# THREONINE DEHYDRATASE ACTIVITY IN HUMANS LACKING CYSTATHIONINE SYNTHASE

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Evidence has been presented that cystathionine synthase (L-serine hydro-lyase (adding L-homocysteine) E.C.4.2.1.21) of rat liver catalyzes two additional reactions: serine deamination (L-serine hydro-lyase (deaminating) E.C.4.2.1.13) (Selim and Greenberg, 1959) and threonine deamination (L-threonine hydro-lyase (deaminating) E.C.4.2.1.16) (Goldstein, et al., 1962). The homogeneous protein possesses all three activities (Greenberg and Nagabhushanam, 1964). In sheep liver there are clearly two enzymes which convert serine to pyruvate and ammonia: a serine dehydratase which does not deaminate threonine (Sayre and Greenberg, 1956) and a threonine dehydratase which attacks serine at a slower rate (Nishimura and Greenberg, 1961).

A recently discovered human syndrome (Carson and Neill, 1962; Field, et al., 1962; Gerritsen, et al., 1962; Carson, et al., 1963; Gerritsen and Waisman, 1964) is due to a lack of activity of cystathionine synthase (Mudd, et al., 1964; Finkelstein, et al., 1964). The known biochemical features of the disease include homocystinuria and abnormally high concentrations of methionine and homocystine in the plasma. We have investigated threonine metabolism in such patients to determine if they have a block in the deamination of this amino acid, and to obtain additional evidence concerning the relationship of cystathionine synthase to threonine dehydratase. In this paper we report results of assays of threonine dehydratase activity in homogenates of liver specimens from four patients lacking cystathionine synthase.

Experimental: Cystathionine synthase was assayed as previously (Mudd, et al., 1964). Tissue was extracted by grinding with sand in potassium phosphate buffer, pH 6.9, 0.03 M. The extract was centrifuged for 7 minutes at 5000 x g and the supernatant fluid used for assay. Proteins were determined by the Lowry method (Layne, 1957). The values for crude extracts were multiplied by 0.8 to correct for dialyzable chromogenic material since the small volumes of the critical extracts available precluded dialysis.

Assay of threonine dehydratase-- We have developed a new highly sensitive method, based on conversion of L-threonine-U-$C^{14}$ to α-ketobutyrate-U-$C^{14}$ and

similar in principle to that of Hayaishi (Hayaishi, et al., 1963). The standard incubation solution contained in a total volume of 0.4 ml (in μmoles): Tris buffer, pH 8.3, 60; EDTA, 1; pyridoxal phosphate, 0.4; L-threonine-U-$C^{14}$, 1, containing 79 x $10^3$ c.p.m.; α-ketobutyrate, 1.4; and enzyme extract. Incubation was at 37°. To end the reaction, 3.3 ml of ice-cold water was added and aliquots of 1.0, 1.0, and 1.5 ml of the resulting solution were successively and rapidly passed over a column of Dowex 50-X4, 200-400 ($H^+$) (0.9 x 2.9 cm). The first two aliquots were sufficient to flush out the bed volume of the column so that the eluate attained a constant concentration of radioactivity. The final 1.5 ml of eluate was collected and the radioactivity determined. When the radioactive product of the enzymatic reaction was to be identified, the specific activity of the L-threonine was increased 5-fold. The total diluted reaction mixture was applied to the column which was then washed with 2 ml of water. The combined eluate and washings were used for product identification. Protein was removed and 2,4-dinitrophenylhydrazones were prepared and purified essentially as described (Cavallini and Frontali, 1954). The 2,4-dinitrophenylhydrazones were studied by paper chromatography and hydrogenation. Hydrogenation was carried out in aqueous solution with a platinum oxide catalyst for 14-18 hours at atmospheric pressure and room temperature. The amino acids resulting from the hydrogenation were identified by paper chromatography. Chromatography was performed on Whatman No. 1 paper by the descending technique. The following solvents were used (a) n-butanol equilibrated with ammonium hydroxide (3%) (Cavallini, et al., 1949); (b) 1-propanol, ammonium hydroxide (28%), water (60:30:10) (Meister and Abendschein, 1956); (c) 1-butanol, potassium phosphate buffer, pH 7.0, 0.02 M, ethanol (5:1:4) (sometimes used with paper dipped in the buffer, 0.1 M and dried); (d) 2-propanol, formic acid (88%), water (70:10:20); (e) tert-butanol, formic acid, water (70:15:15) and (f) 2-propanol, water, ammonium hydroxide (58%) (85:15:1.3). Radioactivity was located by counting segments of the developed chromatogram and was correlated with the location of authentic compounds.

