Altered Kinetic Properties of the Branched-Chain α-Keto Acid Dehydrogenase Complex Due to Mutation of the β-Subunit of the Branched-Chain α-Keto Acid Decarboxylase (E_1) Component in Lymphoblastoid Cells Derived from Patients with Maple Syrup Urine Disease

Yasuhiro Indo, Akito Kitano, Fumio Endo, Izumi Akaboshi, and Ichiro Matsuda
Department of Pediatrics, Kumamoto University Medical School, Kumamoto 860, Japan

Abstract

Branched-chain α-keto acid dehydrogenase (BCKDH) complexes of lymphoblastoid cell lines derived from patients with classical maple syrup urine disease (MSUD) phenotypes were studied in terms of their catalytic functions and analyzed by immunoblotting, using affinity purified anti-bovine BCKDH antibody. Kinetic studies on three cell lines derived from patients with the classical phenotype showed sigmoidal or near sigmoidal kinetics for overall BCKDH activity and a deficiency of the E_1 component activity. An immunoblot study revealed a markedly decreased amount of the $E_{1\beta}$ subunit accompanied by weak staining of the $E_{1\alpha}$ subunit. The E_2 and E_3 component exhibited a cross-reactive peptide. Thus, in at least some patients with MSUD, mutations of the $E_{1\beta}$ subunit might provide an explanation for the altered kinetic properties of the BCKDH complex.

Introduction

The branched chain amino acids leucine, isoleucine, and valine are catabolized through analogous mechanisms for the first three steps: transamination, oxidative decarboxylation of the branched-chain α-keto acid, and dehydrogenation of the resulting branched-chain acyl coenzyme A (CoA)[1] to enoyl CoA (1). The oxidative decarboxylation of branched-chain α-keto acids is performed by a multienzyme complex, branched-chain α-keto acid dehydrogenase (BCKDH), which is associated with the mitochondrial inner membrane and composed of three catalytic components, i.e., branched-chain α-keto acid decarboxylase (E_1), dihydrolipoyl transacylase (E_2) and dihydrolipoyl dehydrogenase (E_3). The E_1 component is further composed of $\alpha(E_{1\alpha})$ and $\beta(E_{1\beta})$ subunits (2). E_1 catalyzes both the decarboxylation of the α-keto acid and the subsequent reductive acylation of the lipoyl moiety that is covalently bound to E_2. E_2 catalyzes a transfer of the acyl group from the lipoyl moiety to coenzyme A (3, 4). The E_3 component is a flavoprotein and reoxidizes the reduced lipoyl sulfur residues of E_2. The E_1 and E_2 components are specific for the BCKDH complex, whereas the E_3 component is identical to that associated with the pyruvate and α-ketoglutarate dehydrogenase complexes (5). These enzyme components presumably catalyze a coordinate sequence of reactions constituting the overall reaction, similar to the mechanism elucidated for the pyruvate dehydrogenase (PDH) complex, as follows (3).

R-CO-COOH + CoA-SH + NAD^+ → R-CO-S-CoA + CO_2 + NADH + H^+. The BCKDH complex activity is deficient in patients with maple syrup urine disease (MSUD).

Currently, four different phenotypes have been distinguished, on the basis of the clinical features; classical (6, 7), intermittent (8, 9), intermediate (10), and thiamine-responsive types (11). The enzyme activities in cultured skin fibroblasts are much lower in the classical than in other types accompanied by a milder clinical course (12). Chuang et al. carried out detailed studies and observed the activities of the E_1, E_2, and E_3 components, separately, in a disrupted cultured skin fibroblast preparation. They proposed that the high affinity component of E_1 was deficient in classical cases of MSUD (13). Danner et al. reported another classical case of E_2 deficiency, demonstrated using the immunoblotting method (14). The genetic heterogeneity of MSUD was also demonstrated in studies involving genetic complementation analysis (15–17). E_3 deficiency was clearly detected in patients together with elevated blood levels of both branched-chain α-keto acids and pyruvate (18, 19). Although MSUD is one of the most common inborn errors of metabolism, first detected by Menkes et al. (6), fundamental knowledge of the biochemical and genetic mechanisms involved in this disease is lacking. We established lymphoblastoid cell lines from patients with different types of this disease as these cells have advantages for studying biochemical genetics (20–24). Using these cell lines, we performed immunochemical and kinetic analyses of the enzyme involved. We found that a deficiency of the $E_{1\beta}$ subunit, observed for the first time in MSUD, may be responsible for the altered kinetic properties of the multienzyme complex.

