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Deficient Fumarylacetoacetate Fumarylhydrolase Activity in Lymphocytes and Fibroblasts from Patients with Hereditary Tyrosinemia

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Summary

Fumarylacetoacetate fumarylhydrolase (E.C.3.7.1.2.), a liver enzyme involved in tyrosine degradation, is shown to be present in many human tissues and cells including lymphocytes, fibroblasts, and cultured amniotic fluid cells. The enzyme activity in lymphocytes from six patients with hereditary tyrosinemia (hepatorenal type) and fibroblasts from three patients, was found to be less than 10% of the activity in control subjects. In lymphocytes and fibroblasts from the parents (n = 16) of the patients the enzyme values were compatible with a heterozygote genotype. The lymphocyte enzyme pattern of the control subjects (n = 97), is complicated, and indicates possible enzyme variants.

Abbreviations

FAH, fumarylacetoacetate fumarylhydrolase
3-HBDH, 3-hydroxybutyrate dehydrogenase
MEM, minimum essential medium (modified)

Hereditary tyrosinemia is a metabolic disorder of autosomal recessive inheritance. The disorder is characterized by hepatocellular damage, renal tubular dysfunction, hypophosphatemic rickets, and abnormal tyrosine metabolism. In 1977, Lindbald *et al.* (10) postulated a deficiency of FAH (E.C.3.7.1.2.), the last enzyme of the tyrosine degradation pathway, to be the primary enzyme defect of the disorder. FAH has been shown subsequently, by several independent groups, to be deficient in liver tissue from patients with hereditary tyrosinemia (1, 4, 6, 8).

The aim of the present investigation was to search for the occurrence of FAH activity in human cells and tissues other than liver, particularly in easily accessible cells such as lymphocytes, fibroblasts, and cultured amniotic fluid cells. Because the enzyme activity was demonstrated in many tissues including the latter cells, the question remained if quantitative determination of FAH activity in lymphocytes and fibroblasts from patients with hereditary tyrosinemia could be used for confirmation of the diagnosis. The present data show that the enzyme activity is deficient in lymphocytes and fibroblasts from the patients with hereditary tyrosinemia (n = 9) and that the parents of the patients (n = 16) have enzyme activities compatible with a heterozygote genotype. A preliminary report of the deficiency of the enzyme activity in lymphocytes from patients with hereditary tyrosinemia has been given previously (9).

MATERIALS AND METHODS

Chemicals. Homogentisic acid and NADH were purchased from Sigma, St. Louis, MO, USA; 3-HBDH from Boehringer, Ingelheim, F.R.G.; metaphosphoric acid and 2,4,6-trimethylpyridine (collodine) from Merck, Darmstadt, F.R.G.; sodium metrizoate/Ficoll from Nyegaard & Co, Oslo, Norway; dextran blue and Sephadex G-25 fine grade from Pharmacia, Uppsala, Sweden; MEM (modified) from Flow lab, Irvine, Scotland and Medium 199 from the National Institute of Public Health, Oslo, Norway. Fumarylacetoacetate was synthesized from homogentisic acid as described by Edwards and Knox (3): 10 ml of collidine buffer 0.1 mole/liter, pH 7.2, 1 ml of ascorbic acid 0.05 mole/liter (neutralized), 5 ml of homogentisate oxidase (prepared from rat liver), 14 ml of distilled water, and 0.3 ml of homogentisic acid 18.2 mmole/liter were incubated in a 250 ml Erlenmeyer bottle for 25 min at 37°C with 100% oxygen saturation. The reaction was stopped on ice by addition of 3.3 ml of metaphosphoric acid 20%. The precipitated protein was removed by filtration through a filter paper (Schleicher & Schull, Wiess band). After 5 days at +4°C the synthetic product was stored at −70°C in small aliquots. Before enzyme analysis, the substrate was neutralized with sodium hydroxide, 4 mole/liter. Under strictly standardized conditions of synthesis, the concentration of fumarylacetoacetate in each batch of neutralized substrate varied between 0.50–0.56 mmole/liter, as calculated from the molar extinction coefficient for fumarylacetoacetate, 13.5×10^3 (3).

