HOMOCYSTINURIA ASSOCIATED WITH DECREASED METHYLENETETRAHYDROFOLATE REDUCTASE ACTIVITY

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SUMMARY:- A new type of homocystinuria is described. A variety of evidence indicates that patients with this type of homocystinuria are not deficient in cystathionine synthase activity. Fibroblasts from these patients were unable to grow as rapidly as control fibroblasts if homocystine replaced methionine in the culture medium. N^5-Methyltetrahydrofolate-homocysteine methyltransferase activity in these cells was not markedly decreased, whereas methylenetetrahydrofolate reductase activity was significantly below normal. A deficiency of this reductase activity can explain the biochemical abnormalities in these patients.

Elevated urinary excretion of homocystine (i.e. homocystinuria) was first discovered in humans in 1962 (1,2). It is not uncommon, more than a hundred cases having been reported. In a majority of the patients studied, homocystinuria has been due to deficient activity of cystathionine synthase, the enzyme which catalyzes the condensation of homocysteine with serine (3). Homocystinuria may also be due to impairment of the methylation of homocysteine by an enzyme which utilizes N^5-methyltetrahydrofolate as methyl donor and a cobalamin (B_{12}) derivative as cofactor. Two known genetic diseases lead to a lack of this B_{12} cofactor and thus to homocystinuria: (a) a disease in which cellular uptake or metabolism of B_{12} is deranged so that the normal intracellular B_{12} derivatives are not accumulated (4-8) and (b) a syndrome in which oral B_{12} is not absorbed normally (9). Finally, a nongenetic form of homocystinuria may be induced by administration of 6-azauridine triacetate (10). We wish now to report upon a hitherto unrecognized form of homocystinuria which our findings

suggest is due to deficient activity of methylenetetrahydrofolate reductase (methylene-THF-reductase; E.C. 1.1.1.68).

Patients:- C.P. is a 16 year-old boy studied at the Massachusetts General Hospital because of muscle weakness, seizures and abnormal electroencephalographic findings. B.M. is a 17 year-old girl studied at Johns Hopkins Hospital because of schizophrenia and mental deterioration. L.M. is a 15 year-old sister of B.M., discovered to be homocystinuric during a survey of the family of B.M.

Experimental:- Methods for obtaining skin biopsies, initiating tissue cultures, and growing and harvesting fibroblasts have been described (6). Two separate biopsies were obtained from B.M. Cells from biopsy I were studied after many generations of growth in tissue culture. Cells from biopsy II were studied after a minimal number of generations. Cystathionine synthase and N^5-methyltetrahydrofolate-homocysteine methyltransferase were extracted from fibroblasts and assayed essentially as described (6) except that in the present work, extracts have been prepared by thawing the frozen cell pellets in suitable buffers, then freezing and thawing once more. The sonication previously used (6) was omitted. For assay of methylene-THF-reductase activity, the frozen cell pellet was extracted with potassium phosphate buffer, pH 7.2, 0.03M. The suspension was centrifuged at 20,000 g for 30 minutes and the resulting supernatant solution was used for enzyme assay after gel filtration through a column of Sephadex G-25 equilibriated with the same buffer. Enzyme assay was performed according to a modification of the method of Kutzbach and Stokstad (11). The oxidative conversion of $^{14}CH_3$-N^5-methyltetrahydrofolate to ^{14}C-formaldehyde was measured. The standard reaction mixture contained the following components in a final volume of 0.423 ml: 80 µmoles of potassium phosphate buffer, pH 6.3; 5 µmoles of ascorbic acid; 0.5 µmoles of neutralized ethylenediaminetetracetic acid; 11.8 mµmoles of $^{14}CH_3$-N^5-methyltetrahydrofolic acid containing 244 x 10^3 DPM; flavin-adenine dinucleotide (FAD) as indicated; 16 mµmoles of menadione (added in 0.003 ml ethanol just prior to initiation of the reaction); gel-filtered cell extract or, in the control, a similar volume of potassium phosphate buffer, pH 7.2, 0.03M (added last to start the reaction). After aerobic incubation for 1 hour at 37° in 12 ml conical centrifuge tubes, the reaction was terminated by addition of 0.3 ml of 1.0M potassium acetate buffer, pH 4.5, containing 0.9 mg dimedone (5',5-dimethyl-1,3-cyclohexanedione). The mixture was incubated at 100° for 5 minutes, then at 0° for 5 minutes. To extract the formaldehyde-dimedone complex, 3 ml toluene was added and the suspension thoroughly mixed with a Vortex Test Tube Mixer for two periods of 15 seconds. After centrifugation a 2 ml aliquot of the clear supernatant layer was removed for determination of radioactivity in a liquid scintillation spectrometer. After subtraction of the value from the non-enzyme control and correction for recovery of formaldehyde during the extraction procedure (82%, not affected by the range of protein concentrations used), the specific enzyme activity was calculated from the radioactivity in the toluene layer.

Materials:- N^5-Methyltetrahydrofolic acid was a gift from Dr. B. T. Kaufman. $^{14}CH_3$-N^5-Methyltetrahydrofolic acid was obtained as the barium salt from Amersham Searle.

