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DEFICIENCIES OF CYSTATHIONASE AND HOMOSERINE DEHYDRATASE ACTIVITIES IN CYSTATHIONINURIA

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Congenital cystathioninuria is a familial disease first reported in 1958.[1] Too few patients with this condition have been studied to allow definition of the clinical syndrome, but it may include various congenital defects and mental retardation or aberration.[1-3] Harris *et al.*,[1] and subsequently Brenton *et al.*,[4] demonstrated abnormally high concentrations of cystathionine in tissue extracts from a cystathioninuric patient. Cystathionine was detected also in the serum of a patient reported by Frimpter *et al.*[2] The presence of increased tissue cystathionine content led Harris *et al.* to advance the hypothesis that this syndrome resulted from a deficiency of the enzyme cystathionase,[5] which normally cleaves cystathionine (reaction 1):[1]

$$\text{Cystathionine} + H_2O \rightarrow \text{cysteine} + \alpha\text{-ketobutyrate} + NH_3. \quad (1)$$

To test this hypothesis directly we assayed hepatic cystathionase, using the methods developed in the course of our studies of human cystathionine synthase (E.C.4.2.1.21)[5] deficiency.[6] Cystathionase activity was markedly reduced in the extract prepared from the liver of a patient with cystathioninuria.

Crystalline rat liver cystathionase also catalyzes homoserine deamination[7] [L-homoserine hydro-lyase(deaminating) E.C.4.2.1.15] (reaction 2):

$$\text{Homoserine} \rightarrow \alpha\text{-ketobutyrate} + NH_3. \quad (2)$$

Patients lacking cystathionase offer a unique opportunity to determine whether the human enzyme, like that of the rat, has this second activity. We developed a microassay for homoserine dehydratase and found that the extract shown to be deficient in cystathionase activity was also markedly deficient in homoserine dehydratase activity. This suggests that cystathionase and homoserine dehydratase are catalyzed by a single enzyme in man and indicates that cystathioninuria is associated with deficiences of at least two enzymatic activities.

Experimental Procedures and Methods.—Assays were performed on extracts of human liver because we failed to detect cystathionase activity in human skin, erythrocytes, or leucocytes.

Although extracts of ileum and rectal mucosa possessed some activity, it was insufficient for adequate assays.

The patient (L.C.) was a mentally retarded 13-year-old white male who has been shown to have cystathioninuria.[3] Dr. Stanely Berlow referred him for the present study. Twenty-four mg of liver tissue were obtained by percutaneous biopsy while the patient was under light nitrous oxide anesthesia. He was receiving chronic treatment with pyridoxine hydrochloride, 100 mg; the phenothiazine tranquilizer, thioridazine, 50 mg; and desiccated thyroid, 100 mg daily. Liver specimens obtained by laparotomy or percutaneous biopsy from patients with various diseases constituted the control group. Some specimens had been frozen for varying periods of time prior to assay. We have shown previously[6] that cystathionase activity is relatively stable under these conditions. Methods for extraction and assay of methionine-activating enzyme [ATP: L-methionine-*S*-adenosyltransferase (E.C.2.5.1.6)], cystathionine synthase, and cystathionase have been published previously.[6] In principle, the cystathionase assay is based upon conversion of cystathionine-2-C^{14} (label in 4-carbon moiety) to α-ketobutyrate-2-C^{14}, and separation of these compounds by ion exchange chromatography.

L-Homoserine-U-C^{14} was prepared from L-methionine-U-C^{14} (New England Nuclear Corp.) by a modification of the method of Flavin *et al.*[8] α-Keto-γ-hydroxybutyrate-C^{14} was prepared with amino acid oxidase. Details of these preparations will be published.[9]

Microassay for homoserine dehydratase: Homoserine dehydratase catalyzes conversion of homoserine to α-ketobutyric acid. The assay depends on separation of the product α-ketobutyrate-C^{14} by use of Dowex 50 (H^+) which retains homoserine. During preliminary experiments, we observed evidence of a second enzyme-dependent reaction of homoserine which produced α-keto-γ-hydroxybutyric acid. This product accounted for 20–40% of the keto compound formed during incubation of homoserine with crude extracts of control human liver.[9] Therefore, when such extracts were assayed for homoserine dehydratase, it was necessary routinely to separate the products of the two reactions by paper chromatography.