Enzyme source. Human tissue from liver, kidney, lung, pancreas, spleen, cardiac muscle and skeletal muscle were obtained from postmortem sections. Liver tissue was, in addition, obtained as biopsies taken at laparotomies. The tissue was homogenized in 4–10 volumes of sucrose 0.25 mole/liter, in a Potter-Elvehjem homogenizer. Fibroblasts were derived from skin. The cells were maintained as monolayers in Falcon tissue culture flasks (75 cm^2) in MEM, supplemented with 17% foetal calf serum. Penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin (2 U/ml), and L-glutamine (1.5 μmole/ml) were added. Fibroblasts from two flasks were used for the enzyme analysis.

Amniotic fluid cells, obtained by amniocentesis at 16–17 wk of gestation, were cultured in Falcon tissue culture flasks (25 cm^2) in medium 199, supplemented with 20% foetal calf serum and 5% bovine embryo extract. Penicillin (100 U/ml) and streptomycin (100 μg/ml) were added. For lymphocyte isolation, 5–10 ml of blood was drawn on evacuated tubes containing heparin (143 units) as anticoagulant. The lymphocytes were isolated within 2 h after the blood was drawn on sodium metrizoate/Ficoll as stated by the manufacturer. A slight contamination of erythrocytes in the lymphocyte preparation was removed by addition of ammonium chloride 0.85% (containing heparin 2500 I.U./liter), for 10–12 min at room temperature. Resedimentation of the lymphocytes was done by centrifugation at $1400 \times g$ for 12 min.

The lymphocytes, fibroblasts, and the amniotic fluid cells were frozen and then disrupted in a small volume (50–100 μl) of sucrose 0.25 mole/liter by sonification for 2×3 sec, using a microtip of a Branson B-12 sonifier. The cytosol fraction of the tissues and cells was prepared by centrifugation in a Sorwall refrigerated centrifuge (0–5°C), $48,000 \times g$ for 120 min.

All samples, except the liver preparation, were gel filtered through a Sephadex G25, fine grade column, bed volume 3 ml, with sucrose as eluting medium.

Dextran blue, added to the cytosol fractions before gel filtration (final concentration 0.1%), was used as marker of the void volume. The complete void volume was collected and for the lymphocytes, fibroblasts, and the amniotic fluid cells this volume was about 400 μl. The protein content of the fractions was determined by the method of Lowry *et al.* (11), and was done immediately after the enzyme analysis was completed. When the lymphocytes and fibroblasts are stored at −20°C the enzyme activity slowly deteriorates. After a month the activity is about 60–70% of the initial value. At +4°C the enzyme activity is markedly unstable. Because of this instability we adapted the standard procedure in the following manner: enzyme analysis was performed 2 days after the cells were collected or isolated. Cytosol fractions were made on the intermediate day, and the samples were kept at −20°C between the steps. Gel filtration was carried out immediately before the enzyme analysis.

Patient and control groups. Fibroblasts from three patients with hereditary tyrosinemia were obtained during the first 6 months of life. These patients presented with a typical picture of hereditary tyrosinemia with excretion of the tyrosine metabolites parahydroxyphenylactate and succinylacetone, as determined by gas chromatography-mass spectrometry. Progressive liver and kidney failure occurred, one of the patients died 7 months old from renal failure with anuria and terminal lung oedema and one died at 4 years of age due to a liver hepatoma.

In the patient group tested for enzyme activity in the lymphocytes, one was 2 years of age and the other five were 11–21 years. The clinical picture of these patients varied considerably. Two of the patients (2 and 11 years) are at present in a good clinical condition, but they still excrete moderate amounts of tyrosine metabolites. The other four patients have varying degrees of liver and kidney pathology. All of the patients have had, in some periods, the complete clinical and biochemical picture of hereditary tyrosinemia.

The control group for the lymphocyte enzyme determination consisted of healthy adults. The control group for the fibroblasts consisted of a 5-year-old boy and four healthy adults.

