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Type I Tyrosinemia: Lack of Immunologically Detectable Fumarylacetoacetase Enzyme Protein in Tissues and Cell Extracts

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ABSTRACT. Type I hereditary tyrosinemia is characterized by the almost complete absence of fumarylacetoacetase in tissues and cells from patients. To investigate the nature of the enzyme deficiency, extracts of tissues (liver and kidney) and cells (lymphocytes and fibroblasts) were immunochemically screened for the presence of fumarylacetoacetase enzyme protein. The antibodies used were raised in rabbits against fumarylacetoacetase purified from beef liver. These antibodies cross-reacted strongly with the human enzyme. No cross-reacting material was found in extracts from liver ($n = 4$) and kidney ($n = 1$) from patients. Extracts from lymphocytes and cultured skin fibroblasts from patients were investigated as well. However, no cross-reacting material was found in extracts of these cells. (*Pediatr Res* 22: 394–398, 1987)

Abbreviations

PAGE, polyacrylamide gel electrophoresis
SDS, sodium dodecylsulfate

Type I hereditary tyrosinemia is an inborn error of tyrosine metabolism. The acute form presents itself shortly after birth as a severe liver and kidney disease and is characterized by failure to thrive, jaundice, hepatosplenomegaly, and tubular dysfunction resulting in a Fanconi-like syndrome (1). The concentrations of tyrosine and methionine in blood are elevated; in the urine the excretion of *p*-OH-phenylpyruvate, *p*-OH-phenyllactate, succinylacetone, succinylacetoacetate, and δ-aminolevulinic acid is increased (2, 3). In the chronic form the symptoms develop more gradually at a later age and are less severe. From the pattern of abnormal metabolites it was postulated that the primary defect is a deficiency of fumarylacetoacetase (EC 3.7.1.2), the enzyme which catalyzes the hydrolysis of fumarylacetoacetate into fumaric acid and acetoacetic acid (2). Recently Berger *et al.* (4, 5) and Kvittingen *et al.* (6) showed that the activity of this enzyme in the liver from patients with type I hereditary tyrosinemia was less than 1% of control activity.

Although only liver and kidney contain the complete set of enzymes in order to degrade tyrosine fully, fumarylacetoacetase is present in most tissues and various celltypes: the enzyme defect can also be shown in muscle, brain, lymphocytes, fibroblasts (7). Besides the acute and chronic phenotype of tyrosinemia a mutation at the fumarylacetoacetase locus has been described which results in a very low activity of the enzyme in lymphocytes but without clinical symptoms (8). Recently it has been shown that liver extracts from patients with the acute form of tyrosinemia lack immunoreactive protein, while in liver from patients with the chronic form variable amounts of enzyme protein could be detected (9). Although these findings may indicate genetic heterogeneity in this disease both the acute and chronic form can occur within the same family (1). A different degree of liver involvement (intoxication) could affect the expression of the fumarylacetoacetase gene to a variable extent. Classification of different (acute and chronic) forms of type I hereditary tyrosinemia has been mainly based on the clinical manifestations (1); only recently it has been suggested that the severity of the disease may be correlated with the remaining enzyme activity and amount of enzyme protein in the liver (9). This study was undertaken to circumvent the possible involvement of the liver by analysing extracts from lymphocytes and cultured fibroblasts by immunochemical methods.

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METHODS

Patients. All patients reported herein had the clinical and biochemical findings of the acute form of tyrosinemia. Patient 1, L. L., was the third child of unrelated healthy parents. Their first child died at the age of 3 months from liver failure. The second child was normal. The patient (female) was born after an uneventful pregnancy. At 3 wk of life she showed jaundice, abdominal distension, diarrhea, glucosuria, hyperammonemia, and elevated concentrations of bilirubin and α-fetoprotein in serum. In the urine the excretion of succinylacetone, *p*-OH-phenylpyruvate, and *p*-OH-phenyllactate was greatly increased. Despite treatment the child died at the age of 1 yr. The clinical and biochemical findings from patient 2, K. V. (10), patient 3, K. H. (3), patient 4. T. de V. (11), and patient 5, R. F. (4) has been published. Deficiency of liver fumarylacetoacetase (<1% of control values) was documented in all cases.