Materials: Commercial L-threonine-U-$C^{14}$ was purified by adsorption on a column of Dowex-50 ($H^+$) (as described above) and elution with 7.7 ml of 0.44 N HCl. Authentic 2,4-dinitrophenylhydrazone of α-keto-β-hydroxybutyrate was a gift from Dr. Alton Meister. Commercial α-ketobutyrate was recrystallized as the sodium salt (Matsuo and Greenberg, 1958). Platinum oxide was purchased (Engelhard, Newark, New Jersey). Reference α-ketobutyrate-$C^{14}$ was prepared by the action of partially purified rat liver cystathionine synthase (Selim and Greenberg, 1959) upon threonine-U-$C^{14}$ or by the action of liver cystathionase on cystathionine-2-$C^{14}$ (label in 4-carbon moiety) (Mudd, et al., 1965).

Results: To validate the assay for threonine dehydratase, this activity was determined in a partially purified preparation of rat liver cystathionine synthase (30-50% ammonium sulfate fraction) (Selim and Greenberg, 1959). The activity found by the determination of radioactivity agreed well with that determined colorimetrically (Sayre and Greenberg, 1956) for the keto acid content of the eluate from the column. Carrier keto acid was not used in this experiment. With the assay described, one can measure activity in the crude extract from less than 5 mg of human liver. Formation of product is proportional to enzyme concentration (Table I, expt. 1a and 1b).

Table I. Effect of Enzyme Concentration and Time

| Experiment | Extract | Protein, mg | Time of incubation, minutes | α-Ketobutyrate formed | |
|---|---|---|---|---|---|
| | | | | mμm | mμm/mg protein/60' |
| 1a | L.H. | 0.56 | 30 | 5.8 | 20.8 |
| | | 0.89 | 30 | 7.5 | 16.9 |
| 1b | L.H. | 0.13 | 90 | 2.1 | 10.5 |
| | | 0.56 | 90 | 11.6 | 13.9 |
| | | 0.89 | 90 | 17.5 | 13.1 |
| 2 | R.S. | 0.68 | 60 | 10.8 | 15.9 |
| | | 0.68 | 120 | 20.4 | 15.0 |

The data indicate the experimental variation encountered and show that with some extracts synthesis of α-ketobutyrate was almost linear with time for 120 minutes (expt. 2) whereas with other extracts there was some decrease in rate with time (expt. 1). The activity was unaffected by the addition of $NH_4Cl$, 4 x $10^{-3}$ M (Holzer, et al., 1964), ADP, 3 x $10^{-4}$ M (Hayaishi, et al., 1963), AMP, 5 x $10^{-4}$ M or reduced glutathionine 5 x $10^{-4}$ M (Sayre and Greenberg, 1956). The concentration of threonine-U-$C^{14}$ used in the assay is not sufficient to attain maximal velocity. The 2,4-dinitrophenylhydrazone of the radioactive reaction product separated from that of pyruvate upon paper chromatography (solvent a) but was inseparable from this derivative of α-ketobutyrate (solvents (a), (b) and (c)). After hydrogenation its radioactivity migrated chiefly with authentic α-aminobutyrate upon chromatography with solvents (c), (d), (e) and (f). During chromatography with solvents (d) and (e) the hydrogenated product gave rise to two additional spots which migrated at rates of 1.11 and 1.25 relative to α-aminobutyrate (solvent d) and which contained approximately 10 and 20-30% of the total radioactivity respectively. The 2,4-dinitrophenylhydrazone of reference α-ketobutyrate-$C^{14}$ was shown to yield the same three radioactive areas as a result of hydrogena-

tion. α-Ketobutyrate is not metabolized significantly under the standard assay contitions. Reference α-ketobutyrate-$C^{14}$ was recovered quantatively after incubation with crude human enzyme. The possibility that the apparent threonine dehydratase activity might be due to transamination from threonine was excluded since the 2,4-dinitrophenylhydrazone of the radioactive reaction product separated chromatographically from that of α-keto-β-hydroxybutyrate and yielded no threonine upon hydrogenation.

