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Brain
homocyst(e)ine
homocystinuric
kidney
liver
plasma
2-mercaptoethanol

Protein-Bound Homocyst(e)ine in Normal Subjects and in Patients with Homocystinuria

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Summary

A method was developed to quantitate protein-bound homocyst(e)ine using 2-mercaptoethanol. Protein-bound homocyst(e)ine was discovered in the plasma from normal individuals, ranging from 0.5–2.2 nmole/ml. In two obligatory heterozygotes for classical homocystinuria, plasma protein-bound homocyt(e)ine was 3.5 and 4.8 nmole/ml, respectively. Untreated homozygotes showed approximately a 40-fold increase of plasma protein-bound homocyst(e)ine. Furthermore, using conventional methods, no free homocystine was detectable in the supernatant of plasma precipitate from two classical homocystinuric patients treated with pyridoxine, but plasma protein-bound homocyst(e)ine showed a 10-fold increase. Protein-bound homocyst(e)ine was also demonstrated in the liver, kidney, and brain tissues from a patient with methylenetetrahydrofolate reductase deficiency.

Speculation

The results in this study suggest that determination of protein-bound homocyst(e)ine using 2-mercaptoethanol may provide a more reliable assessment of treatment in patients with homocystinuria and a potentially useful tool for the definition of the carrier state.

Demonstration of protein-bound homocyst(e)ine in various tissues of homocystinuric patients suggests the possibility that this compound may be directly associated with the development of some of the pathologic changes in the tissues.

Homocysteine is a metabolite of methionine metabolism and is not detectable in tissue fluids from normal individuals by conventional methods of amino acid determination. It is found in the plasma and urine from patients with a metabolic block in the synthesis of cystathionine from homocysteine and serine (4, 16, 21), or from patients with defects in the remethylation of homocysteine to methionine (8, 12, 17, 18, 24). In approximately half of the patients with cystathionine synthase deficiency, administration of large doses of pyridoxine eliminates plasma and urinary homocystine when conventional methods of detection are used (1, 13, 23). However, some clinical abnormalities such as ectopia lentis and abnormal morphology in the hepatocytes may persist or develop in spite of treatment (9–11). Thus, if homocystine is directly responsible for the pathogenesis in homocystinuric patients, a significant quantity of homocystine must have escaped detection by conventional methods. This postulate is supported by the failure to detect homocystine in various tissues from patients with homocystinuria due to cystathionine synthase deficiency (2, 20) or due to methylenetetrahydrofolate reductase deficiency (14, 24), and by the finding of a compound bound to plasma proteins and behaving like homocystine in homocystinuric patients (2).

This paper describes the finding of a significant portion of homocyst(e)ine in protein-bound form in the plasma and various tissues from patients with homocystinuria. In addition, detectable quantities of protein-bound homocyst(e)ine have been demonstrated in the plasma from normal subjects and heterozygotes for homocystinuria.

MATERIALS AND METHODS

Plasma samples were obtained from heparinized venous blood by immediate centrifugation and precipitation with four volumes of 3.75% sulfosalicylic acid in 0.3M lithium citrate buffer, pH 2.0. Tissue specimens were obtained at autopsy within 2 hr after death and were frozen at −70°C until analysis.

Amino acid concentrations in the supernatant fractions of plasma were determined by a Beckman Model 121-M Amino Acid Analyzer, using a single column. For the measurement of protein-bound amino acids, washed precipitates of plasma were resuspended in water to the original plasma volume, neutralized to pH 7 with lithium hydroxide, mixed with 1/10 volume of 2-mercaptoethanol, and incubated at 37°C for 120 min. The protein was reprecipitated with sulfosalicylic acid to a final concentration of 3.75%, and the clear supernatant was used for amino acid analysis. Tissue samples were washed with 10 mM Tris HCl buffer, pH 7.5, blotted, weighed, and then disrupted by sonication in distilled water. The suspensions were processed in the same manner as in plasma for the determination of both free and protein-bound amino acids. Protein was determined by the method of Lowry *et al.* (15). An informed consent was obtained for all research procedures.