Methods

Radioisotopes and chemicals. α-Keto[1-^{14}C]isovaleric acid was purchased from the Radiochemical Center, Amersham, UK, and stored at −20°C. The sodium salt of α-ketoisovaleric acid and pig dihydrolipoyl dehydrogenase (E_3) were purchased from Sigma Chemical Co. (St. Louis, MO). Scintisol EX-H was obtained from Wako Pure Chemicals (Osaka, Japan). Complete and incomplete Freund's adjuvant, and gelatin were obtained from Difco Laboratories (Detroit, MI). Nitrocellulose paper was purchased from Schleicher & Schuell (Dassel, West Germany). Peroxidase-conjugated swine immunoglobulins to rabbit immunoglobulins were obtained from Dakopatts (Glostrup, Denmark). CNBr-activated

Presented at the 29th Annual Meeting of the Japanese Society of Inherited Metabolic Disease in Nagoya, October 1986, and at the 6th International Neonatal Screening Symposium, Austin, TX November 1986.

Address reprint requests to Dr. I. Matsuda, Department of Pediatrics, Kumamoto University Medical School, Honjo 1-1-1, Kumamoto 860, Japan.

Received for publication 23 July 1986 and in revised form 20 February 1987.

1. *Abbreviations used in this paper:* BCKDH, branched-chain α-keto acid dehydrogenase; E_1, decarboxylase component of BCKDH; $E_{1\alpha}$, α-subunit of E_1; $E_{1\beta}$, β-subunit of E_1; E_2, acyl-transferase component of BCKDH; E_3, dihydrolipoyl dehydrogenase; PDH, pyruvate dehydrogenase; MSUD, maple syrup urine disease.

J. Clin. Invest.

0021-9738/87/07/0063/08 $2.00
Volume 80, July 1987, 63–70

Sepharose and marker proteins were purchased from Pharmacia Fine Chemicals (Tokyo, Japan). 4-Methoxy-1-naphthol was obtained from Aldrich Chemical Co. (Milwaukee, WI). Bovine kidneys and hearts were obtained from a slaughterhouse, shipped to the laboratory on ice and processed immediately. The human liver and kidney tissues were obtained at autopsy.

Cell strains. Lymphoblastoid cell lines derived from two disease-free Japanese and four with MSUD, two with the classical (K.Y., Y.T.) and two with the intermittent type (R.F., K.F.), were established after incubation with Epstein-Barr virus, as described (20, 21). Lymphoblastoid cell lines (GM 1366, GM 1655) and skin fibroblast cell lines (GM 1364, GM 1654) derived from individuals with MSUD were purchased from the Human Mutant Cell Repository, Camden, NJ. Data on K.Y., R.F., and K.F. have been reported, respectively (25–27).

Cell culture and preparation of cell samples. Lymphoblastoid cells were grown in RPMI 1640 medium containing penicillin (100 IU/ml) and streptomycin (100 μg/ml) supplemented with 20% fetal calf serum in a CO_2 incubator at 37°C. A subculture was performed every 3 to 4 d by adding fresh medium to adjust the cell count to 3 to 4 × 10^5/ml. Exponentially growing cells, of which the viability was determined to be > 90% by the trypan blue dye exclusion method, were harvested and washed three times with Dulbecco's Ca^{2+}, Mg^{2+} free phosphate buffer solution (28). The washed cells were rapidly frozen (−75°C) and kept for 15 h to 3.5 mo, and thawed before the assay. For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), frozen lymphoblasts (1.5 × 10^8) were suspended in ~ 9 vol of 0.25 M sucrose/1 mM EDTA/0.2 mM phenylmethylsulfonyl fluoride/1.0 mM benzamidine/10 mM Tris-HCl, pH 7.5 (buffer A). The disrupted cells were further homogenized in an electrically driven Potter-Elvehjem homogenizer with a Teflon pestle, with 5 strokes, on ice. The following centrifugation steps were a modification of the method of Loewenstein et al. (29). Whole cells and nuclei were removed by centrifugation at 3,000 *g* for 1 min, and the mitochondrial fraction was precipitated by centrifugation at 15,000 *g* for 2 min. The homogenization and 3,000 *g* centrifugation steps were repeated. All centrifugation times exclude the acceleration and deceleration times. The mitochondrial pellet was suspended in the original volume of buffer B (30 mM potassium phosphate, pH 7.4/0.1 mM EDTA/0.1 mM EGTA/0.2 mM phenylmethylsulfonyl fluoride/1 mM benzamidine/1 mM dithiothreitol) and then sonicated three times for 10 s each with 30-s intervals, on ice. A soluble fraction was obtained from the mitochondrial suspension, using an Eppendorf centrifuge 5414S (Hamburg, West Germany) at top speed for 2 min. The fraction was subjected to SDS-PAGE.

Human skin fibroblasts in culture (150 cm^2 × 4) were harvested, using a plastic scraper. Cells were washed twice with Dulbecco's Ca^{2+}, Mg^{2+} free phosphate buffer solution. Crude mitochondrial pellets were prepared in buffer A using digitonin (30) then were stored at −75°C. The frozen pellets were resuspended in ice-cold buffer B, 1% Triton X-100. The samples were sonicated three times for 10 s each with 30-s intervals, on ice. A soluble fraction was obtained by using the Eppendorf centrifuge for 2 min. The fraction was subjected to SDS-PAGE.

