Homocystinuria

An Observation on the Inheritance of Cystathionine Synthase Deficiency

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Homocystinuria is an inborn error of the metabolism of methionine, which is associated with a deficiency of cystathionine synthase activity in liver and brain. It is inherited as an autosomal recessive trait; characteristic clinical stigmata usually include mental retardation and ectopia lentis (Carson, Dent, Field, and Gaull, 1965; Schimke, McKusick, Huang, and Pollack, 1965; Mudd, Finkelstein, Irreverre, and Laster, 1964; Brenton, Cusworth, and Gaull, 1965a, b).

Incipient glaucoma secondary to complete dislocation of the optic lens necessitated operative removal of the lens in a patient. It seemed of genetic interest, therefore, to examine this tissue for evidence of cystathionine synthase activity. The lens is of special interest since it is derived embryologically from superficial ectoderm which is induced by the optic vesicle of the neural ectoderm.

Methods

The lens from this 7-year-old patient with homocystinuria (Case 5 of Carson *et al.*, 1965) was removed at operation intact, and placed immediately in ice-cold physiological saline for transport to the laboratory. It weighed 123 mg., was crystal clear, but was spherical rather than typically lens-shaped. It was incubated with shaking in air for four hours at 36.7° C. in a flask containing 10 ml. Krebs-Ringer phosphate solution with 1 mg. per ml. glucose plus 9 µc of ³⁵S L-methionine,† containing 0.2 µmoles of L-methionine in 0.2 ml. H₂O (specific activity = 45 mc/mM). Paper chromatography of 100 µl. radioactive methionine solution revealed no other ninhydrin-positive material (isobutanol: 90% formic acid: H₂O, 75:15:10, v/v). As control, a

normal lens was similarly incubated on another day. This lens was obtained at operation from a 50-year-old man who underwent enucleation because of a tumour of the retina. This lens weighed 228 mg. and was clear. In contrast to the lens from the patient with homocystinuria, it was typically lens-shaped. At the end of the incubation period the lenses, still intact, were homogenized in ice-cold 6% perchloric acid and separated into a protein fraction and an acid-soluble fraction. After this, operations were carried out in parallel.

Protein Fraction. The protein fraction was washed four times with ice-cold perchloric acid followed by two washings of acetone, two washings of chloroform, and methanol 2:1, and finally two washings of ether. The dried protein was hydrolysed in 6 ml. 6N hydrochloric acid in sealed tubes overnight, and the acid was removed by evaporation under reduced pressure. The hydrolysate was then oxidized for two hours with performic acid which was similarly removed. This was transferred to a column of Dowex 1 CO₃⁻ (0.9 × 15 cm. 200-400 mesh, X 10). After washing with 50 ml. water, 100 ml. 0.1 N acetic acid were put over the column, converting it to the acetate form and giving a neutral eluate containing methionine sulphone. This was followed by 100 ml. 0.1 N hydrochloric acid, giving an intermediate acetate eluate, and, finally, an acidic eluate containing cysteic acid was collected at the stage when excess free HCl emerged from the column. The eluates containing methionine sulphone and cysteic acid were evaporated to dryness, made up to volume, and an aliquot transferred to Whatman No. 1 filter paper. The neutral eluate was developed in two directions: first, butanol-acetic acid-water (4:1:5, v/v) for 40 hours, followed by acetone-water (4:1, v/v) for 12 hours. The acidic eluate was developed only in the first direction. The chromatograms were sprayed with 0.5% ninhydrin in methanol (pH 5.0) and methionine sulphone and cysteic acid spots eluted with 60% ethanol. The optical density of the solution was measured at 570 mµ and the amounts of amino acids calculated from the optical densities of the known amounts of these amino acids run under the same conditions. The radioactivity was determined

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† Obtained from the Radiochemical Centre, Amersham.

the same solution by liquid scintillation counting in Bray's solution (Bray, 1960) at 60% efficiency.