RESULTS

IDENTIFICATION OF PROTEIN-BOUND HOMOCYST(E)INE

Using column chromatography, pure L-homocystine appeared as a single ninhydrin-positive peak at 196 min (Fig. 1A). In contrast, L-homocystine incubated with 2-mercaptoethanol appeared as a new peak at 126 min (Fig. 1B). A similar peak was found at 126 min when L-homocysteine alone or L-homocysteine incubated with 2-mercaptoethanol was chromatographed, demonstrating the reduction of homocystine to homocysteine in the presence of 2-mercaptoethanol. Two minor peaks were also detected, but they had not been identified definitively (Fig. 1B). After incubation with 2-mercaptoethanol, supernatants of plasma precipitates from patients with homocystinuria showed a peak at 126 min, corresponding to homocysteine instead of at 196 min, corresponding to homocystine. This peak was demonstrated to cochromatograph with pure L-homocysteine (data not shown). In addition, peaks of reduced glutathione and cysteine were found at 43 and 67 min, respectively.

When normal human plasma was incubated with 0.02–0.1 mM homocystine (Table 1), more than 40% of exogenous homocystine was recovered as homocysteine from the the acid precipitate after treatment with 2-mercaptoethanol. Total recovery from the supernatant and precipitate was close to 100%. These results demonstrated that large portions of exogenous homocystine were bound

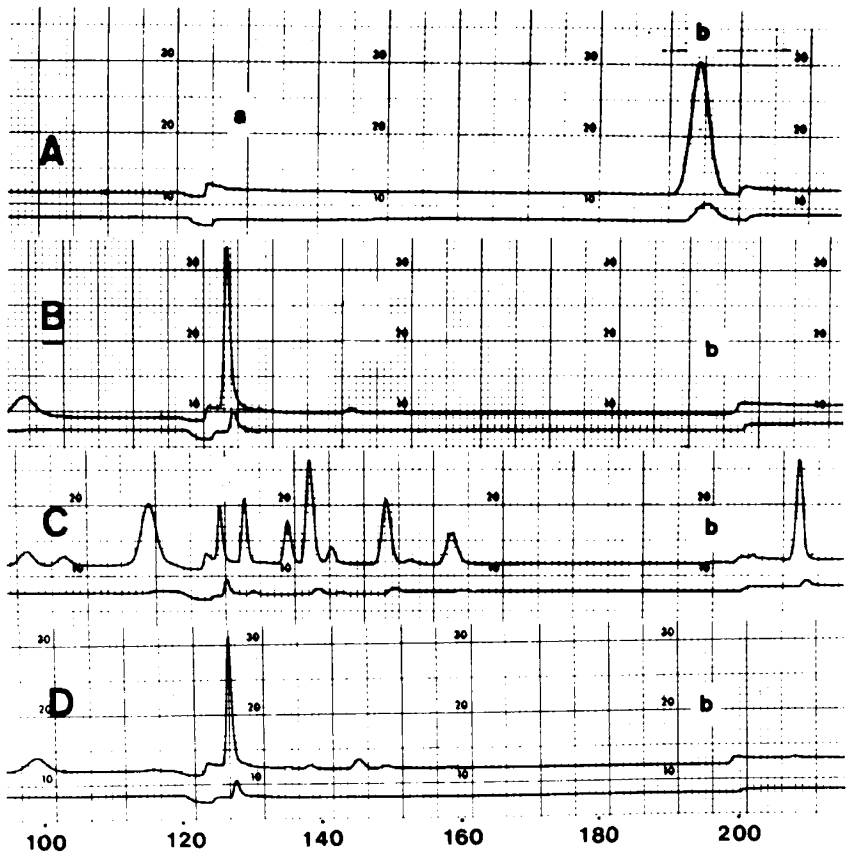

Fig. 1. Amino acid profile of (*A*) L-homocystine alone, (*B*) homocystine incubated with 2-mercaptoethanol, (*C*) supernatant of acid precipitate of plasma from a homocystinuric patient treated with pyridoxine, and (*D*) plasma precipitate from the above patient incubated with 2-mercaptoethanol. *a*: L-homocysteine, *b*: L-homocystine.

Table 1. *Distribution of exogenous homocystine in plasma*[1]

Homocystine added (nmole/ml)	Homocystine recovered (nmole/ml)		
	Supernatant	Precipitate	Total
100	55.4	46.9	102.3 (calculated)
50	23.3	28.8	52.1 (calculated)
50[2]			51.0 (measured)
20	4.0	15.8	19.8 (calculated)

[1] One ml of plasma was mixed with 1 ml of L-homocystine in 10 mM Tris HCl, pH 7.5, and incubated at 37°C for 120 min. The reaction was terminated by the addition of buffered sulfosalicylic acid and the amount of homocystine was determined as described in the *Methods* section.

