cerebrum, and cerebellum) of the rat (Table I). The organs were either frozen *in vivo*

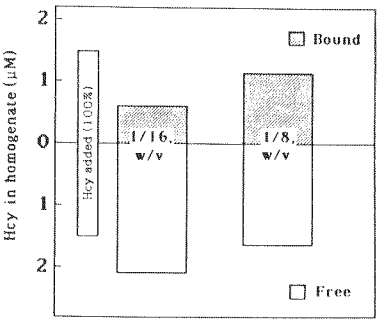

FIG. 4. **Recovery of exogenous Hcy added to liver homogenate.** Liver was homogenized in 50 mM phosphate buffer (pH 7.4), containing 10 mM DTE, and the homogenate was incubated for 20 min at 37 °C. Then, the solution was saturated with ammonium sulfate, and left for 10 min at 0 °C, and the precipitated protein was collected and washed on glass fiber filters. The protein was resuspended in phosphate buffer and the incubation and ammonium sulfate treatment were repeated. The liver proteins were then resuspended in phosphate buffer (pH 7.4), containing no DTE. Exogenous L-Hcy (final concentration of 3 μM) was added to the liver homogenate subjected to dilutions corresponding to 1/8 or 1/16 (w/v), and incubated for 10 min at 37 °C. Free and protein-bound Hcy were determined as described in the text. The height of the *bar* to the left indicates the amount of Hcy added to the extract, *i.e.* 100%.

TABLE I

The concentrations of free and protein-bound Hcy in rat tissues frozen in vivo or frozen after death of the animal

Mean values (5–6 determinations) with their 95% confidence intervals in parentheses. Differences within groups (frozen *in vivo* and frozen after death of the animal) are given by the Footnotes a–o.

Tissue	Free Hcy	Bound Hcy	Ratio (free/bound)
	nmol/g	*nmol/g*	
Frozen *in vivo*			
Liver	4.57 (2.22–7.40)[b,d,l]	3.04 (2.35–3.79)[b,d,e]	1.47 (0.81–2.29)
Kidney	2.10 (1.40–2.88)[b,m]	1.73 (0.75–2.88)[b,d]	1.52 (0.79–2.39)
Heart	1.70 (1.13–2.34)[g,h,k]	1.10 (0.76–1.49)[b]	1.60 (1.06–2.22)
Lung	1.87 (1.40–2.39)[b,m]	1.40 (1.07–1.77)[b,d]	1.36 (1.02–1.74)
Spleen	1.62 (0.89–2.49)[c]	0.53 (0.47–0.59)[c]	3.15 (1.42–5.30)
Cerebrum	0.78 (0.31–1.46)[a,f]	0.34 (0.23–0.46)[a]	2.72 (0.69–6.20)
Cerebellum	5.15 (2.63–8.18)[b,d,i,o]	0.29 (0.20–0.40)[a]	17.81 (9.99–27.34)[e]
Frozen after death of the animal			
Liver	1.31 (0.73–2.10)[b]	2.91 (1.98–4.07)[b,d,e]	0.45 (0.28–0.69)[a]
Kidney	1.54 (1.31–1.78)[b]	1.21 (0.73–1.85)[b,d]	1.38 (0.82–2.12)[b,f]
Heart	0.90 (0.53–1.40)	0.68 (0.48–0.93)[b]	1.34 (0.82–2.02)[b,f]
Lung	1.03 (0.71–1.44)	1.28 (0.78–1.91)[b,d]	0.83 (0.58–1.15)[c]
Spleen	1.18 (0.63–1.95)[g]	0.45 (0.39–0.51)[b,c]	2.68 (1.33–4.62)[b,d,h]
Cerebrum	0.57 (0.35–0.86)[a,f]	0.19 (0.13–0.26)[a]	3.03 (2.24–3.95)[b,d,g,h]
Cerebellum	4.62 (3.08–6.53)[b,e]	0.21 (0.13–0.32)[a]	23.71 (11.29–42.34)[b,d,e,i]

[a] Significant differences from *b* ($p < 0.01$).
[c] Significant differences from *d* ($p < 0.01$).
[e] Significant differences from other values in the same group ($p < 0.01$).
[f] Significant differences from *g* ($p < 0.05$).
[h] Significant differences from *i* ($p < 0.01$).
[k] Significant differences from *l* ($p < 0.05$).
[m] Significant differences from *o* ($p < 0.05$).