Acid-soluble Fraction. After neutralization with KOH and removal of the potassium perchlorate precipitate, the acid-soluble fraction was transferred quantitatively to Dowex 2 Cl^- (0.9×15 cm., 200-400 mesh, X 10). The column was then washed with 50 ml. water, and the wash was evaporated to near dryness and transferred quantitatively to a similar Dowex 1 CO_3^- . This column was washed with 50 ml. water. The column was then eluted with 100 ml. 0.1 N acetic acid, giving a neutral eluate containing the free sulphur amino acids. Oxidation by performic acid was performed after, rather than before, this fraction was collected. The neutral fraction then underwent two-dimensional paper chromatography as described except that 10 μg . each of the appropriate amino acids were added at the origin as carrier. The column was further eluted with 100 ml. 0.1 N HCl in the same manner as the protein hydrolysate. Since the oxidation was performed after collection of the neutral fraction, cyst(e)ine appeared in the neutral fraction and then was converted to cysteic acid. Therefore, it did not appear in the acid fraction as it did in the protein hydrolysate. The acetate and acid fractions contained insignificant amounts of radioactivity and were not further studied.

Results

Labelled methionine and cyst(e)ine were incorporated into the proteins in both lenses. Under these conditions, the specific radioactivities of the cysteine and methionine in the proteins of the lens of the patient with homocystinuria were the same as those of the patient with a normal lens (Table I). This suggested that the conversion of methionine to cysteine in the lens of the patient with homocystinuria was unimpaired. Direct confirmation of this was sought in the acid-soluble fraction.

A pilot experiment with a cataractous lens had shown that the amounts of the sulphur-containing free amino acids in the acid-soluble neutral fraction were too small to be detected chemically by these methods. Thus, because of the rarity and small size of the specimens, it was deemed prudent to add carrier amino acids to the paper chromatograms and to determine total radioactivity of the various compounds rather than specific activities. This seemed justified in view of the very small pool sizes involved. Since the unique opportunity to do the experiments arose suddenly and at a time when our assessment of the recoveries of radioactivity from the various fractions by this new method was incomplete, a tally of all the counts into the various acid-soluble sub-fractions was done, to be certain that the results in the two lenses were comparable. The results

TABLE I
INCORPORATION OF ^{35}S FROM METHIONINE INTO LENS PROTEIN

	Control	Homocystinuric
Methionine	6605	6278
Cyst(e)ine	232	406

The data give specific activity: counts per minute per μmole

TABLE II
DISTRIBUTION OF RADIOACTIVITY INTO VARIOUS LENS ACID-SOLUBLE SUB-FRACTIONS

Sub-fraction	Control	Homocystinuric
Dowex 1 effluent	90,825 (18%)	18,809 (16%)
Dowex 1 neutral eluate	393,730 (79%)	97,022 (81%)
Dowex 1 intermediate eluate	5,249 (1%)	1,921 (2%)
Dowex 1 acidic eluate	6,937 (1%)	2,668 (1%)
Total	497,741	120,420

The data give counts per minute and percentage distribution of radioactivity in the acid-soluble fraction.

(Table II) demonstrated that the distribution of radioactivity in the various sub-fractions was similar. The major portion of the radioactivity appeared in the neutral eluate which contained the neutral sulphur-containing amino acids. The percentage of the acid-soluble fraction contained in the neutral eluate was approximately 80% in each lens. The other sub-fraction containing a significant portion of the radioactivity was the effluent which contained about 17% of the total radioactivity in each lens. Evidence will be presented elsewhere showing that a considerable amount of the radioactivity appearing in the Dowex CO_3^- effluent represents S-adenosylmethionine ('active methionine').

An estimate of the conversion of the immediate precursor homocysteine into its product cystathionine and the next product cysteine was determined by comparing the total counts in each of the products with the total counts in the precursor. This comparison showed that, under the conditions of the experiment, the ability to convert homocysteine to cystathionine and cysteine in the acid-soluble fraction was the same in the lens from the patient with homocystinuria as in the control lens (Table III). Similar comparisons with regard to methionine and taurine could not be calculated because these two are the fastest running constituents in the second direction, and due to an error they ran to the

edge of the paper and were partially lost. This is one of the many factors that contributed to losses of ^{35}S after paper chromatography (cf. Tables II and III).

TABLE III
INCORPORATION OF ^{35}S FROM METHIONINE INTO
LENS ACID-SOLUBLE FRACTION

	Homo- cysteine	Cysta- thionine	Cysteine	Cysta- thionine/ Homo- cysteine	Cysteine/ Homo- cysteine
Control	26,769	1725	1800	0.06	0.07
Homocystinuric	7738	719	675	0.09	0.09

The data give counts per minute.