Assay of FAH. The rate of hydrolysis of fumarylacetoacetate was determined spectrophotometrically at 330 nm, essentially as described previously (8). All measurements were done at 37°C by the standard procedure: 0.1 ml of sodium phosphate buffer 0.1 mole/liter, pH 7.5, and 0.02 ml of the fumarylacetoacetate solution were preincubated in a micro-cuvette with 1 cm light path. When the absorbance level was stable (after about 2 min) the reaction was started by adding 0.1 ml of the enzyme solution. After a nonlinear phase of about 1–2 min the enzyme reaction proceeded

Table 1. *Fumarylacetoacetate hydrolysing activity in various human tissues and cells*[1]

Enzyme source	FAH[2] U/g protein (mean ± S.D.)
Liver, biopsy material (7)	52.8 ± 10.3
Liver, autopsy material (12)	41.2 ± 11.0
Kidney (8)	5.1 ± 1.8
Spleen (2)	1.3–1.6
Lung (2)	1.0–0.9
Pancreas (2)	1.1–1.3
Skeletal muscle (2)	1.0–1.1
Cardiac muscle (2)	1.2–2.8
Fibroblasts (5)	3.4 ± 0.3
Lymphocytes (96)	3.6 ± 0.6
Amniotic fluid cells, cultured (5)	4.1 ± 1.6

[1] For assay conditions see text. Number in parenthesis represent number of specimen. Standard deviation has been calculated whenever the number exceeded two, otherwise individual values are given.
[2] FAH, fumarylacetoacetate fumarylhydrolase.

Table 2. *Fumarylacetoacetate hydrolysing activity in fibroblasts from patients with hereditary tyrosinemia, parents of the patients, and in control persons*[1]

	FAH[2] U/g protein
Controls (n = 5)	3.4 ± 0.3
Parents of patients (n = 4)	1.7 ± 0.2
Patient 1	0.3
Patient 2	0.4
Patient 3	0.3

[1] For assay conditions see text.
[2] FAH, fumarylacetoacetate fumarylhydrolase.

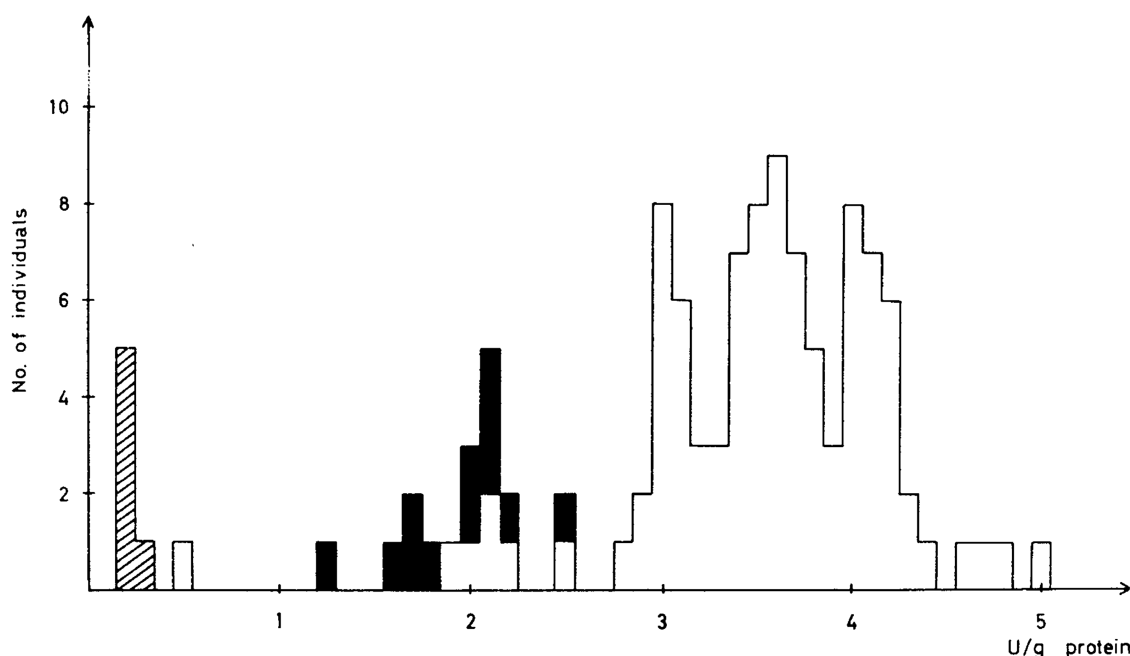

Fig. 1. Fumarylacetoacetate hydrolysing activity in lymphocytes from patients with hereditary tyrosinemia, ▨; parents of the patients, ■; and control subjects, □. For assay conditions see text.