The reaction mixture was similar to that of Matsuo and Greenberg[7] and contained the following, in micromoles: *Tris*-HCl, pH 8.4, 25; α-ketobutyric acid, 0.9; pyridoxal phosphate, 0.2; EDTA, 2; L-homoserine-U-C^{14}, 0.52 containing 2×10^5 cpm. The final volume, after the addition of the tissue extract, was 0.25 ml and incubation was at 37° for 60 min. The reaction mixture was diluted with 2.75 ml of iced water and was passed through a column of Dowex 50-X4 (H^+), 200–400 mesh, 0.9×4.0 cm. The column was washed with 5 ml of water and the total eluate was collected. The radioactivity of an aliquot was determined and the remainder of the eluate was used to prepare the 2,4-dinitrophenylhydrazone derivatives.[6] These derivatives were chromatographed on Whatman #1 paper by use of *n*-butanol saturated with 3% ammonium hydroxide. After development, the chromatogram was cut into 1-cm strips and the radioactivity of each strip was determined. The radioactivity separated into four areas: two corresponding to the 2,4-dinitrophenylhydrazone of α-keto-γ-hydroxybutyric acid, and two to the 2,4-dinitrophenylhydrazone of α-ketobutyric acid. The fraction of the total radioactivity corresponding to the 2,4-dinitrophenylhydrazone of α-ketobutyrate was determined. This fraction multiplied by the total radioactivity in the column eluate gave a value for α-ketobutyrate production. Recoveries of the 2,4-dinitrophenylhydrazones of the products were comparable during the extraction procedure.

Results.—Cystathionase activity in the liver of a patient with cystathioninuria: The activities of methionine-activating enzyme and cystathionine synthase in the extract of liver from the patient with cystathioninuria were within the control range (Table 1). In contrast, the value for cystathionase activity was 5 per cent of the mean control value and 13 per cent of the lowest control value. Since the control group contained some individuals with hepatic disease, it is possible that the normal mean value for hepatic cystathionase is different from 14 mμmoles/mg protein/30 min. When added pyridoxal phosphate was omitted from the standard reaction mixture, both control extract[6] and the extract from L.C. showed approximately a 25 per cent decrease in cystathionase activity.

The extract of the patient's liver did not inhibit cystathionase activity of control liver. Most of the radioactive material remaining in the incubation mixture after

TABLE 1

ACTIVITIES OF CYSTATHIONASE AND CONTROL ENZYMES IN THE EXTRACT OF LIVER FROM A PATIENT WITH CYSTATHIONINURIA

	Methionine-activating enzyme (mμmoles/mg protein/60 min)	Cystathionine synthase (mμmoles/mg protein/135 min)	Cystathionase (mμmoles/mg protein/30 min)
Control group			
Number of patients	17	15	12
Mean result	7.1	258	14
Range of values	4.3–14.5	133–610	6.0–24
Cystathioninuria	4.6	142	0.75

Assays were performed under standard conditions. The values for controls are taken from our previous publications.[10-13]

TABLE 2

FACTORS INFLUENCING HOMOSERINE DEHYDRATASE ACTIVITY IN EXTRACTS OF HUMAN LIVER

Conditions	α-Ketobutyrate formed (mμmoles)
Complete system	10.6
Omit pyridoxal phosphate	4.4
Increase pyridoxal phosphate to 1.0 μmole	10.3
Omit α-ketobutyric acid	7.2
Omit EDTA	5.4
Add 2-mercaptoethanol, 0.0125 μmole	10.4
Increase L-homoserine to 2.0 μmoles	24.6

The complete system is described in the text. Extracts of human liver containing 0.56 mg protein were used. Incubation was for 60 min. These tissues had been stored frozen prior to extraction.

the assay of the patient's liver extract was proved to be cystathionine. A calculation based on the data of Brenton *et al.*[4] showed that the amount of cystathionine present in the extract of the patient's liver was probably no more than 10–15 per cent of that routinely added to the reaction mixture. These observations indicate that the cause of the low cystathionase activity in the liver extract from L.C. was not (*a*) the presence of a dissociable inhibitor, (*b*) consumption of cystathionine by a side reaction, or (*c*) a lowering of the specific activity of the substrate by endogenous cystathionine.

The small apparent activity in the cystathioninuric liver extract was indeed cystathionase, since the radioactive reaction product was characterized as α-ketobutyrate by incubation of 0.24 mg of liver protein with five times the standard cystathionine-C^{14} concentration for 60 min. The product's 2,4-dinitrophenylhydrazone derivative was prepared, isolated, and hydrogenated[6] and the resultant amino acid was shown to migrate with α-aminobutyrate during paper chromatography using 2-propanol, 70; formic acid (88%), 10; water, 20 and *tert*-butanol, 70; formic acid (88%), 15; water, 15.