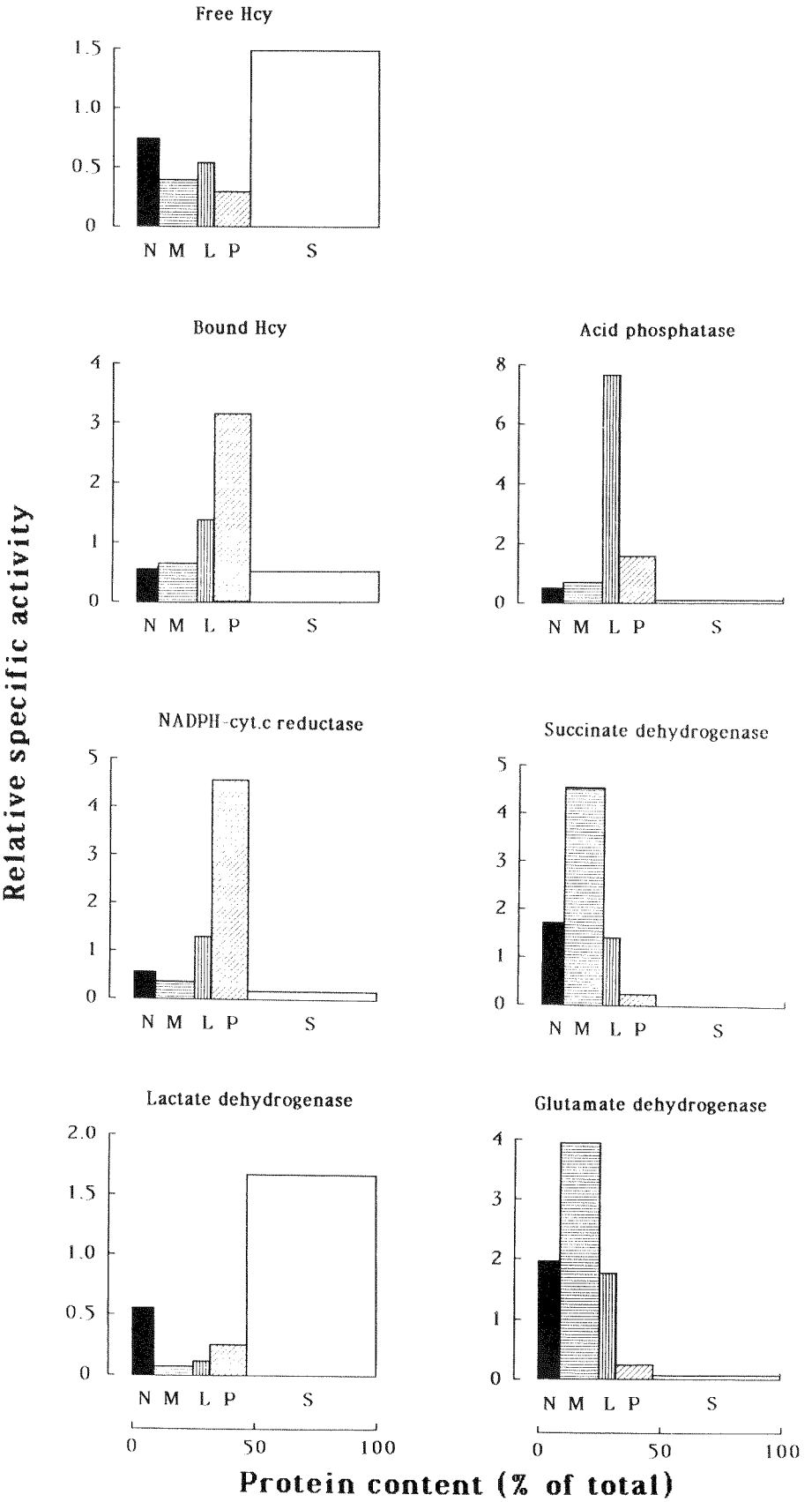

FIG. 5. **Subcellular distribution of free Hcy, protein-bound Hcy, and marker enzymes in rat liver.** The *abscissa* shows the percentage of total protein in each fraction presented as cumulative values. The *ordinate* gives the relative specific activity, which represents the percentage of the activity recovered divided by the percentage of the protein in each fraction, taking the specific activity of the cumulative fractions N, M, L, P, and S as 1. *N*, nuclear fraction; *M*, mitochondrial fraction; *L*, lysosomal fraction; *P*, microsomal fraction; *S*, soluble fraction (cytosol).