Table 3. *Acetoacetate formation calculated from the disappearance of fumarylacetoacetate and from the consumption of NADH*[1]

Enzyme source	Acetoacetate formation (nmole)	
	Calculated from fumarylacetoacetate disappearance (mean ± S.D.)	Calculated from NADH consumption (mean ± S.D.)
Lymphocyte, cytosol fraction	33.8 ± 1.1	31.1 ± 1.3

[1] A deproteinized aliquot of hydrolysed fumarylacetoacetate was mixed with 0.3 ml of sodium phosphate buffer 0.3 mole/liter, pH 7.0, 250 nmoles of NADH and 0.02 ml of sodium hydroxide, 4 mole/liter, in a total volume of 970 μl. Excess 3-HBDH was added and from the decrease of absorbance at 340 nm the consumption of NADH was calculated using the molar extinction coefficient for NADH, 6.3×10^3.

in a linear manner for at least 10 min or until nearly all the substrate had been hydrolysed. The concentration of protein in the assay varied between 0.28–0.82 g/liter and the enzyme reaction is proportional to the protein content up to at least 1.24 g/liter. Enzyme activity is expressed in units (U), one unit being the amount of enzyme hydrolysing 1 μmole of substrate per min.

RESULTS

As shown in Table 1, all tissues investigated including lymphocytes, fibroblasts, and cultured amniotic fluid cells, catalyze the hydrolysis of fumarylacetoacetate. In postmortem liver tissue from three patients with hereditary tyrosinemia there was no detectable enzyme activity present nor was there any enzyme activity in the kidney tissue from these patients. Control liver and kidney samples showed mean activities of 41.2 and 5.1 U/g protein, respectively (Table 1). The samples from the patients with hereditary tyrosinemia were obtained at 15 min, 8 h, and 12 h after death. The autopsies of the control patients were performed from 12–36 h after death.

In Table 2 the enzyme activity in fibroblasts from three patients, the parents of two of these patients and in controls, are given. Figure 1 shows the pattern and level of the enzyme activity in lymphocytes from six patients with hereditary tyrosinemia, the parents of these patients, and in 97 control persons. Each value on the diagram is mean of two parallel determinations. The total standard deviation of the analysis is 0.17, determined by drawing duplicate blood samples from 17 control persons (enzyme values between 3–4 U/g protein) on two successive days. Control values below 2.8 U/g protein were redetermined a second time and in all cases reproduced within ± 5%. The individual lymphocyte and fibroblast enzyme values of the patients with hereditary tyrosinemia have been given, although a value of 0.3 U/g protein (zero + 2 S.D.) must be considered the lowest measurable value. One control person has an enzyme value in lymphocytes of 0.5 U/g protein. Also, in fibroblasts, this person has a very low enzyme activity, 0.8 U/g protein.

3-HBDH and NADH were used for accurate quantitation of the acetoacetate formed by the lymphocyte enzyme reaction (2). Table 3 shows that the estimated amount of acetoacetate formed is slightly higher when calculated from the apparent disappearance of fumarylacetoacetate than when calculated from the NADH consumption. The discrepancy may be due to a slight side reaction being present. The Km value of the lymphocyte and fibroblast enzyme reaction was determined and found to be 0.9 and 1.0 μmole/liter, respectively. Optimum pH for the lymphocyte enzyme reaction was 7.5. A slight increase of the reaction rate occurred from pH 7.5–8.5, but at pH values of 8.0 and above the reaction became nonlinear. The increasing reaction rate at high pH values was therefore attributed to increasing contribution of side reactions. Under standardized conditions, using pH 7.5, the side reaction has minor practical implications.