Homoserine dehydratase: Homoserine dehydratase activity in extracts of human liver is maximal at pH 8.4. Enzymatic activity was reduced significantly in the absence of pyridoxal phosphate or EDTA (Table 2). Increase in the standard concentration of pyridoxal phosphate did not increase the reaction rate; 2-mercaptoethanol had no effect. Omission of the carrier α-ketobutyrate reduced activity about 30 per cent. In the presence of carrier, α-ketobutyrate-C^{14} recovery was greater than 90 per cent. The standard concentration of homoserine is insufficient to attain maximal velocity. Product formation was linearly proportional

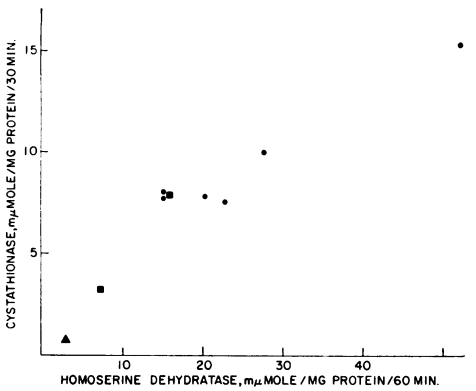

FIG. 1.—Homoserine dehydratase and cystathionase activities in liver extracts prepared from control patients and a patient with cystathioninuria. The assays were performed under the standard conditions. Homoserine dehydratase results are given as mμmoles/mg protein/60 min and cystathionase results as mμmoles/mg protein/30 min. The cystathioninuric patient is represented by ▲. The values obtained from extracts of two biopsy specimens from patient C.B. are designated as ■. The first biopsy (lower values) was performed before therapy was instituted, and the second biopsy (higher values) was obtained after 1 month of treatment (see text).

to amount of added liver extract from 0.14 to 0.56 mg protein and to the duration of incubation up to 90 min.

Homoserine dehydratase activity in extracts of control specimens of human liver ranged from 15.0 to 72.2 mμmoles/mg protein/60 min. A lower value, 7.1 mμmoles/mg protein/60 min, was found in a specimen obtained from C.B., an alcoholic with a markedly fatty liver. At the time of biopsy, physical examination revealed evidence of severe vitamin deficiency. A repeat liver biopsy after 1 month of improved nutritional intake with vitamin supplementation showed considerable improvement histologically, and repeat enzyme studies demonstrated an increase in cystathionase from 3.2 to 7.9 mμmoles/mg protein/30 min and in homoserine dehydratase to 15.8 mμmoles/mg protein/60 min. Hope reported cystathioninuria in rats depleted of pyridoxal phosphate and attributed it to deficient cystathionase activity.[14] Possibly, at the time of the initial biopsy C.B. was deficient in vitamin B_6. We have excluded the values obtained for this specimen from the control series.

Values for homoserine dehydratase are proportional to simultaneously determined cystathionase values (Fig. 1). This is compatible with the hypothesis that the two enzymes are identical in human liver.

Homoserine dehydratase activity of the liver extract from the cystathioninuric patient was 2.9 mμmoles/mg protein/60 min, 8 per cent of the mean control value and 19 per cent of the lowest acceptable control value (Table 3). The ratio of activity of cystathionase to homoserine dehydratase in the extract of the liver of the cystathioninuric patient was similar to the ratio found in the control group.

During these experiments, we also found that the liver extract of the patient with cystathioninuria formed α-keto-γ-hydroxybutyrate at a rate of 31 mμmoles/mg protein/hr, a value within the present control range of 9.2–48.1 mμmoles/mg protein/hr.[9]

Discussion.—These studies demonstrate that congenital cystathioninuria in man is secondary to a specific defect in the activity of cystathionase. This enzymatic defect explains the increased tissue[1–4] and serum[2] levels of cystathionine found in such patients and the presence of cystathionine in the urine. Evidence from the study of patients with cystathionine synthase deficiency suggests that cystathionine is an obligatory intermediate in the major pathway for conversion of the sulfur of methionine to inorganic sulfate.[15] Therefore, a deficiency of cystathionase

TABLE 3

ACTIVITY OF HOMOSERINE DEHYDRATASE IN THE EXTRACT OF LIVER FROM A PATIENT WITH CYSTATHIONINURIA

	Homoserine dehydratase (mμmoles/mg protein/60 min)	α-Ketobutyrate (% total keto acid)
Controls		
M.E.	15.0	60
C.B.	15.8	89
P.C.	22.8	64
C.F.	27.4	76
M.B.	49.6	68
L.H.	72.2	60
Cystathioninuria		
L.C.	2.9	8