[2] After incubation with exogenous homocystine, the plasma mixture was treated with 2-mercaptoethanol before protein precipitation for total recovery in the supernatant. Recovery was expressed as homocystine for comparison.

to plasma protein(s) and that the bound homocyst(e)ine was readily released by 2-mercaptoethanol as homocysteine.

PROTEIN-BOUND HOMOCYST(E)INE IN PLASMA

For all subjects, heparinized venous blood was immediately centrifuged and the plasma was immediately precipitated for amino acid determination. The exception was T. C. a patient with methylenetetrahydrofolate reductase deficiency (14, 24). For the latter, only stored plasma was available. Table 2 shows that only protein-bound homocyst(e)ine was found in the plasma from normal subjects and heterozygotes for cystathionine synthase deficiency. In Homozygotes I and II with pyridoxine therapy, protein-bound homocyst(e)ine was the only form detectable or was higher than the free form at low concentrations. On the other hand, in Homozygote III protein-bound homocyst(e)ine is lower than the free form, suggesting saturation of the binding capacity at high plasma concentrations. Only protein-bound homocyst(e)ine was detected in the frozen plasma from subject T.C. However, previous studies on the same subject showed free homocystine when the plasma was immediately processed (24).

Table 2. *Free and protein-bound homocyst(e)ine in plasma (nmole/ml)*

Enzyme defect	Treatment (mg B_6/day)	Free homocystine	Bound homocyst(e)ine
Normal subjects (8 cases)		N. D.[1]	1.4 ± 0.68[2]
Cystathionine synthase deficiency			
Heterozygote I		N. D.	3.5
Heterozygote II		N. D.	4.8
Homozygote I	600	11.7	25.9
	700	N. D.	17.1
Homozygote II	600	14.5	28.7
	700	N. D.	17.1
Homozygote III		113.2	59.0
Methylenetetrahydrofolate reductase deficiency			
Homozygote T. C.[3]		N. D.	48.3

[1] N. D. = not detectable.

[2] Mean ± SD.

[3] Stored plasma.

PROTEIN-BOUND HOMOCYST(E)INE IN TISSUES

Table 3 shows the concentrations of protein-bound homocyst(e)ine in tissues from the patient with methylenetetrahydrofolate reductase deficiency at autopsy. No free homocystine wa

Table 3. *Protein-bound homocyst(e)ine (nmole/g protein)*

Tissue	Homocystinuric patient	Nonhomocystinuric subjects[1, 2]
Liver	76.1	N. D.
Brain	403.1	N. D.
Kidney	55.2	N. D.

[1] N. D. = not detectable.

[2] Tissues from three nonhomocystinuric subjects were analyzed.

detectable. In this patient, brain tissue contained the highest concentration of protein-bound homocyst(e)ine. No protein-bound homocyst(e)ine was detectable in the brain, liver, or kidney from subjects who died of unrelated disorders.

DISCUSSION

It has been reported that significant "loss" of plasma cystine or homocystine occurred during prolonged storage (5, 6, 19, 22). On the other hand, no apparent loss of plasma homocystine was observed during short storage of a few hours (19). However, homocystine had never been detected in the plasma from normal subjects and heterozygotes for homocystinuria, even when the plasma was immediately processed by conventional methods.

Using our newly developed method, a small quantity of homocyst(e)ine was found to be bound to plasma protein(s) from normal subjects and a somewhat higher concentration was found in the plasma from heterozygotes for homocystinuira. The mechanism of binding and whether the binding involved homocystine or homocysteine had not been determined. It was reported, however, that half-cystine molecules were bound to serum proteins by disulfide linkage, and that homocystine interfered with the formation of this linkage (7). Nevertheless, the authors' method proved to be highly sensitive qualitatively and quantitatively, as few amino acids were released by 2-mercaptoethanol from protein precipitates (Fig. 1D). Using this method, a 10-fold increase of protein-bound homocyst(e)ine was demonstrated when none was found in the plasma supernatant from patients under treatment (Fig. 1C and D). In addition, this method also provided a reliable quantitation of tissue homocyst(e)ine which had never been found in organs from homocystinuric patients (2, 14, 20, 24).

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