using liquid nitrogen or removed and frozen immediately after the animal was killed. All values fulfilled the criteria of normal distribution and identical variance after logarithmic transformation. Freezing *in vivo* gave significant higher mean values for both free ($p < 0.005$) and bound ($p < 0.005$) Hcy, whereas the mean ratio between free and bound Hcy was not dependent on the isolation procedure ($p > 0.10$). In most tissues free Hcy seemed somewhat more dependent on the isolation procedure than bound Hcy. This was particularly pronounced for liver where free Hcy was 3-fold higher when frozen *in vivo*

compared to liver frozen after death of the animal. Cerebrum and cerebellum were exceptions to the general conclusion, in that bound Hcy was somewhat more dependent on the isolation procedure than free Hcy (Table I).

Highest concentrations of both free (about 5 nmol/g, wet weight) and bound Hcy (about 3 nmol/g) were found in liver and lowest concentrations (free 0.8 nmol/g; bound 0.3 nmol/g) in cerebrum. The ratio between free and bound was about 1.5 in most tissues (frozen *in vivo*) (Table I). Details on the statistical evaluation of Hcy content in organs within groups isolated by the same procedure, are given in this table. We have previously communicated that the amount of Hcy in cerebellum is remarkably high (27). The protein-bound fraction was low in this region of the brain, giving a free/bound ratio of about 18 (Table I).

Subcellular Distribution of Free and Bound Hcy in Rat Liver—The amount of free and protein-bound Hcy was determined in subcellular fractions of rat liver. Free Hcy was almost exclusively localized to the cytosol fraction, whereas bound Hcy was associated with the endoplasmatic reticulum (50%) and the cytosol fraction (30%) (Fig. 5). Notably, the recovery of both free and bound Hcy was almost 100% relative to the whole homogenate, which in turn contained free and bound Hcy in amounts equal to that determined directly in whole liver (data not shown).

Hcy in Isolated Rat Hepatocytes—The amount of free Hcy in isolated rat hepatocytes was about 5–20 pmol/10^6 cells and the bound fraction 5–30 pmol/10^6 cells (Fig. 6). The values correspond to a free/bound ratio of about 0.6 which equals that found in whole liver. Large amounts were exported into the extracellular medium. Both free and bound Hcy and the Hcy egress increased markedly after the medium was supplemented with methionine. The amount of Hcy released into the medium during 90 min was more than 20-fold higher than intracellular Hcy in liver cells suspended in a medium supplemented with 200 μM methionine (Fig. 6).

Both free and bound Hcy, the Hcy egress, and the increase

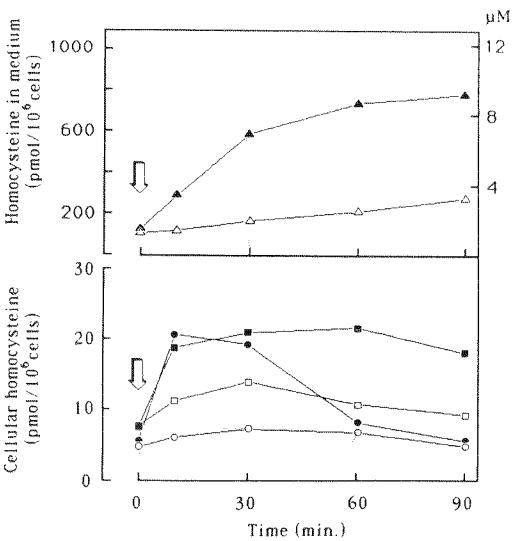

FIG. 6. **Cellular and extracellular Hcy of isolated rat hepatocytes.** Isolated rat hepatocytes (11.7 × 10^6 cells/ml) were preincubated in a medium containing 1 μM methionine for 30 min. At this time point, one-half of the cell suspension was supplemented with 200 μM methionine (*arrow*). The amount of Hcy exported into the medium (△, ▲) is shown in the *upper panel* whereas free Hcy (○, ●) and protein-bound Hcy (□, ■) are shown in the *lower panel. Closed symbols* show the cells suspended in a medium supplemented with 200 μM methionine and *open symbols* the control cells.