Mitochondrial extracts from liver and kidney. Mitochondria from tissues obtained at autopsy were prepared by conventional differential centrifugation of the homogenates in buffer A, as described (31). The soluble mitochondrial fraction after sonication was subjected to SDS-PAGE.

Preparation of the BCKDH (E_1 and E_2) and PDH complexes from bovine tissue. The BCKDH (E_1 and E_2) complex was purified from bovine kidney, basically as described by Lawson et al. (32). The prepared sample was further subjected to sucrose density gradient centrifugation. The density gradient was formed from 15 ml of 10% sucrose in 30 mM potassium phosphate, pH 7.4/0.1 mM EGTA/0.2 mM phenylmethylsulfonyl fluoride/5 mM 2-mercaptoethanol (buffer C) and 15 ml of 30% sucrose in buffer C on a layer of 50% sucrose in buffer C (5 ml) placed at the bottom. After centrifugation in a rotor (RPS 27-2, Hitachi Corp., Tokyo, Japan) for 12 h at 18,000 rpm, the gradient was fractionated into 1-ml aliquots. The fractions showing BCKDH activity were combined, and the complex was collected by centrifugation at 180,000 *g* for 3.5 h. The precipitate was dissolved in 1 ml of buffer B and a soluble fraction was obtained with an Eppendorf centrifuge 5414S, at full speed for 2 min. The sucrose density gradient centrifugation was repeated twice and the specific activity of the obtained BCKDH was 3 to 7 U/mg protein. As shown by Lawson et al. (32), a complex of E_1 and E_2 without the E_3 component, was obtained. The PDH complex was purified from bovine heart, according to Matuda et al. (33), and the maximum specific activity of the enzyme was determined to be 3.8 U/mg protein.

Preparation of antibodies against bovine BCKDH, the PDH complex and pig dihydrolipoyl dehydrogenase. Antibody was raised in a female New Zealand White rabbit by injecting 100 μg of the purified BCKDH complex mixed with Freund's complete adjuvant into the lymph nodes of the hind legs (34). 2 wk after the fifth injection, blood was obtained from the marginal ear vein. The serum was divided into small aliquots and kept frozen at −75°C until use. A similar regimen was followed for the preparation of the anti-bovine PDH complex and pig dihydrolipoyl dehydrogenase.

Preparation of affinity purified antibodies against the bovine kidney BCKDH complex. An affinity column was prepared with the bovine BCKDH complex as the bound ligand. The procedure for coupling the ligand to CNBr-activated Sepharose was as follows. The gel matrix (0.3 g) was activated and washed according to the manufacturer's instructions. Approximately 0.5 mg of the ligand was bound to the gel matrix. The gel was packed into a column (1 × 1 cm), and the preparation washed with 1 M NaCl, 10 mM sodium phosphate, pH 7.2, and 150 mM NaCl, 10 mM sodium phosphate, pH 7.2, alternatively several times. The gel was further washed with 0.5 M NaCl, 0.1% Tween 20, 50 mM sodium phosphate, pH 7.5 (buffer D). The rabbit anti-bovine BCKDH complex serum was loaded onto the affinity column repeatedly. The affinity gel was extensively washed with buffer D, and then eluted with 0.1 M Na_2CO_3. The immunoglobulin fractions were neutralized to an apparent pH of 7–8 with 5 M HCl. The fractions were pooled, dialyzed against 150 mM NaCl, 10 mM sodium phosphate, pH 7.2, and then concentrated with a collodion bag (Sartorius, West Germany). The antibody was used within 3 mo.

Immunotitration of the BCKDH and PHD complexes. We used the direct immunotitration method, in which a constant amount of an enzyme is incubated with various amounts of a monospecific antibody (35). In the control incubation, the same amount of nonimmune immunoglobulin was incubated with the BCKDH complex.

Immunotitration of the PDH complex was performed by the method of Matuda et al. (33).

SDS-PAGE. Electrophoretic analysis of proteins was carried out on 10% (wt/vol) polyacrylamide-gel slabs with a 5% (wt/vol) stacking gel, using the discontinuous buffer system of Laemmli (36) and a Bio-Rad apparatus (Protean dual slab cell, Bio-Rad Laboratories, Richmond, CA).