Assay of fumarylacetoacetase. The activity of fumarylacetoacetase in tissue and cell extracts was measured as described earlier (12, 13). Human skin fibroblasts were cultured in HAM's-F10 (GIBCO, Paisley, Scotland) medium, harvested with 0.1% trypsin, and washed with phosphate buffered saline. Leucocytes were prepared according to the method of Wyss *et al.* (14). Lymphocytes were isolated from blood with the Ficoll-Paque (Pharmacia, Uppsala, Sweden) method according to the manufacturer's instructions. Cells and tissues were homogenized in 0.025 mM K-phosphate buffer, pH 7.2 and centrifuged. Protein was determined by the method of Lowry *et al.* (15) using bovine serum albumine as a standard.

Electrophoresis. Proteins were separated by discontinuous PAGE (3% stacking gel, 7.5% separating gel) or by discontinuous SDS-PAGE (3% stacking gel, 10.0% separating gel). Gels were stained with either Coomassie brilliant blue or silver.

Immunological detection of fumarylacetoacetase protein. Fu-

marylacetoacetase protein in tissue and cell extracts was characterized by Western blot analyses. Samples were prepared by boiling for 5 min in 10 mM dithiothreitol and 1% SDS and subjected to SDS-PAGE. After electrophoresis the proteins were transferred to nitrocellulose sheets by electroelution. Gels were stained with silver to ensure quantitative transfer of proteins. After transfer of the proteins the nitrocellulose sheets were incubated overnight with fumarylacetoacetase antibody (dilution 1:5000). The antigen-antibody complex was visualized either by treating the sheet with ^{125}I-protein A followed by exposure to X-omat XRP-5 or XAR-5 films (Eastman Kodak Co., Rochester, NY) or by treating the sheets with a second antibody (antirabbit IgG, Sigma Co., St. Louis, MD) conjugated with alkaline phosphatase.

Preparation of antiserum. Purified fumarylacetoacetase was emulsified with complete Freund's adjuvant (1:1). Three rabbits were injected subcutaneously with 0.3 mg of protein per animal. The injections were repeated with 0.3 mg of protein per animal with incomplete Freund's adjuvent (1:1) after 28, 37, and 50 days. Blood was drawn before every injection and 9 days after the last injection the animals were bled, and the serum was stored at −70° C until analysis. The serum was tested for the presence of antibodies against beef liver fumarylacetoacetase by immunoblotting.

Purification of fumarylacetoacetase. The method used for the isolation of fumarylacetoacetase from beef liver was based on that described by Mahuran *et al.* (16). Unless otherwise specified, all the steps in the purification of the enzyme was carried out at 4° C. Centrifugation was carried out at 15,000 × *g*.

Step 1. Crude Extract. A fresh beef liver was cut into small cubes and connective tissue was removed. A 200-g portion was homogenized for 2 min in a blender with 400 ml 0.85% KCl solution. The homogenate was filtered through cotton gauze and stirred gently for 2 h.

Step 2. Ethanol Precipitation (50/70%). Five hundred ml of 100% ethanol precooled to −20° C were slowly added to 500 ml solution of the previous step to obtain a final concentration of 50% ethanol and the temperature of the mixture was kept below 0° C. After stirring for 1 h the precipitate was removed by centrifugation at −20° C. The concentration of ethanol was brought to 70% by slowly adding 550 ml of 100% ethanol to 800 ml of the supernatant over a period of 10 min. The solution was allowed to stand for 1 h and was then centrifuged at −10° C. The crude enzyme paste was stored overnight at −20° C.

Step 3. Ammonium Sulfate Precipitation. The paste (14 g) was stirred for 30 min in 95 ml of 0.025 M K-phosphate buffer at pH 7.2. The supernatant was recovered after centrifugation of the suspension. Solid ammonium sulfate (8 g) was slowly added to 20 ml of the supernatant fluid. After standing for 1 h the solution was centrifuged. The precipitated protein was dissolved in a minimal volume of 0.025 M K-phosphate pH 7.2 and stored overnight at 4° C.