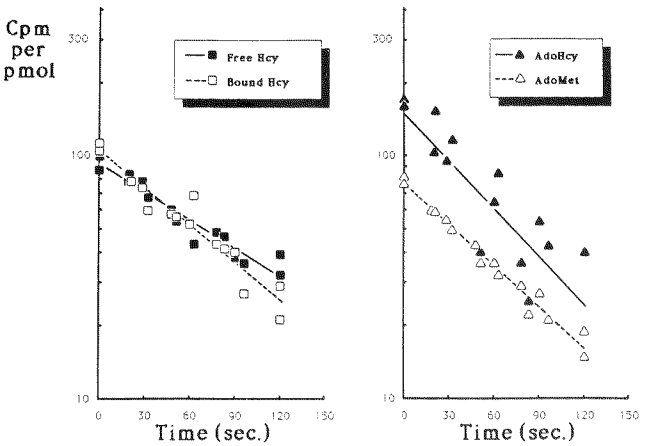

FIG. 7. **Turnover rate of sulfur in Hcy and related compounds determined by pulse-chase experiment.** Hcy, AdoHcy, and AdoMet in rat hepatocytes (9.6 × 10^6 cells/ml) were labeled by incubating the cells with [^{35}S]methionine, and radioactive methionine was then chased by addition of unlabeled methionine, as described under "Experimental Procedures." The figure shows semilogarithmic plots of the specific activities of free Hcy, protein-bound Hcy (*left panel*), AdoHcy, and AdoMet (*right panel*) *versus* time after start of the chase. The straight lines drawn for free Hcy (R = 0.9618), bound Hcy (R = 0.9603), AdoHcy (R = 0.8672), and AdoMet (R = 0.9873) are obtained by log-linear least square regression analysis.

in these parameters after addition of methionine, varied about 2-fold from one cell preparation to another (data not shown).

Pulse-chase Experiments with [^{35}S]Methionine—Radioactive sulfur in free and protein-bound Hcy, AdoHcy, and AdoMet was determined after pulse labeling with [^{35}S]methionine followed by a chase with excess unlabeled methionine. Log-linear least square regression analysis of the decay curve gave a half-life of 53.4 s for AdoMet (R = 0.9873). The half-lives for radioactivity associated with free Hcy, bound Hcy, AdoHcy, and AdoMet were not significantly different (Fig. 7).

Estimation of Turnover Rates—The assumption is made that there is no synthesis of [^{35}S]AdoMet in the chase period (Fig. 7). Therefore, the half-life of the radioactivity in AdoMet is a measure of the half-life of this metabolite. Nearly all AdoMet in rat liver is demethylated to AdoHcy and further catabolized to Hcy (28), and the small fraction used for polyamine biosynthesis could be disregarded. The turnover rates (k) of each of the metabolites in the following reaction sequence

$$\text{AdoMet} \rightarrow \text{AdoHcy} \rightarrow \text{Hcy} \rightarrow$$

can be estimated from the metabolic flux, determined from the amount of AdoMet and its turnover rate, and the concentration of each metabolite:

$$[\text{AdoMet}] \cdot k_{\text{AdoMet}} = [\text{AdoHcy}] \cdot k_{\text{AdoHcy}} = [\text{Hcy}] \cdot k_{\text{Hcy}} \quad (1)$$

$$t_{1/2\text{AdoHcy}} = t_{1/2\text{AdoMet}} \cdot [\text{AdoHcy}]/[\text{AdoMet}] \quad (2)$$

$$t_{1/2\text{Hcy}} = t_{1/2\text{AdoMet}} \cdot [\text{Hcy}]/[\text{AdoMet}] \quad (3)$$

The half-life for AdoHcy was calculated as 2 s, and $t_{1/2}$ for total Hcy (free plus bound Hcy regarded as a single pool) as 3 s under conditions of the chase experiment (Fig. 7). Assuming that free Hcy is formed via a unidirectional reaction from bound Hcy, or vice versa, $t_{1/2}$ for free Hcy is 1.2 s and $t_{1/2}$ for bound Hcy 1.8 s.