Protein blot analysis. BCKDH immunoreactive protein in cultured cell extracts was detected after SDS-PAGE. The gel was soaked for 30 min in 25 mM Tris, 190 mM glycine, pH 8.3. Electroblotting was performed in an Bio-Rad electroblot apparatus (Trans-Blot Cell, Tokyo, Japan) onto nitrocellulose, according to the manufacturer's instructions. After the electrophoretic transfer, the blot (nitrocellulose paper with immobilized proteins) was soaked in 100 ml of buffer E (150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2, containing 1% gelatin and 0.1% Tween 20) for 1 h at 40°C. After rinsing with buffer F (150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2, containing 0.25% gelatin and 0.1% Tween 20) for 1 h, the protein blot was incubated overnight at room temperature with shaking in 100 ml of buffer F containing 200 μl of the specific anti-BCKDH complex antibody. After incubation with the antibody, the blot was washed three times for 20 min each with 200 ml of buffer F. The protein blot was then incubated with 100 ml of buffer F containing 300 μl of peroxidase-conjugated swine immunoglobulins to rabbit immunoglobulins for 1 h, with shaking. The blot was washed as described above. Peroxidase activity was detected using freshly prepared 4-methoxy-1-naphthol (1 ml of a 1% methanol solution in 50 ml of 50 mM Tris-HCl, pH 7.4) plus 50 μl of 30% H_2O_2. The color reaction was halted by washing in distilled water. The same procedure was used for the blotting of the PDH complex and dihydrolipoyl dehydrogenase (E_3).

Enzyme assays

Spectrophotometric assaying of the BCKDH complex (overall) activity. Spectrophotometrically, the activity of the bovine BCKDH complex was assayed at 30°C in the presence of excess dihydrolipoyl dehydrogenase (E_3) as described (3). One unit of enzyme catalyzes the formation of 1 μmol NADH per min (3).

Radiochemical assaying of the BCKDH activity

Preparation of a radiolabeled substrate. α-Keto [1-^{14}C]isovaleric acid is unstable under the storage conditions used and is contaminated by volatile radioactive compounds. Because it is necessary to use a substrate with a high specific activity at low substrate concentrations, we modified the radioactive substrate preparation to obtain more precise kinetic data. Volatile radioactive contaminants were removed from the substrate before incubation, as described for radioactive branched-chain amino acids by Dancis et al. (12). Immediately before use, the radioactive substrate was dissolved in deionized water and nitrogen was bubbled through the solution for 30 min on ice. The solution was then shaken for 1 h at room temperature in a test tube connected to a mini-counting vial with a thick rubber tube, containing filter paper (Whatman Inc., Clifton, NJ, 4 × 5 cm) immersed in 0.2 ml of 20% β-phenethylamine in methanol. With this procedure, the radioactive contaminants were fairly well minimized. The volume of the radioactive substrate solution was appropriately adjusted with deionized water. Approximately 0.1 μCi of radioactive α-ketoisovaleric acid was used per assay, with the addition of the nonradioactive substrate to obtain various specific activities.

BCKDH overall assay. The activity of BCKDH (overall) was assayed as described (13, 37) using a disrupted lymphoblastoid cell suspension, except that calf serum was omitted. The assay mixture contained a disrupted cell suspension (equivalent to 0.8–1.0 mg) in 0.05 ml of Dulbecco's Ca^{2+}, Mg^{2+} free phosphate buffer solution and α-keto[1-^{14}C]isovaleric acid (~ 0.1 μCi) in a final volume of 0.37 ml. The reaction was carried out at 35°C in a test tube connected to a mini-counting vial with a thick rubber tube, as described by Ichiyama et al. (38). The vial contained a filter paper strip that was immersed in 0.2 ml of 20% β-phenethylamine in methanol. After equilibration for 1 min, the reaction was started by addition of the labeled substrate. At the end of incubation, 0.1 ml of 15% trichloroacetic acid was injected through the rubber tube. This acidified reaction mixture was then left to stand for 1 h at 35°C to remove the residual $^{14}CO_2$. After incubation, 7 ml of a scintillation cocktail (Scintisol EX-H) was added to the vial to determine the radioactivity. All kinetic data were obtained in duplicate. In all experiments, a duplicate blank incubation was carried out, for which all the ingredients, except the disrupted cell suspension, were used. The blank value was within 1 to 4% of the total sample counts, which ranged from 2,000 to 7,000 dpm for normal lymphoblastoid cells, depending on the substrate concentration. The nonenzymatic evolution of $^{14}CO_2$ was subtracted from the value obtained with the experimental incubation. The activity of frozen cells was stable for at least 3.5 mo at −75°C.

Assay for the E_1 component. The radiochemical assay used was essentially that described by Chuang et al. (13), except that calf serum was omitted. The reaction mixture contained α-keto[1-^{14}C]isovaleric acid (~ 0.15 μCi) and the disrupted cell suspension (equivalent to 0.8–1.0 mg) in 0.05 ml of Dulbecco's Ca^{2+}, Mg^{2+} free phosphate buffer solution in a final volume of 0.37 ml.