Step 4. Anion Exchange Chromatography. The solution of the previous step was clarified by centrifugation. The supernatant was passed through Sephadex G-25 Coarse (Pharmacia) column (1.6 × 58 cm), equilibrated with 0.02 M Tris/HCl buffer pH 8.8. Fractions (5 ml) were collected at a flow speed of 3 ml/min. The fractions having enzyme activity (10–15) were combined and applied to a DEAE-Trisacryl (LKB, Bromma, Sweden) column (2.6 × 34 cm), equilibrated with 0.02 M Tris/HCl pH 8.8. After application of the enzyme solution to the column 230 ml of 0.02 M Tris/HCl pH 8.8 was passed through the column at a flow rate of 2.5 ml/min. After 23 fractions of 10 ml the enzyme was eluted from the column with 0.08 M Tris/HCl buffer pH 8.3. The fractions having enzyme activity (39–48) were combined and sufficient ammonium sulfate was added to give a final concentration of 45% (w/v). A white precipitate was centrifuged off, dissolved in a minimal volume of 0.025 M K-phosphate pH 7.2, and stored overnight at 4° C.

Step 5. Chromatofocusing. The solution of the previous step was clarified by centrifugation and desalted as in the previous step except that the Sephadex G-25 column was equilibrated with 0.025 M Tris/HCl pH 8.3. The fractions having enzyme activity were combined and applied to a column containing Poly Buffer Exchange (Pharmacia) (1.6 × 20 cm) and on top of a layer of 2 cm Sephadex G-25 Coarse. Equilibration and elution were carried out according to the manufacturer's instructions procedure. The pH interval was 8–6.8. The start buffer was 0.025 M Tris/HCl pH 8.3. The elution was performed with 400 ml of Polybuffer 96-HCl pH 6.8 (1:10 diluted). The flow rate was 1.0 ml/min and fractions of 5 ml were collected. The enzyme eluted around pH 7.7. The fractions containing enzyme activity (44–53) were combined and solid ammonium sulfate was added to obtain a final concentration of 50% (w/v). The solution was allowed to stand for 1 h. The precipitated protein was collected by centrifugation and washed three times with saturated ammonium sulfate (in 0.025 M K-phosphate pH 7.2) to remove Polybuffer. The resulting precipitate was dissolved in 1 ml of 0.025 M K-phosphate pH 7.2 and stored at −70° C.

RESULTS

In Table 1 summarizes the data of fumarylacetoacetase activities, measured in various tissues, and cell types from normal individuals and patients with type I hereditary tryrosinemia. In normal individuals liver and kidney contain the highest activity of fumarylacetoacetase, in patients the activity is below the detection limit (less than 1% of control activity).

In fibroblasts, amniotic fluid cells, leucocytes, and lymphocytes derived from patients, the activity of fumarylacetoacetase is higher (7–15%). In normal chorionic villus biopsy specimens the activity is comparable to that of amniotic fluid cells. However, in cultured chorionic cells the activity is about five times higher.

A typical purification of fumarylacetoacetase from beef liver is summarized in Table 2. Usually enzyme preparations were obtained which were purified 400–500× with respect to crude

Table 1. *Activity of fumarylacetoacetase in tissues and cells from normal human and patients with type I hereditary tyrosinemia**

Tissue/cell type	Normal	Range	Type I hereditary tyrosinemia	Range
Liver	20.45 ± 6.29 (11)	11.10–32.00	ND (14)	
Kidney	5.35 ± 2.14 (12)	2.10–10.50	ND (6)	
Skeletal muscle	0.89 ± 0.24 (3)	0.70–1.16	ND (1)	
Placenta	0.84 (2)	0.38–1.29	0.14 (1)	0.03–0.26
Leucocytes	1.79 ± 0.30 (8)	1.45–2.36	0.09 ± 0.09 (4)	0.02–0.21
Lymphocytes	1.54 ± 0.38 (8)	1.03–2.09	0.24 ± 0.18 (3)	0.05–0.40
Fibroblasts	1.28 ± 0.66 (20)	0.34–3.22	0.20 ± 0.12 (4)	0.02–0.30
Amniotic fluid cells	1.00 ± 0.16 (4)	0.85–1.22		
Chorionic villi	0.97 ± 0.32 (14)	0.38–1.40		
Cultured chorionic villi cells	6.29 ± 2.09 (8)	4.39–9.75		

* Activity in mU/mg (±SD); number of individuals in parentheses.