DISCUSSION

The existence of mixed disulfides between cellular proteins and thiols like glutathione or cysteine has been reported for

various tissues (29–34), including rat tissues (30–34). The activities of numerous enzymes are modified by thiol-disulfide exchange (35). These observations form the basis of the view that protein-mixed disulfides are occurring *in vivo* and biological disulfides may play a role in metabolic regulation (36, 37). However, objections to this possibility exist. Oxidation of thiols or reduction of disulfides during extraction of cells or tissues are sources of erratic results. Furthermore, most reports on protein-mixed disulfides in tissues are based on extraction of tissues in strong acids, followed by liberation of thiols in the presence of a reducing agent (29–33). Under these conditions, liberation of protein fragments may account for most thiols, and the high content of protein-mixed disulfides in liver, lung, and heart may be artifacts. In addition, the *in vivo* experiments supporting a regulatory role are hampered by the general lack of specificity of agents affecting the thiol-disulfide redox balance (36).

The present paper describes a method for the determination of Hcy bound to tissue proteins. The method is based on a radioenzymic assay for free Hcy (and its disulfides) in solution (14). In addition, we exploited the finding that Hcy is readily released from native, soluble tissue proteins in the presence of DTE (Fig. 1).

The major advantages of this method are the high specificity and sensitivity, and the latter is required for the determination of the small amounts of Hcy in tissues and isolated cells under physiological conditions (6). A limitation is that chemicals inhibiting AdoHcy hydrolase, which may be present in the extraction medium, must be removed from Hcy prior to the enzymic conversion of Hcy to AdoHcy.

Precipitation of tissue proteins with perchloric acid was not found useful, because there was only a slight liberation of Hcy from proteins denatured in acid (Fig. 1). Similar observations have recently been made for the interaction of Hcy with native plasma protein(s). A method based on acid precipitation of proteins (38) gave lower values for protein-bound Hcy in human plasma than a radioenzymic assay involving dissociation of Hcy from native plasma proteins (13). These observations may be explained by trapping of Hcy or cyclization of Hcy at low pH to form Hcy thiolactone, which readily acylates proteins (39).

Hcy added to crude liver extract treated with ammonium sulfate and DTE was almost totally recovered (Fig. 4). However, this finding does not ensure a total recovery of endogenous Hcy. We observed that a fraction of endogenous Hcy was slowly released from proteins in crude liver homogenate, whereas a rapid release was obtained from soluble proteins of liver cytosol (Fig. 1). This problem was not overcome by including 0.05% of Triton X-100 in the incubation medium (data not shown). Furthermore, treatment of liver homogenate with DTE and ammonium sulfate must be repeated twice to remove 70% of measurable endogenous Hcy, whereas DTE immediately released all Hcy from plasma proteins (13). It is conceivable that a fraction of endogenous Hcy is trapped in and slowly released from tissue particles or clusters of hydrophobic protein in crude liver extract. In addition, a portion may be noncovalently but tightly bound to native proteins, including enzymes involved in Hcy metabolism.

We determined the amount of protein-bound Hcy in various tissues of rat (Table I). Free Hcy was determined in the same tissue specimens, and these values were in accordance with data published previously (14). The organs were either frozen *in vivo* using liquid nitrogen or frozen after death of the animal. In this way one may obtain a measure of the metabolic alterations taking place immediately after death. It has been shown previously that freezing *in vivo* prevents the postmortem elevation of AdoHcy and adenosine (40). Conceivably, high concentrations of adenosine may reduce the amount of Hcy via reversal of the AdoHcy hydrolase reaction. This possibility has been investigated previously in mice, but free Hcy was not dependent on the isolation procedure in this species (14). Therefore, it was unexpected to find higher amounts of both free and bound Hcy in rat tissues frozen *in vivo* (Table I). This difference between rat and mouse may be related to different properties of AdoHcy hydrolase from these two species. Similarly, difference in the *in vivo* response to the administration of the adenosine analogue, 3-deazaadenosine, to hamster and rat has been explained by interspecies variations in the catalytic properties of AdoHcy hydrolase (41).