Assay for the E_3 component. The E_3 component was assayed in the direction of lipoamide reduction, as described by Ide et al. (39) with the following modification. The assay mixture contained 50 mM potassium phosphate, pH 6.5, 1.2 mM EDTA, 0.1 mM NADH, 0.1 mM NAD^+, 1.0 mM DL-lipoamide and the sonicated cell extract. The sonicated cell extract was prepared as follows: frozen and thawed cells were suspended in Dulbecco's Ca^{2+}, Mg^{2+} free phosphate buffer solution, sonicated three times for 10 s each at 30-s intervals on ice, and then centrifuged with an Eppendorf centrifuge 5414S for 2 min. The supernatant was used for assaying the E_3 component. The reaction was started by the addition of lipoamide and followed spectrophotometrically at 30°C and 340 nm. Blanks without lipoamide were run.

Protein determination. The protein concentration during purification of the bovine BCKDH complex and SDS-PAGE was determined by the method of Bradford (40) with bovine gamma globulin as a standard. For other samples, the protein concentration was measured by the method of Lowry et al. (41) with bovine serum albumin as a standard.

Statistical methods. The statistical significance was determined using Student's *t* test.

Results

BCKDH enzyme activity

Kinetics of BCKDH overall activity. Linearity of the enzyme function was seen over a protein concentration range of 0.3 to 1.0 mg cell protein per reaction, in the dose dependency curve for BCKDH from normal subjects at 2.0 mM α-ketoisovaleric acid. The time courses of the enzyme activity at 0.1 and 2.0 mM α-ketoisovaleric acid concentration were linear up to 10 and 40 min, respectively, with ~ 1.0 mg of disrupted cell protein (data not shown).

All kinetic data were obtained under conditions under which a linear relationship with time and the amount of protein added was observed. Disrupted normal lymphoblastoid cells showed hyperbolic Michaelis-Menten kinetics over the substrate range tested, 0.05–2.0 mM (Fig. 1). The V_{max} and apparent K_m values were 13–15 nmol per h/mg of protein and 0.05–0.06 mM, respectively. In contrast, cells from MSUD subjects showed two different kinetic patterns (Fig. 2). One group A showed hyperbolic kinetics for BCKDH over the substrate concentration of 0.05–2.0 mM, whereas group B exhibited sigmoidal kinetics or near sigmoidal kinetics. The group A cell lines were derived from intermittent MSUD patients (K.F., R.F.) and GM 1366. The group B ones were from classical MSUD patients (K.Y., Y.T.) and GM 1655. Both group A and B showed significantly reduced enzyme activities measured at 2.0 mM or lower (0.054 mM) substrate concentration ($P < 0.01$), which corresponds to the apparent K_m value of the highly purified bovine BCKDH complex (42). The enzyme activities in group A were higher than

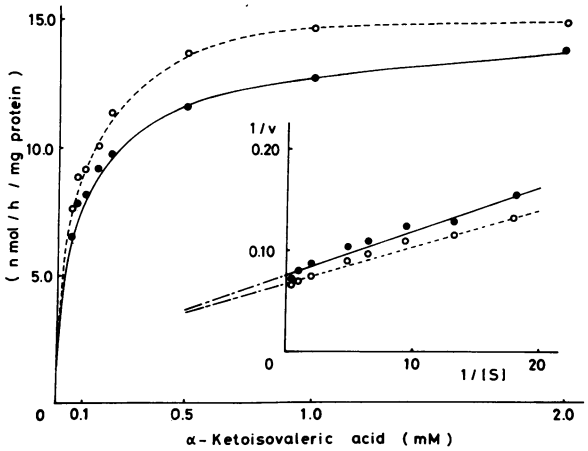

Figure 1. Activities of BCKDH complex in disrupted lymphoblastoid cell lines from disease-free Japanese. The rate of the overall reaction catalyzed by the multienzyme complex was measured in the presence of cofactors, as described in Methods, with α-keto-[1-^{14}C]isovaleric acid as the variable substrate. Disrupted cell suspensions (equivalent to 0.8–1.0 mg of protein) were used as the enzyme source. All kinetic data were determined under conditions with which a linear relationship with time and the amount of protein added observed. Lineweaver-Burk plots are shown in the insert. ○, control 1; ●, control 2.