† Not detectable.

Table 2. *Purification of fumarylacetoacetase from beef liver*

Step	Total activity (U)	Yield (%)	Specific activity (mU/mg protein)	Purification
1. Crude extract	1662	100	12	1
2. Ethanol precipitation	524	32	231	19
3. Ammonium sulphate precipitation	448	27	689	57
4. DEAE-Trisacryl	304	18	1665	139
5. Chromatofocusing	71	4	6076	506

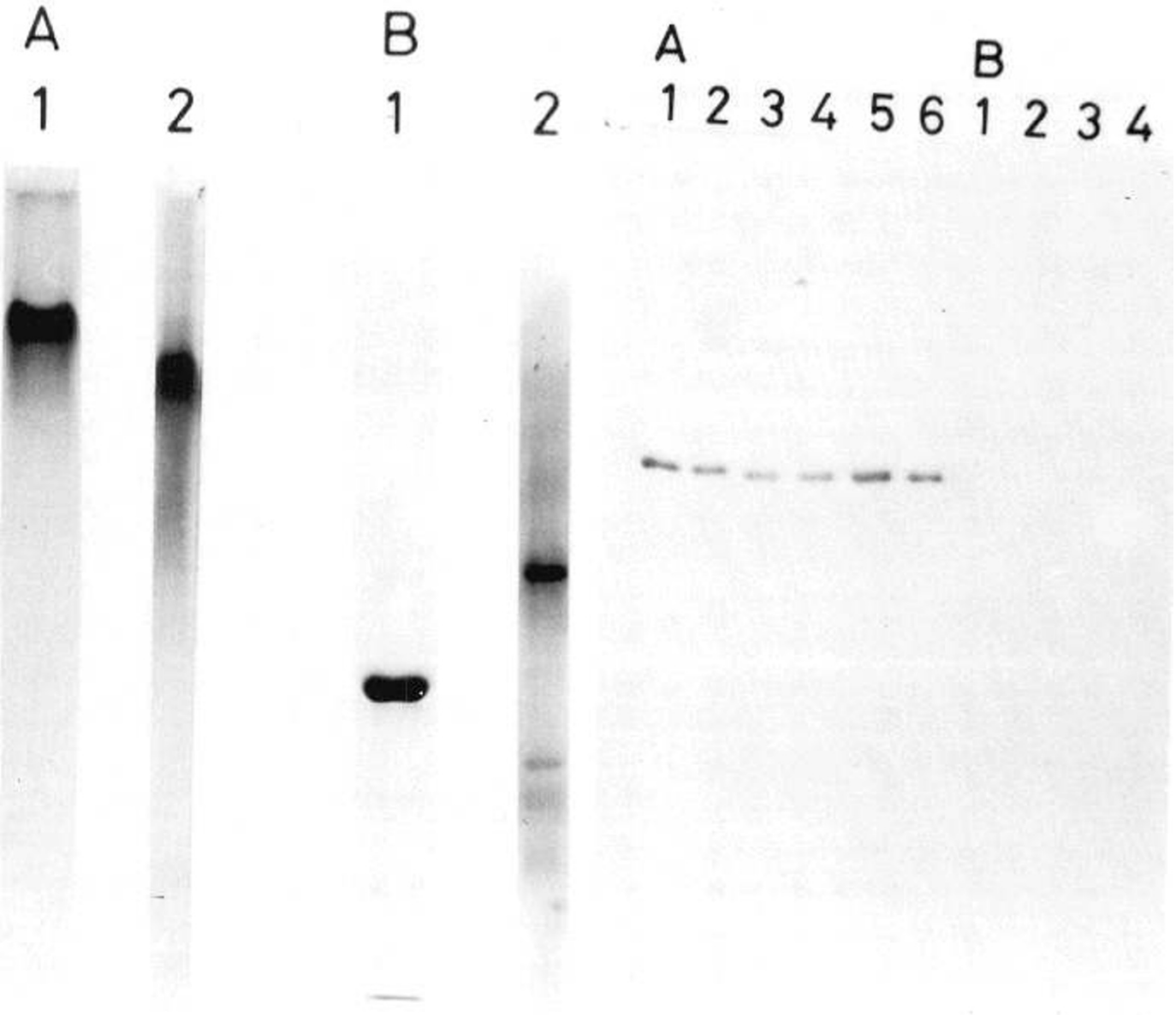

Fig. 1. PAGE of purified beef liver fumarylacetoacetase. *A*, PAGE at pH 8.3. *Lane 1*: 36 μg, Coomassie brilliant blue stain. *Lane 2*: 4 μg, silver stain. *B*, SDS-PAGE. *Lane 1*: 4.0 μg, Coomassie brilliant blue stain. *Lane 2*: 1 μg, silver stain.

Fig. 2. Immunoblot analysis of normal human liver and in liver from type I hereditary tyrosinemia patients. *A*, normal liver. *Lane 1*: purified beef liver fumarylacetoacetase (0.02 μg protein). *Lane 2*: beef liver extract (4 μg total protein). *Lanes 3–6*: extracts from normal liver (8 μg total protein). *B*, liver from type I hereditary tyrosinemia patients. *Lane 1*, patient LL; *lane 2*, KV; *lane 3*, KH; *lane 4*, TdV (10 μg total protein).

homogenates. The purity of the final enzyme preparation was determined by PAGE under native as well as under reducing and denaturating conditions. Furthermore different stains were used. From Figure 1*A* it can be seen that after native PAGE single bands were observed; subjecting the enzyme to SDS-PAGE contaminating polypeptides of higher and lower molecular weight were observed (Fig. 1*B*). In order to analyze tissue and cell extracts from patients by immunoblotting techniques antibodies against beef liver fumarylacetoacetase were raised in rabbits. The antibodies obtained cross-react with the liver enzyme; in Figure 2*A* it is shown that immunoblots from both beef liver and normal human liver reveal a single band at a molecular weight of 43,000.

Immunoblots from liver extracts from type I hereditary tyrosinemia patients do not show cross-reacting material (Fig. 2*B*). Single protein bands were also observed in kidney, lymphocytes and fibroblasts (Fig. 3*A*, *lanes 1–3*, respectively). No cross-reacting material was also observed in extracts from kidney, lymphocytes and fibroblasts from patients (Fig. 3*B*, *lanes 1–3*, respectively). The very weak lines at the position of fumarylacetoacetase were also observed when immunoblots were treated with preimmune serum instead of specific antibody.

DISCUSSION

The analysis of tissue and cell extracts by immunoblotting techniques has been a powerful tool in the molecular-biological characterization of inborn errors of metabolism. Using this technique Tanguay *et al.* (9) reported the lack of immunoreactive protein in liver extracts from type I hereditary tyrosinemia patients. In the study reported herein their results could be confirmed (Fig. 2). However, the severe hepatointoxication typical for this disease might have caused the specific loss of biologically unstable proteins. An example of this phenomenon is the finding that the activity of *p*-OH-phenylpyruvate dioxygenase, the enzyme that catalyses the first step in the degradation of tyrosine, is depressed not only in the liver from type I hereditary tyrosinemia patients but more generally in severe liver diseases from different etiology (5, 17).