Discussion

Cystathionine synthase activity had been shown previously to be deficient in the liver and brain of patients with homocystinuria (Mudd *et al.*, 1964; Brenton *et al.*, 1965a, b). Under the conditions of these experiments, the activity of cystathionine synthase was found to be present to an equal extent in the lens from a patient with homocystinuria as compared to a control lens. These results suggest that either a structural gene controlling the development of cystathionine synthase, or a regulator gene controlling its synthesis or inactivation, is different in the lens from the corresponding genes in liver and brain.

Well-studied instances of multiplicity of gene control of an enzyme activity are few. In the hereditary myopathy due to deficiency of muscle phosphorylase, the liver phosphorylase is normal, whereas in the hereditary deficiency of liver phosphorylase, the muscle phosphorylase is normal (Rowland, Fahn, and Schotland, 1963). These two enzymes show a number of clear differences in properties of inactivation and reactivation so that this would seem to be an instance of distinct enzymes produced under the influence of different genes. Another apparent instance of the multiplicity of gene control is the inheritance of glucose-6-phosphate dehydrogenase deficiency in which the deficiency exhibited by the erythrocytes may or may not be accompanied by a similar deficiency in the leucocytes; here as well, present evidence favours molecular heterogeneity (Marks, 1964). Since the number of enzymes examined for such tissue differences is small, it is not possible to say whether this multiplicity of gene control is general or exceptional. The comparative properties of cysta-

thionine synthase in various tissues should be explored, especially with regard to possible differences in isoenzyme subunits.

It is of embryological interest that the lens, which differentiates later than the brain but by induction of the superficial ectoderm by the optic vesicle of the developing brain, should exhibit such a difference in enzyme phenotype.

Comparison of the radioactivity (counts/min.) in acid-soluble fraction of the lens from the homocystinuric patient with that of the control lens indicated that the latter incorporated three to four times as much radioactivity as the former. This is not entirely unexpected in that the control lens was larger and therefore had more actively metabolizing cells. Furthermore, it is known that the intracellular pool of methionine in the erythrocytes of these patients is larger, reflecting the raised concentration of methionine in the plasma (unpublished results of Dr. G. Winston Barber, Wills Eye Hospital, Philadelphia, Pennsylvania). Since the lens is known to transport amino acids actively from the surrounding aqueous humour (Kinsey and Reddick, 1963), it seems likely that the smaller total amount of radioactivity entering the lens is a reflection of a relatively large pool of unlabelled methionine in the lens. Although the plasma methionine level at the time of operation was not determined, the patient (G. McC.; Brenton *et al.*, 1965a, b) was known to run raised methionine levels.

This potentially larger pool of unlabelled methionine may also account for the similarity of the incorporation of ^{35}S into methionine and cyst(e)ine of protein. In the lens from the child, compared to the older adult, a larger protein incorporation would have been anticipated (personal communication, Dr. Abraham Spector, Institute of Ophthalmology, New York). In any case, this would be consistent with the early cataracts known to occur in these patients (Carson *et al.*, 1965).

Certain tacit assumptions deserve comment. First, it is assumed that the trans-sulphuration pathway remains active in the ageing lens, since it might be argued that had a control lens from a younger patient been used, the cystathionine synthase activity might have been greatly reduced. No firm information is available on this point, but the similar distribution of radioactivity in the various sub-fractions (Table II) tends to support the idea that the pathway is active. Secondly, this experiment assumed that no alternative pathways for the conversion of methionine to cystathionine and cysteine existed in the lens. This seems likely on present knowledge but cannot be ruled out unequivocally. Finally, steady-state kinetics were

assumed. Since this was a tracer experiment, the question of saturation of the enzyme by substrate was not relevant. In any case it seems likely that substrate was not rate-limiting, since the incubation medium was sampled periodically during the incubations, and it was determined that the net utilization of substrate was less than the error of direct scintillation counting of the media. Had substrate been rate-limiting in this system, it would seem likely that a four-hour incubation would have largely depleted it, since it is known that methionine is actively taken up by lens (Mandel, Dardenne, and Lessinger, 1957) and has the highest affinity for the active transport sites of neutral amino acids in lens (Kinsey and Reddy, 1963). Simply adding a greater concentration of unlabelled methionine to the incubation medium would not be definitive, since with surviving lens, the uptake of tracer amino acid decreases asymptotically with the total concentration of amino acids present in the surrounding media.

Summary

Cystathionine synthase activity, previously shown to be absent from liver and brain of patients with homocystinuria, was found to be present in the optic lens of such a patient. The biological implications are discussed briefly.

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