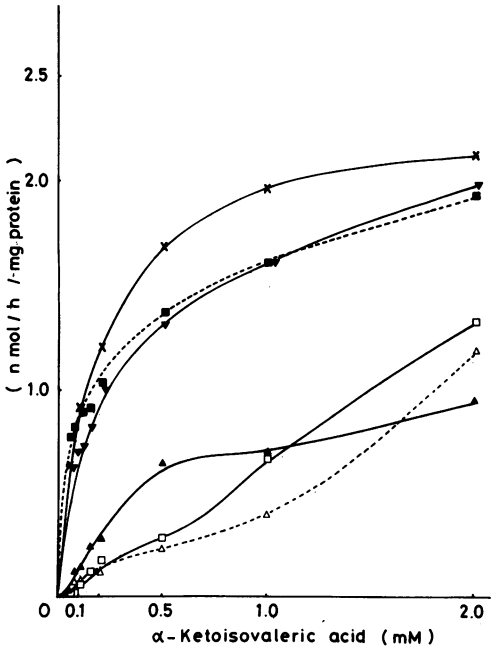

Figure 2. Activities of BCKDH complex in disrupted lymphoblastoid cell lines from MSUD subjects. The incubation and protein added were the same as for Fig. 1. GM 1655 (□) and GM 1366 (▼) are MSUD cell lines obtained from the Human Mutant Cell Repository, Camden, NJ. K.Y. (△) and Y.T. (▲) are cell lines from classical type MSUD patients. R.F. (■) and K.F. (×) are cell lines from intermittent type MSUD patients.

those in group B at 2.0 mM ($P < 0.01$) or 0.054 mM ($P < 0.01$) substrate concentration. Fig. 3 *a* is a Lineweaver-Burk plot for the BCKDH complex. The apparent K_{ms} for R.F., K.F., and GM 1366 were 0.067, 0.123, and 0.097 mM, respectively. The same plots for K.Y., Y.T., and GM 1655 showed no linearity. In contrast, Hill plots (Fig. 3 *b*) showed that the concentration of substrate needed for half-maximal velocity ($K_{0.5}$) was 0.4–2.0 mM for K.Y., Y.T., and GM 1655. The Hill coefficients (h) were 1.2–1.4, from the same plots.

Kinetics of E_1 activity. Determination of the E_1 activity and the kinetics of the reaction in disrupted cells from normal and MSUD patients are shown in Fig. 4. Disrupted cells from two normal subjects showed hyperbolic kinetics, in the range of 0.05 to 0.2 mM. Similar to the control pattern, the kinetic pattern of the R.F., K.F., and GM 1366 cell lines (group A) was hyperbolic, and E_1 activities at 0.05–0.2 mM substrate concentration were 30–40% and 60–65% of the control level for the R.F., GM 1366, and K.F. cell lines, respectively. The K.Y., Y.T., and GM 1655 cell lines (group B) exhibited essentially no E_1 enzyme activity in the concentration range of this substrate. The differences of E_1 activities between normal and group A at substrate concentration 0.056 or 0.206 mM ($P < 0.05$) were significant. The enzyme activities in group A were higher than those in group B, at the same substrate concentration ($P < 0.05$). When the activities were measured at a higher substrate concentration and plotted on a large scale, a kinetic pattern that was nearly sigmoidal was found for these cell lines (group B), and for the overall enzyme kinetics (data not shown).

E_3 component activity. The activities of the E_3 component derived from normal lymphoblast cell lines were 37.3±7.1 (means±SD) ($n = 4$) mU/mg of protein. The levels of E_3 activity in all the MSUD cell lines were within a normal range, as shown; 34.0 in R.F., 30.6 in K.F., 32.5 in K.Y., 32.4 in Y.T., 41.2 in GM 1655, and 41.8 in GM 1366.

Immunotitration of bovine BCKDH complex activity with a specific antibody. Immunoglobulin from a rabbit sensitized with the highly purified bovine kidney BCKDH ($E_1 + E_2$) complex specifically inhibited the catalytic activity of this enzyme complex, when NADH production was measured by means of the overall assay for the enzyme complex. 50% inhibition of the activity occurred in the presence of the antibody at a ratio of 1.2:1 (immunoglobulin/BCKDH, wt/wt), when 2.0 μg of BCKDH protein (5 U/mg of protein) was used (data not shown).

Antiserum prepared against pig heart dihydrolipoyl dehydrogenase (E_3) was tested in terms of catalytic inhibition of the enzyme. 50% inhibition was observed when 2 μg of bovine BCKDH protein (2 U/mg) and 2 U of pig dihydrolipoyl dehydrogenase were incubated in the presence of 5 μl of the antiserum against E_3 (data not shown).

Immunotitration of bovine PDH complex activity with the antiserum. 50% inhibition occurred when 35 μg of PDH protein (1.3 U/mg of protein) was incubated with 20 μl of the prepared antiserum against the PDH complex.