The present work demonstrates, for the first time, protein-bound Hcy in various tissues of the rat under physiological conditions (Table I). In all tissues, except cerebellum, the amount of protein-bound Hcy equalled free Hcy. This finding is not in agreement with the current view (8, 15) that nearly all intracellular Hcy is associated with proteins.

Subcellular fractionation of rat liver showed that free Hcy was localized in the cytosol, whereas bound Hcy was associated with the microsomes and cytosol fraction (Fig. 5). Therefore, a substantial fraction of proteins forming a complex with Hcy are probably microsomal. Hcy bound to cytosol proteins is most likely in rapid equilibrium with Hcy associated with the endoplasmic reticulum, since a monoexponential decay curve for radioactive sulfur in protein-bound Hcy was observed (Fig. 7).

The subcellular fractionation experiments (Fig. 5) gave nearly 100% recovery of both free and bound Hcy. This finding was in fact unexpected. We anticipated that redistribution of Hcy would occur during tissue processing and preparation of subcellular fractions since Hcy readily forms mixed disulfides with proteins (42). The apparent stability of the free and bound Hcy may be explained by the low temperature during preparation of subcellular fractions. The possibility that free Hcy exists as an acid-soluble mixed disulfide should also be considered (14).

We have recently communicated the remarkable finding that cerebellum from several mammalian species contains relatively high levels of free Hcy. No other regions of the brain were rich in Hcy (27). Notably, the amount of protein-bound Hcy in cerebellum of the rat is low and equals that in other areas of the brain, giving a free/bound ratio in cerebellum of about 18 (Table I). Several interesting explanations of these findings should be considered. Cerebellum may contain limited amounts of protein(s) serving as acceptors for Hcy, or, alternatively, may be rich in thiols forming acid-soluble, mixed disulfide(s) with Hcy. A major portion of free Hcy in cerebellum may reside in a compartment not in equilibrium with bound Hcy. Whatever the reason, the metabolism and function of Hcy in cerebellum obviously deserve further attention.

Isolated hepatocytes contain free and bound Hcy in proportions equal to that observed in whole liver (Fig. 6, Table I), and exported large amounts of Hcy into the extracellular medium (Fig. 6), as previously demonstrated for other cells (6, 43). Both free and bound Hcy, and the Hcy egress increased markedly when the medium was supplemented with methionine (Fig. 6). This may be related to increased flux through the AdoHcy hydrolase pathway in the presence of excess methionine. In addition, the methionine effect shows that the Hcy level and the Hcy egress are dependent on the metabolic status of the cell.

The data presented in Fig. 7 provide information on turn-

over rates of AdoMet and AdoHcy, in addition to Hcy. AdoMet in hepatocytes showed a half-life of 54 s. This is lower than the half-life reported by Farooqui *et al.* (44) in rat liver cells but equals that published by Hoffman in mouse liver (45). Furthermore, the turnover rate for AdoHcy obtained by us ($t_{1/2}$ = 2 s) was below that reported for AdoHcy in mouse liver by Hoffman (45). The high turnover rates derived from data of Fig. 7 relative to those reported in the literature (44, 45) may be explained by the fact that the latter studies were performed in the presence of inhibitors of biosynthesis or catabolism of AdoMet. These compounds are expected to retard, whereas excess methionine may enhance, the turnover of AdoMet and AdoHcy.

The isotope, ^{35}S, in methionine was essentially equilibrated between AdoMet, AdoHcy, and free and protein-bound Hcy throughout the rapid chase period (Fig. 7). During this period there was a continuous production of AdoHcy as well as Hcy. This indicates that the turnover rate of AdoHcy and Hcy is production rate limited. The isotope equilibrium between AdoHcy and Hcy also supports the current view (7) that hydrolysis of AdoHcy is the source of Hcy.

The isotope equilibrium between free and protein-bound Hcy is consistent with the following interpretations. Free Hcy may be produced *in vivo* by a unidirectional reaction from bound Hcy (or vice versa), and in this case the half-life of bound Hcy is about 1.8 s. Alternatively, there is a rapid exchange between free and bound Hcy, and the rate of formation and dissociation of the Hcy-protein complex greatly exceeds the turnover rate of total Hcy. Finally, isotope equilibrium would also result if bound Hcy is formed from free Hcy during extraction of cells in ammonium sulfate. The latter possibility seems unlikely, however, because protein-bound Hcy was not affected by inclusion of Hcy or *N*-ethylmaleimide or DTE in the extraction medium.