Our experience with human liver threonine dehydratase is summarized in Table II. The control values do not establish a normal range because the

Table II. Threonine Dehydratase and Cystathionine Synthase Activities

| Patients | Age, Years, Race and Sex* | Clinical features | Acquisition of tissue | Threonine dehydratase** | Cystathionine synthase† |
|---|---|---|---|---|---|
| D.D. | 6/WM | Cystic fibrosis | Post-mortem | 7.0 | 241 |
| I.H. | 60/NF | Adenocarcinoma of uterus | Laparotomy | 5.0 | 334†† |
| C.B. | 32/WM | Fever unknown etiology | Laparotomy | 5.0 | 184†† |
| P.C. | 47/NF | Inactive adrenal adenoma; treated hypothyroidism; cholelithiasis | Laparotomy | 5.9 | 215 |
| H.B. | 72/WM | Carcinoma of stomach; no hepatic metastases | Laparotomy | 4.5 | 302 |
| M.B. | 53/NF | Benign gastric ulcer; minimal portal fibrosis | Laparotomy | 12.7 | 219†† |
| L.H. | 62/NM | Carcinoma of rectum | Laparotomy | 17.3 | 518 |
| K.K. | 56/WF | Aortic aneurysm; myocardial infarction | Post-mortem | 3.9 | 62 |
| L.S. | 39/WF | Carcinoma of cervix | Laparotomy | 5.2 | 149 |
| J.H. | 25/WF | Homocystinuria | Menghini needle | 28.8 | 5 |
| R.S. | 1/M | Homocystinuria | Post-mortem | 15.5 | 2 |
| L.B. | 24/WM | Homocystinuria | Menghini needle | 25.7 | 6 |
| K.B. | 21/WF | Homocystinuria | Menghini needle | 6.3 | 3 |

*N, Negro; W, white; F, female; M, male.
**α-Ketobutyrate formation, mμmole/mg protein/60 minutes.
†Cystathionine formation, mμmole/mg protein/135 minutes.
††Value published previously (Finkelstein, et al., 1964).

Some of the tissues were stored at -60° before extraction. Some of the extracts were stored at -60° before assay. The specimen from R.S. had been thawed at least once prior to extraction. The values for threonine dehydratase are the averages of all determinations, normalized to 60 minutes assuming linearity with time.

specimens were obtained from patients with various diseases. Changes may also have occurred post-mortem and during storage of tissue or extracts. The extracts of liver specimens from the four patients with the clinical syndrome associated with homocystinuria had by direct assay no more than 3% of the mean control concentration of cystathionine synthase. In contrast, in none of these cases was hepatic threonine dehydratase activity reduced to the same extent and indeed there is no evidence of any decrease at all. The radioactive product of the reaction with extracts J.H. and R.S. was identified as α-ketobutyrate by paper chromatography of the 2,4-dinitrophenylhydrazone derivatives and of the products of hydrogenation of these derivatives. A portion of the extract from the liver of R.S. was passed through Sephadex to remove low molecular weight material. By a colorimetric assay this extract was shown to form keto acid during incubation with threonine. In the absence of threonine little or no keto acid was formed. With serine rather than threonine, keto acid formation was of borderline significance. Similar experiments could not be carried out with the extracts from the other cystathionine synthase deficient patients since too little material was available. Attempts to develop a more sensitive radioactive assay for serine dehydratase were unsatisfactory, presumably because of side reactions and further metabolism of the product in the crude extracts.

Discussion: We demonstrate in this paper that four patients with proven lack of cystathionine synthase do not have a similar deficiency of hepatic threonine dehydratase. Although these two enzymatic activities are thought to be catalyzed by a single protein in rat liver, the situation for human liver has not been investigated. We have been unable to demonstrate a strict parallelism between the threonine dehydratase and cystathionine synthase activities of crude extracts of non-homocystinuric human liver. However no conclusion is warranted until the human enzyme(s) have been purified. The present findings are consistent with two possibilities: (a) Threonine dehydratase and cystathionine synthase are different proteins in the human liver. (b) These two reactions are catalyzed by a single protein which in the patients with the syndrome of homocystinuria has been modified so as to impair only cystathionine synthase activity. Further investigation of threonine and serine metabolism in these patients is indicated.

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