DISCUSSION

FAH activity has not been reported previously in human tissues other than liver. In this study we have shown that many human tissues and cells can hydrolyse fumarylacetoacetate enzymatically.

The patients with hereditary tyrosinemia show defective enzyme activity in all tissues investigated. Defective enzyme activity in autopsy material can always be due to postmortem changes. In the present case the liver and kidney samples from the patients with hereditary tyrosinemia were obtained at an earlier stage after death than the materials from the control persons. This indicates that the lack of enzyme activity in the tissue samples from the patients with hereditary tyrosinemia is probably not due to postmortem changes. Furthermore, the deficiency of the enzyme both in liver and kidney tissue from the patients is as expected when considering the pathology of the disorder affecting principally the liver and kidneys. The method of substrate synthesis does not result in a completely pure substrate (8) and the slight residual activity (5–10% of controls) in fibroblast and lymphocytes from patients with hereditary tyrosinemia may in part be due to a slight side reaction. In most tissues and cells, apart from liver, compounds of low molecular weight must be removed in order to obtain a linear reaction. The explanation for this may be the removal of NADH-mediated enzyme reactions and thiol interaction with the substrate. In an enzyme preparation from granulocytes, however, the enzyme reaction does not proceed in a linear manner even after gel filtration. This unusual behaviour may be due to hydrolytic activity of proteases. The lymphocyte enzyme preparation, which is not totally devoid of granulocytes, and even the fibroblast enzyme preparation may contain some protease activity and thus give rise to possible side reactions. Gel filtration of the lymphocyte and fibroblast enzyme preparation does, however, substantially eliminate side reactions, as implied by the result in Table 3, and the enzyme reaction proceeds in a linear manner after a short initial nonlinear phase. In fibroblasts and lymphocytes, all parents of the patients have enzyme values compatible with a heterozygote genotype. This result strongly suggests that the deficiency of the enzyme activity in the patients is due to genetic alterations and not to an inhibition of the enzyme activity. The lymphocyte enzyme pattern in the control group is complicated and implies the presence of an enzyme variant.

Six control persons have enzyme values comparable with the parents of the patients. If they were all carriers of hereditary tyrosinemia the disorder would be far more common in Scandinavia than it is presently estimated to be (7). The enzyme values of these six persons may arise from a different genetic constellation than for the obligate carriers. One control person, a 31-year-old female, has enzyme values in lymphocytes and fibroblasts just above that of the patients. Apart from the low enzyme activity, no clinical or biochemical abnormality can be found in this person, and even after L-tyrosine loading (300 ml/kg body weight) she excretes no abnormal tyrosine metabolites. When interpreting the results of this person, the existence of isoenzymes must be considered; however, a wellfounded explanation based on isoenzymes is difficult to conceive because the enzyme is deficient in all tissues and cells from the patients and in both lymphocytes and fibroblasts from the control person.

A possible explanation for our findings is that the control person with the low enzyme value is homozygote for a variant gene to the "tyrosinemia gene." The frequent encounter of a "heterozygote" enzyme value may then in fact represent a heterozygote genotype for the variant gene. As the control person with the low enzyme activity evidently have enough enzyme activity for adequate hydrolysis of fumarylacetoacetate, the variant gene may either give a low but sufficient enzyme activity in liver tissue or it may produce an enzyme which is abnormally unstable *in vitro*.

In spite of the unsettled questions discussed above, we have shown that the diagnosis of hereditary tyrosinemia can be established by enzyme determination in easily accessible materials such as lymphocytes and fibroblasts. The genetic nature of the FAH deficiency in the patients supports the hypothesis of this enzyme

defect being the primary cause of the disorder. Determination of succinylacetone in amniotic fluid of pregnancies at risk of tyrosinemia was recently reported to be a successful method in identifying affected fetuses (5). The presence of the enzyme FAH in cultured amniotic fluid cells furthermore opens the possibility of prenatal diagnosis of hereditary tyrosinemia by enzyme measurement in these cells. Combined determination of succinylacetone and of the enzyme activity will give substantial security to the prenatal diagnosis.

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