The turnover rate of the intracellular Hcy-protein complex is remarkably high, and the presence of enzyme(s) responsible for the reduction of disulfides containing the homocysteinyl residue seems likely. This possibility should be related to the finding of Racker (46) reporting on the presence in beef liver of a glutathione-homocystine transhydrogenase preferring homocystine as a hydrogen acceptor. In the liver, the parenchymal cells contained the highest activity of thiol-protein disulfide oxidoreductase, and this enzyme was mainly localized to the microsomal fraction (47) which contained a large portion of protein-bound Hcy in liver (Fig. 5).

The short half-life of protein-bound Hcy adds some interesting aspects to the possible biological implications of the interaction of Hcy with intracellular proteins. Protein-bound Hcy may represent a Hcy compartment which is readily mobilized. Assuming that interaction of Hcy with proteins alters their biological function(s), the half-life of the Hcy-protein complex is such that *in vivo* regulation of some cellular function by thiol-disulfide interchange involving Hcy is feasible.

In conclusion, a substantial fraction of intracellular Hcy in various tissues of the rat is associated with proteins, and in the liver cells at least, the Hcy-protein complex is characterized by a high turnover rate, and seems to be in a rapid equilibrium with free Hcy. The physiological implications of protein-bound Hcy as well as its presence and possible role in disease states, like homocystinuria (1), methionine-dependent cancer (4), and during chemotherapy with antifolate drugs (6), are subjects for further investigations.

Acknowledgments—The technical assistance of Gry Kvalheim and Halvard Bergesen is highly appreciated. We would like to thank Per Eystein Lønning for performing the statistical calculations.