Assays were performed under the standard conditions described in the text. The column eluate was treated with 2,4-dinitrophenylhydrazine and the resultant derivatives were extracted and chromatographed. The percentage of the radioactivity applied to the paper which was inseparable from the 2,4-dinitrophenylhydrazone of α-ketobutyric acid is given as α-ketobutyrate, per cent total keto acid. α-Keto-γ-hydroxybutyric acid 2,4-dinitrophenylhydrazone accounted for the remainder of the radioactivity.

might lead to subnormal concentrations of cyst(e)ine in the plasma and to decreased excretion of inorganic sulfate. Both these findings have been reported.[2] Neither homocyst(e)ine nor methionine accumulate[2] nor would they be expected to, since the enzymatic synthesis of cystathionine is essentially irreversible.

The possibility that the hepatic cystathionase activity of L.C. was low due to an environmental factor rather than to a genetic lesion was considered. At the time of biopsy, L.C. was receiving desiccated thyroid. Doses of L-thyroxine which cause severe thyroid toxicity decrease cystathionase activity in rat liver.[16-18] However, the amount of thyroid L.C. was receiving (100 mg/day) is likely to have resulted only in suppression of production of an equivalent amount of endogenous hormone. Clinically, L.C. appeared euthyroid and his serum protein-bound iodine was normal (5.1 μg per 100 ml). Furthermore, rats given five times the equivalent of the above dose of thyroid do not have a decreased specific activity of hepatic cystathionase.[19] At the time of biopsy, L.C. was receiving thioridazine. Control experiments have shown that administration of equivalent or higher doses of this drug to rats does not change hepatic cystathionase.[19] The activity of cystathionase in rat liver is increased by a high casein diet[20] and by high doses of methionine.[20] L.C. was taking a normal diet when studied. Therefore, the evidence suggests it is most unlikely that environmental factors caused the deficiency of hepatic cystathionase activity in L.C.

Our studies indicate that a second enzyme activity, homoserine dehydratase, is deficient in cystathioninuria. This finding, and the observation that the ratio of cystathionase to homoserine dehydratase in human liver is relatively constant, suggests that a single protein catalyzes both reactions in man (as in the rat[7]). In the mammal, homoserine is formed from methionine[21] and may accumulate in the liver after methionine administration.[22] Deamination of homoserine to α-ketobutyrate by homoserine dehydratase is the primary reaction in the catabolism of homoserine.[23] The metabolic effects of a deficiency of this enzymatic activity are unknown, but cystathioninuric patients offer a means to investigate this question.

Evidence has been advanced to indicate that rat liver cystathionase catalyzes also the desulfuration of cystine[24] and cysteine.[25-27] Sufficient material was not available to enable us to investigate whether the patient deficient in cystathionase

also has defects in these enzymatic activities. However, this possibility should be considered in future studies of cystathioninuria.

Pyridoxal phosphate is a cofactor for rat liver cystathionase.[28] Frimpter[2] observed that administration of pyridoxine to a cystathioninuric patient decreased cystathionine excretion and increased sulfate excretion. During our present studies, we assayed the extract of liver from the cystathioninuric patient both without added pyridoxal phosphate and with pyridoxal phosphate added at a concentration sufficient to achieve a maximal rate with the enzyme from control liver specimens. The stimulation due to added pyridoxal phosphate was about 33 per cent, the same as that noted in extracts from control patients. Since the patient with cystathioninuria was being treated with pyridoxine at the time of the liver biopsy, our studies show that in spite of both *in vitro* and *in vivo* administration of vitamin B_6, cystathionase activity in this patient could not be restored beyond a level far below normal. It is possible, although unlikely, that the extract from our patient would have been further stimulated upon addition of pyridoxal phosphate to a concentration beyond that required to attain a maximal velocity with extracts from control patients. This point could not be tested due to lack of tissue. Nonenzymatic degradation of cystathionine in the presence of pyridoxal phosphate has been reported.[29] It is possible that the reported[2] decrease in cystathionine excretion after pyridoxal administration reflects this reaction rather than an increase in cystathionase activity.