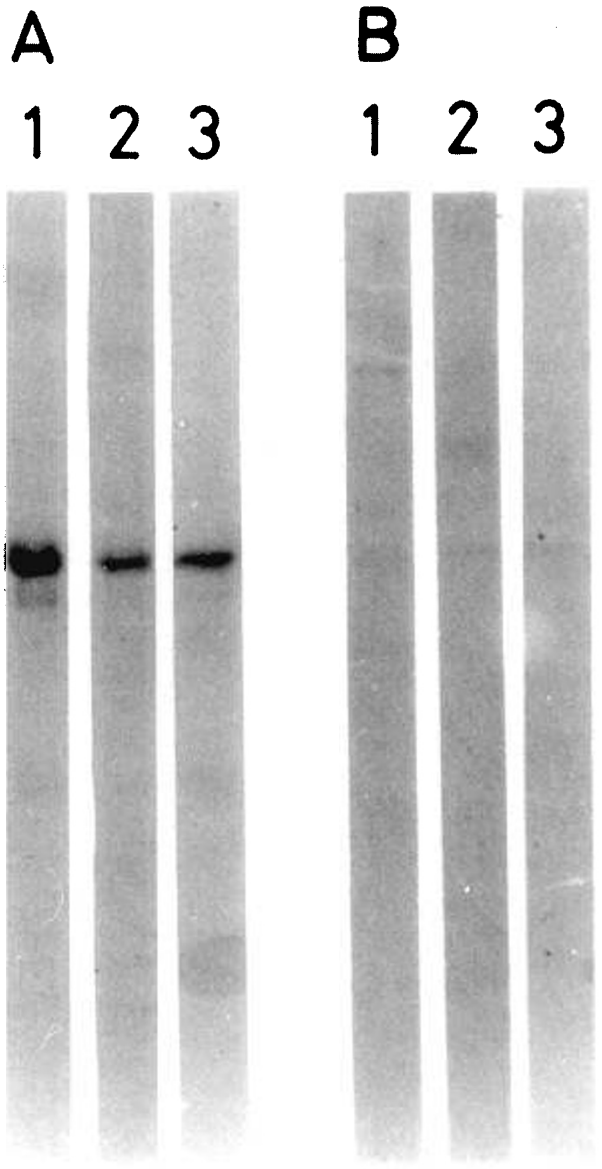

Fig. 3. Immunoblot analysis of kidney, lymphocytes, and fibroblasts from normal humans and type I hereditary tyrosinemia patients. *A*, normal human. *Lane 1*: kidney (10 μg total protein). *Lane 2*: lymphocytes (15 μg of total protein). *Lane 3*: fibroblasts (25 μg of total protein). *B*, type I hereditary tyrosinemia. *Lane 1*: kidney KV (20 μg of total protein). *Lane 2*: lymphocytes RF (15 μg of total protein). *Lane 3*: fibroblasts KV (50 μg).

A decisive answer to the question whether the absence of immunoreactive protein in liver from patients is caused by the intoxication of the liver could be obtained from the analysis of cells and tissues not affected by the disease, *e.g.* peripheral blood cells and cultured skin fibroblasts. As shown in Figure 3, no protein cross-reactive with antibody against fumarylacetoacetase was found in cells obtained from patients with the acute form of tyrosinemia as compared to cells from normal individuals. The absence of cross-reacting material might be caused by the impairment of protein synthesis. A disturbance of gene expression to such an extent implies a rather drastic mutation in the structural part of the gene or a mutation affecting the regulatory sites such as promotors or ribosome binding sites. An alternative explanation for the absence of immunoreactive material might be the synthesis of a very unstable protein. A similar case has been reported for adenosine deaminase deficiency. No immunologically detectable adenosine deaminase was found in tissues and cells from patients. However, its presence was demonstrated in a cell-free translation system supplemented with poly A-mRNA from patients (18).

Fumarylacetoacetase purified from beef liver has been shown to exist of two identical monomers of a molecular weight approximately 43,000 (16). From Figure 2*A* it can be concluded that the human liver enzyme contains monomers of about the same molecular weight. If the enzyme preparations used in this study (Table 2) are considered to be highly purified, the abundance of fumarylacetoacetase protein can be calculated to be approximately 0.2% of total liver protein. This is in agreement with the finding of Nicole *et al.* (19) who reported a relative abundance of rat liver fumarylacetoacetase mRNA of about 0.14%. These findings indicate that the abundance of fumarylacetoacetase in liver is appreciable.

In conclusion, the impairment of fumarylacetoacetase activity in the acute form of tyrosinemia can be accounted for by a complete reduction in the amount of enzyme protein. Studies of enzyme synthesis in cultured fibroblasts by pulse labeling, by pulse-chase experiments, *in vitro* translation, and the use of specific cDNA probes should further clarify the nature of the mutation and provide a basis for the classification of different forms of type I hereditary tyrosinemia.

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