Results:- Homocystinuria without cystathionine synthase deficiency. The three patients C.P., B.M., and L.M. were homocystinuric and homocystinemic. However, in contrast to typical cystathionine synthase deficient patients, these subjects did not have abnormal elevations of methionine in plasma or urine, C.P. and B.M. excreted most of the sulfur of an oral dose of L-methionine as inorganic sulfate (12), and extracts from fibroblasts of the three patients had

Table 1

Cystathionine Synthase Activity in Extracts of Fibroblasts

Controls	Cystathionine synthase deficient	C.P.	B.M.		L.M.
			Biopsy I	Biopsy II	
mμmoles cystathionine formed per mg protein per 135 minutes					
35 (3.7-65)	0-0.5	23.2	37.6	25.9	36.7

The mean and range (in parentheses) for controls are based on a study of 36 control cell lines. The range of values for cystathionine synthase deficient subjects represents 29 homocystinuric individuals whose fibroblasts exhibit typical cystathionine synthase deficiency (3,13).

normal specific activities of cystathionine synthase (Table 1).

Methylation of homocysteine. The ability of fibroblasts to methylate homocysteine was assessed by growth of cells in media containing either methionine or homocystine (6). Representative results are shown in Table 2. Control cells are able to generate methionine from homocystine rapidly enough to allow growth at an almost normal rate when homocystine replaces methionine in the medium. In contrast, cells from C.P., B.M., and L.M. grow no better upon homocystine than in a medium containing neither homocystine nor methionine. These cells do grow well upon methionine. For comparison, the results obtained with cells from E.M., which are known to be unable to methylate homocysteine normally (6), are presented also.

Assays of N^5-methyltetrahydrofolate-homocysteine methyltransferase. Results of assays of the methyltransferase which catalyzes homocysteine methylation in fibroblasts are shown in Table 3. Extracts from cells of C.P. contained normal activities of this enzyme under all conditions tested. Extracts from cells of B.M. were also close to normal, although some activities fell slightly below the control range. For comparison, values for cells of E.M. are included to illustrate the results obtained with cells which are unable to form the coenzyme needed for this methyl transfer reaction (6,8).

Assay of methylene-THF-reductase activity (Table 4). Extracts from cells of homocystinuric patients with cystathionine synthase deficiency or with

Table 2

Growth of Fibroblasts in Medium Containing Homocystine or Methionine

	Relative number of cells		
	Addition to unsupplemented medium		
	None	Homocystine	Methionine
Experiment 1			
Control A	0.9	3.4	4.1
E.M.	0.6	0.7	2.1
C.P.	0.9	0.9	3.0
B.M. (biopsy I)	0.6	0.6	1.6
Experiment 2			
Control B	1.1	2.3	2.9
Control C	1.0	2.4	2.5
B.M. (biopsy II)	1.1	0.9	3.3
L.M.	0.8	0.9	4.1

The unsupplemented medium contained dialyzed fetal bovine serum and no methionine. To this medium was added methionine or homocystine in final concentrations of 0.1 mM and 0.05 mM, respectively. *Experiment 1.* Each flask received 3.75×10^5 cells. After 3 days incubation in unsupplemented medium, the media were changed to those indicated and the incubation continued for 5 days. The numbers in the table are the final cell counts relative to the number of cells in the innoculum, taken as 1.0. *Experiment 2.* Flasks were inoculated and the cells were incubated for 2 days in unsupplemented medium. At this time flasks were harvested to give initial cell counts for each cell line. These varied from 0.54 to 1.01×10^6. Cells in replicate flasks were then incubated in the indicated media for 6 days. Numbers in the table are the final cell counts relative to the initial cell count taken as 1.0. E.M. was homocystinuric due to lack of N^5-methyltetrahydrofolate-homocysteine methyltransferase activity secondary to deranged B_{12} metabolism. C.P., B.M. and L.M., are the subjects of the present communication.

deranged B_{12} metabolism have activities of this enzyme which are not decreased compared to the activities observed in extracts from control cells. Extracts from cells of C.P., B.M., and L.M. were each significantly low in activity of methylene-THF-reductase. The decreased activity was found in cells grown from two separate biopsies from B.M. which were tested after very different intervals of growth in tissue culture.

Table 3

N^5-Methyltetrahydrofolate-homocysteine Methyltransferase Activity in Extracts of Cells from Controls and from Patients with Homocystinuria

Assay conditions	Specific enzyme activity			
	Controls	Present patients C.P.	B.M.	E.M.*
Cells grown in basal medium				
Without methyl-B_{12}	4.2 *(2.9-7.3)*	4.1	3.5	0.03
With methyl-B_{12}	7.9 *(6.1-11.3)*	7.0	5.5	4.5
Cells grown in basal medium + hydroxy-B_{12} (1µg/ml)				
Without methyl-B_{12}	15.6 *(11.5-18.7)*	15.6	11.2	0.9
With methyl-B_{12}	18.8 *(14.3-23.4)*	16.7	12.4	5.9

*Homocystinuric patient with deranged B_{12} metabolism.