After our experiments with the extract from L.C. had been completed,[30] Frimpter reported cystathionase assays on extracts of liver tissue from two cystathioninuric patients.[31] He found both these extracts to possess a greatly decreased activity of cystathionase. *In vitro* addition of pyridoxal phosphate, at an unspecified concentration, stimulated the cystathionase activities of these extracts, in one case to a value within the control range. This difference from our findings may reflect true differences between the patients studied or may be due merely to differences in the assay conditions. Frimpter used a 3-hr incubation. Matsuo and Greenberg reported that rat liver cystathionase activity is not linearly proportional to time during such a period,[7] and we have confirmed their observation using extracts from human liver. Frimpter determined the rate of reaction by measuring the accumulation of cystine. No data was presented concerning the recovery of this compound under the conditions of his experiments. On the basis of his findings, Frimpter suggested the important possibility that there is a "structural alteration of the apoenzyme, resulting in failure to combine normally with the coenzyme."[31]

Cystathionase deficiency is the second instance in which an enzymatic lesion has been defined in the transsulfuration pathway in man. Previously, it has been shown that patients with the syndrome of homocystinuria are defective in cystathionine synthase.[11] The availability of mutants lacking discrete steps in this pathway makes it possible to clarify a number of biochemical problems. For example, the finding of normal cystathionase activity in patients with cystathionine synthase deficiency suggests that the level of cystathionase is not affected by a substantial decrease in the load of cystathionine presented for metabolism.[6] In cystathioninuria there is a marked accumulation of cystathionine in the tissues.[1, 4] The level of cystathionine synthase activity in such tissue offers an index of the degree of control of accumulation of this enzyme by its metabolic product. The value of the specific

activity of hepatic cystathionine synthase in L.C. was toward the lower extreme of the control range, suggesting that this enzyme is not markedly repressed by an abnormally high concentration of cystathionine. Further studies are needed to determine if a moderate degree of repression exists.

Summary.—(1) Cystathionase activity was markedly reduced in an extract from the liver of a patient with idiopathic cystathioninuria. Activities of methionine activating enzyme and cystathionine synthase were within the normal range in this extract.

(2) A sensitive, radiochemical assay for homoserine dehydratase has been developed. By use of this assay, the liver extract from the cystathioninuric patient was shown to be deficient also in homoserine dehydratase.

(3) The ratio of homoserine dehydratase/cystathionase activities in liver extracts is relatively constant, a finding which is consistent with the hypothesis that the two activities are properties of a single enzyme.

(4) The cystathionase activity of the extract from the liver of the patient with cystathioninuria was not stimulated more than were controls by the *in vitro* addition of pyridoxal phosphate. The role of pyridoxal phosphate in this syndrome is discussed.

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*ALTERATIONS OF A MATERNALLY INHERITED MITOCHONDRIAL STRUCTURAL PROTEIN IN RESPIRATORY-DEFICIENT STRAINS OF NEUROSPORA**

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One approach to an understanding of the nature of extrachromosomal heredity, subcellular morphology, and nucleocytoplasmic relationships is through the investigation of the genetics of enzymes and proteins of mitochondria. Genetic information for the primary structure of some mitochondrial-localized enzymes resides in nuclear DNA;[1, 2] no direct demonstration of a gene-protein relationship involving mitochondrial DNA has been previously reported.

Concepts developed in several areas of independent investigation led to the working hypothesis of this study. First, although concepts of extrachromosomal heredity have been evolving for many years, only one class of extrachromosomal mutations affecting mitochondria are well known; these are the respiratory-deficient mutants of yeast and *Neurospora.*[3] Extensive analyses of such mutants have not produced results interpretable in terms of either the classical one gene–one enzyme hypothesis or related corollaries regarding pathways of intermediary metabolism. Rather, several of the components of the electron transport chain are found to occur in either excess or deficit in those mutants. In addition, Green and associates[4] have proposed that polymacromolecular assemblies called elementary particles, with a fixed set of proteins in invariant proportions, are associated with the mitochondrial inner membrane. Among the principal components of those particles are the enzymes of the electron transport system and the mitochondrial structural protein. The importance of the structural protein is emphasized not only by its quantitative preponderance in the mitochondria, but also by its critical role in the organization and assembly of the enzymes of the particles. Moreover, recent investigations indicate that mitochrondria from all organisms contain DNA.[5] That this DNA may play a hereditary role is indicated by observations that *Neurospora* mitochondria replicate by division of pre-existing mitochondria[6] and exhibit genetic continuity after interhyphal transplantation.[7]

In this communication, the results of experiments stimulated by the foregoing concepts support the hypothesis that two different extrachromosomal mutations of (presumably) mitochondrial DNA lead to alterations of the primary structure