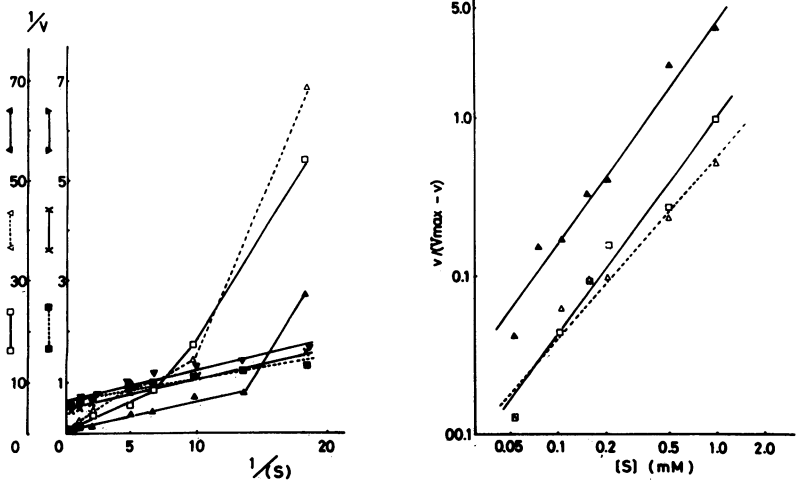

Figure 3. Kinetics of the BCKDH complex in disrupted lymphoblastoid cell lines from MSUD subjects. The incubation and protein added were the same as for Fig. 1. The cell lines were the same as for Fig. 2. (*a*) Lineweaver-Burk plots of the BCKDH complex activities in the cell lines; K.F. (×), R.F. (■), K.Y. (△), Y.T. (▲), GM 1655 (□) and GM 1366 (▼). (*b*) Hill plots of the BCKDH activities in the cell lines; K.Y. (△), Y.T. (▲) and GM 1655 (□).

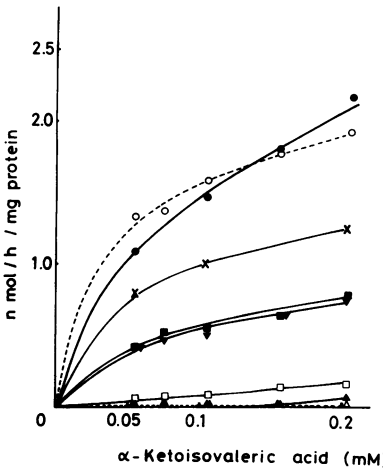

Figure 4. Kinetics of the E_1 component activity in disrupted lymphoblastoid cell lines from disease-free and MSUD subjects. Radiochemical ferricyanide assays were carried out as described in Methods in the presence of 0.2 mM-TPP with α-keto-[1-^{14}C]-isovaleric acid as the variable substrate. The disrupted-cell suspensions used were the same as for Fig. 1. The cell lines from MSUD subjects are the same as in Fig. 2. (Control 1, ○; control 2, ●; K.F., ×; R.F. ■; K.Y., △; Y.T., ▲; GM 1655, □; GM 1366, ▼.

Immunoblotting. As shown in Fig. 5, similar antigen peptide patterns were observed in all except lane 2, which contained the bovine PDH complex. The three main immunogenic peptides were, in decreasing size, the E_2 (M_r 52,000), $E_{1\alpha}$ (M_r 46,000), and $E_{1\beta}$ (M_r 37,000) peptides of the BCKDH complex. The bovine PDH complex on the nitrocellulose blot was not recognized by the anti-BCKDH complex immunoglobulin. The antibody raised against the bovine BCKDH complex could detect cross-reacting peptides of the human tissues. The three peptides were similar in size to the corresponding bovine BCKDH peptides.

The E_3 peptide was also identified on immunoblots, using the antibody against pig heart dihydrolipoyl dehydrogenase (data not shown).

To delineate the molecular basis for the low BCKDH activity in MSUD lymphoblasts, enzymes were studied, using anti-bovine BCKDH complex immunoglobulin. This experiment was performed at the same time and under the same condition, except for lane 7. A typical immunoblot is shown in Fig. 6, in which three antigenic proteins corresponding to E_2, $E_{1\alpha}$, and $E_{1\beta}$ of purified bovine kidney BCKDH were clearly stained, and two additional bands, besides the latter, were seen for both normal and MSUD cell lines. Since these bands did not appear in case of the purified bovine BCKDH complex, the origin of these proteins is unknown. All three cell lines (K.Y., Y.T., and GM 1655) derived from classical MSUD patients lacked a single antigenic protein that corresponded to the $E_{1\beta}$ subunit of BCKDH. In addition, a protein corresponding to $E_{1\alpha}$ was weakly stained in all, particularly in the K.Y. cell line.

In contrast, an antigenic protein that corresponded to the E_2 component was moderately reduced in the R.F. cell line derived from an intermittent type patient, and the protein was absent in the GM 1366. Another cell line (K.F.) contained three proteins.

To rule out the possibility of an artifact from Epstein-Barr virus transformation, we compared the BCKDH complex of lymphoblastoid cells and skin fibroblasts derived from the same patients. As shown in Fig. 7, the same structural abnormalities of BCKDH complex were observed in two MSUD subjects, determined using both lymphoblastoid cells and skin fibroblasts. We also noted absence of the E_2 protein in the GM 612 skin fibroblast, as a reference, since the observation was described by Eisenstein et al. (43).