REFERENCES

1. Mudd, S. H., and Levy, H. L. (1983) in *Metabolic Basis of Inherited Diseases* (Standbury, J. B., ed) Ed. 5, pp. 522–559, McGraw-Hill Publications, New York
2. McCully, K. S. (1983) *Atheroscler. Rev.* **11,** 157–246
3. Boers, G. H., Smals, A. G., Trijbels, F. J., Leermakers, A. I., and Kloppenborg, P. W. (1983) *J. Clin. Invest.* **72,** 1971–1976
4. Hoffman, R. M. (1984) *Biochim. Biophys. Acta* **738,** 49–87
5. Barak, A. J., Tuma, D. J., and Beckenhauser, H. C. (1984) *J. Am. Coll. Nutr.* **3,** 93–96
6. Ueland, P. M., Svardal, A., Refsum, H., Lillehaug, J. R., Schanche, J.-J., and Helland, S. (1986) in *The Biochemistry of S-Adenosylmethionine as a Basis for Drug Design* (Borchardt, R. T., Creveling, C. R., and Ueland, P. M., eds) pp. 263–274, Humana Press, Clifton, NJ
7. Ueland, P. M. (1982) *Pharmacol. Rev.* **34,** 223–253
8. Finkelstein, J. D., and Martin, J. J. (1984) *J. Biol. Chem.* **259,** 9508–9513
9. Foreman, J. W., Wald, H., Blumberg, G., Pepe, L. M., and Segal, S. (1982) *Metab. Clin. Exp.* **31,** 613–619
10. Wilcken, D. E. L., and Gupta, V. J. (1978) *Clin. Sci.* **57,** 211–215
11. Kredich, N. M., Kendall, H. E., and Spence, F. J., Jr., (1981) *Anal. Biochem.* **116,** 503–510
12. Kredich, N. M., Hershfield, M. S., Falletta, J. M., Kinney, I. R., Mitchell, B., and Koller, C. (1981) *Clin. Res.* **29,** 541A
13. Refsum, H., Helland, S., and Ueland, P. M. (1985) *Clin. Chem.* **31,** 624–628
14. Ueland, P. M., Helland, S., Broch, O. J., and Schanche, J.-S. (1984) *J. Biol. Chem.* **259,** 2360–2364
15. Wallwork, J. C., and Duerre, J. A. (1984) *Fed. Proc.* **43,** 1693
16. de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R., and Appelmans, F. (1955) *Biochem. J.* **60,** 604–617
17. Schmidt, E. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed) Vol. 2, Ed. 2, pp. 650–656, Academic Press, New York
18. Arrigoni, O., and Singer, T. P. (1962) *Nature* **193,** 1256–1258
19. Walter, K., and Schütt, C. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed) Vol. 2, Ed. 2, pp. 856–860, Academic Press, New York
20. Sottocasa, G. L., Kuylenstierna, B., Ernster, L., and Bergstrand, A. (1967) *J. Cell Biol.* **32,** 415–438
21. Bergmeyer, H. U., and Bernt, E. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed) Vol. 2, Ed. 2, pp. 574–579, Academic Press, New York
22. Berry, M. N., and Friend, D. S. (1969) *J. Cell Biol.* **43,** 506–520
23. Helland, S., and Ueland, P. M. (1982) *Cancer Res.* **42,** 1130–1136
24. Johnson, R. A., and Wichern, D. W. (eds) (1982) *Applied Multivariate Statistical Analysis*, p. 594, Prentice Hall Inc., Englewood Cliffs, NJ
25. Rosner, B. (ed) (1982) *Fundamentals of Biostatistics*, p. 547, Duxbury Press, Boston
26. Schanche, J.-S., Schanche, T., Ueland, P. M., Holy, A., and Votruba, I. (1984) *Mol. Pharmacol.* **26,** 553–558
27. Broch, O. J., and Ueland, P. M. (1984) *J. Neurochem.* **43,** 1755–1757
28. Eloranta, T. O., and Kajander, E. O. (1984) *Biochem. J.* **224,** 137–144
29. Harding, J. J. (1970) *Biochem. J.* **117,** 957–960
30. Harrap, K. R., Jackson, R. C., Riches, P. G., Smith, C. A., and Hill, B. T. (1973) *Biochim. Biophys. Acta* **310,** 104–110
31. Isaacs, J., and Binkley, F. (1977) *Biochim. Biophys. Acta* **497,** 192–204
32. Keeling, P. L., Smith, L. L.,, and Aldridge, W. N. (1982) *Biochim. Biophys. Acta* **716,** 249–257
33. Meredith, M. J. (1983) *Anal. Biochem.* **131,** 504–509
34. Grimm, L. M., Collisin, M. W., Fisher, R. A., and Thomas, J. A. (1985) *Biochim. Biophys. Acta* **844,** 50–54
35. Kosower, N. S., and Kosower, E. M. (1978) *Int. Rev. Cytol.* **54,** 109–160
36. Ziegler, D. M. (1985) *Annu. Rev. Biochem.* **54,** 305–329
37. Gilbert, H. F. (1982) *J. Biol. Chem.* **257,** 12086–12091
38. Kang, S.-S., Wong, P. W. K., and Becker, N. (1979) *Pediatr. Res.* **13,** 1141–1143
39. Dudman, N. P. B., and Wilcken, D. E. L. (1982) *Biochem. Med.* **27,** 244–253
40. Helland, S., and Ueland, P. M. (1983) *Cancer Res.* **43,** 1847–1850
41. Kim, I.-K., Zhang, C.-Y., Chiang, P. K., and Cantoni, G. L. (1983) *Arch. Biochem. Biophys.* **226,** 65–72
42. Smolin, L. A., and Benevenga, N. L. (1984) *Am. J. Clin. Nutr.* **39,** 730–737
43. German, D. C., Bloch, C. A., and Kredich, N. M. (1983) *J. Biol. Chem.* **258,** 10997–1103
44. Farooqui, J. Z., Lee, H. W., Kim, S., and Paik, W. K. (1983) *Biochim. Biophys. Acta* **757,** 342–351
45. Hoffman, J. L. (1980) *Arch. Biochem. Biophys.* **205,** 132–135
46. Racker, E. (1955) *J. Biol. Chem.* **217,** 867–874
47. Ansorge, S., Wiederanders, B., Riemann, S., Brouwer, A., and Knook, D. L. (1984) *Biomed. Biochim. Acta* **43,** 1213–1221