Cell free extracts were prepared and subjected to gel filtration and then assayed without added B_{12} or with an optimal concentration of methyl-B_{12} (5×10^{-6}M). Values for controls and for E.M. are taken from a previous publication (8). The values listed are mean specific enzyme activities expressed as mµmoles methionine formed per mg protein per hour with the range of observed values in parentheses.

Discussion:- We report here homocystinuria of a new type. For the patients in question, cystathionine synthase deficiency is ruled out by the lack of hypermethioninemia, their ability to convert the sulfur of a large oral dose of methionine to inorganic sulfate at a normal rate, and by the presence of adequate concentrations of cystathionine synthase activity in their cultured fibroblasts. Nutritional tests indicate cells from these patients are unable to form methionine from homocystine at a normal rate, yet direct assay of N^5-methyltetrahydrofolate-homocysteine methyltransferase activities showed no striking abnormality. All the results would be explicable were the patients in question deficient in their ability to reduce methylenetetrahydrofolate to N^5-methyltetrahydrofolate. N^5-Methyltetrahydrofolate is the methyl donor in one pathway for conversion of homocysteine to methionine, but is not known to participate in other enzymatic reactions, so it would not be unexpected if the major consequence of inability to form N^5-methyltetrahydro-

Table 4

Methylenetetrahydrofolate Reductase Activity in Fibroblasts from Control and Homocystinuric Subjects

	Specific activity* Conditions of assay		$\frac{-FAD}{+FAD} \times 100$
	without FAD	with FAD	
Non-homocystinuric controls			
Range (no. of subjects)	1.04-4.64 (8)	2.81-7.09 (12)	36.8-74.6 (10)
Mean ± 1 SD	2.94±1.10	5.04±1.36	61.1±12.6
Homocystinuric controls			
Cystathionine synthase deficient			
S.H.	3.34	4.22	78.8
B.H.	5.25	8.95	58.8
Deranged B_{12} metabolism			
E.M.	2.02	3.03	66.9
J.R.	3.85	5.88	65.5
M.R.	5.07	7.71	65.9
Present patients			
B.M. (biopsy I)	0.34+	0.62+	54.9
(biopsy II)	0.54+	0.82+	66.3
L.M.	0.61+	0.77+	78.4
C.P.	0.27+	1.39+	19.4++

*Expressed as mμmoles formaldehyde formed per mg protein per hour.
+P<0.02, by the one-tailed test (14), in comparison with the non-homocystinuric controls or with all controls.
++P<0.01 by the two-tailed test.

Fibroblasts were grown and extracted as described in the text. All assays were carried out under standard conditions. FAD (25 mμmoles) was added ar not, as indicated. The control subjects included 5 normal volunteers and 7 patients, or relatives of patients, with diseases not known to involve amino acid or folic acid metabolism. S.H. and B.H. are unrelated. J.R. and M.R. are brothers. B.M. and L.M. are sisters. In several instances (including B.M. and C.P.) values were obtained from two or more cell pellets from a single individual. These values generally agreed well and were averaged to give a single value for each subject. All specific activities listed were measured in fresh, unstored extracts. In a few cases the ratio of activities was determined by use of extracts stored at -45°. Such storage did not affect this ratio.

folate were deficient cellular methylation of homocysteine. The results reported in Table 4 directly demonstrate that cells from C.P., B.M., and L.M. have abnormally low activities of methylene-THF-reductase. In this work, methylene-THF-reductase activity has been measured by following the oxidation of N^5-methyltetrahydrofolate to formaldehyde and tetrahydrofolate in the presence of an artificial electron acceptor, menadione (15). This reverse reaction is thought to occur only slowly, if at all, under normal in vivo conditions (16). While the pattern of activities observed might have been different if the more physiological "forward" (i.e. reductive) reaction had been measured, the present results are highly indicative that a lack of methylene-THF-reductase activity explains the homocystinuria of C.P., B.M., and L.M.*

The results of assays of methylene-THF-reductase activity suggest that the specific genetic lesion in C.P. may be different from that in the M. sisters. Under the standard assay conditions, the reductase in extracts of C.P. cells is stimulated 5.2-fold by addition of FAD to the reaction medium, whereas the activities in extracts from cells of the M. sisters or control subjects are stimulated only 1.3-2.7-fold. Preliminary results indicate this finding in C.P. may be due to instability of his reductase activity when the assay is performed without added FAD. However, it remains possible that the decrease in methylene-THF-reductase activity in C.P. is related primarily to an abnormality of flavin metabolism rather than to an abnormality of the reductase apoenzyme.

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* Preliminary results from studies of a liver biopsy from B.M. generally support this hypothesis. Neither cystathionine synthase nor N^5-methyltetrahydrofolate-homocysteine methyltransferase activity was sufficiently low to account for the homocystinuria of B.M. Activity of betaine-homocysteine methyltransferase (E.C.2.1.1.5), an enzyme catalyzing an alternate route for homocysteine methylation, was normal. Further studies of this tissue have been deferred pending completion of investigations of the fibroblasts so that the very limited amount of liver available can be utilized with maximum efficiency.

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