In all the cell lines examined, the E_3 peptide, which is similar in size to porcine dihydrolipoyl dehydrogenase, was identified on immunoblots (data not shown).

We also studied the PDH complex, using rabbit antibodies raised against the bovine heart enzyme, as a reference for the mitochondrial multienzyme complex. Fig. 8 shows the immunoblot pattern of the PDH complex on SDS-PAGE. Similar antigen peptide patterns were observed for all the cell lines examined. The six main immunogenic peptides were, in decreasing size, dihydrolipoyl transacetylase, dihydrolipoyl dehydrogenase, X, the α-subunit of pyruvate dehydrogenase, Y, and the β-subunit of pyruvate dehydrogenase. An additional band, X, that migrated below the E_3 component, was consistently found in the purified preparation of the complex, when the electrophoretic analysis was performed using Laemmli's buffer system, as noted previously by Stanley and Perham (44). The extra band, Y of undetermined origin, was seen only when immunoblotting was performed.

Discussion

The BCKDH complexes derived from patients with two different MSUD phenotypes were studied in terms of their catalytic functions (substrate dependent kinetics) and the properties of the

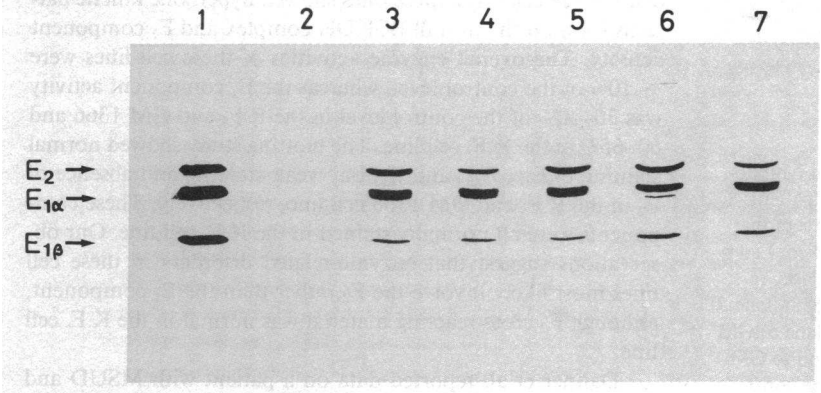

Figure 5. Immunoblots of the BCKDH complex. Various protein samples were resolved by SDS-PAGE and then transferred electrophoretically onto nitrocellulose paper, as described in Methods. BCKDH immunoreactive proteins were detected by the protein blot technique, using affinity purified antibody. The BCKDH (E_1 + E_2) complex peptides were, in decreasing size: (E_2) dihydrolipoyl transacylase (M_r 52,000), ($E_{1\alpha}$) the α-subunit of branched-chain α-keto acid decarboxylase (M_r 46,000), and ($E_{1\beta}$) the β-subunit of branched-chain α-keto acid decarboxylase (M_r 37,000). (Lane *1*) The purified bovine kidney BCKDH complex (0.5 μg) with its component peptides, as indicated; (lane *2*) the purified bovine heart PDH complex (3.3 μg); (lanes *3–5*) mitochondrial extracts (120 μg) from disease-free lymphoblastoid cell lines; (lanes *6* and *7*) mitochondrial extracts of human liver and kidney, respectively (120 μg of each protein).

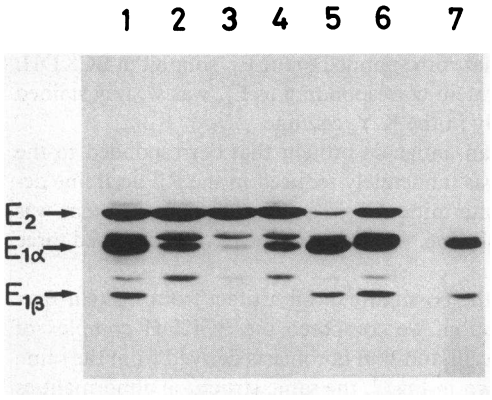

Figure 6. Immunoblots of the BCKDH complex in lymphoblastoid cell lines from MSUD subjects. Mitochondrial extracts containing 120 μg protein each were resolved by SDS-PAGE. The BCKDH peptides were identified by immunoblotting, as described under Methods. The BCDKH ($E_1 + E_2$) complex peptides were the same as for Fig. 5. (Lane *1*) A disease-free cell line; (lanes *2–4*) GM 1655 and cell lines from classical type MSUD patients (K.Y. and Y.T.); (lanes *5–6*) cell lines from intermittent type MSUD patients (R.F. and K.F.); (lane *7*) GM 1366. The blot was intentionally overexposed to a chromogenic substrate to demonstrate absence of the $E_{1\beta}$ subunit protein, except for lane *7*. These experiments were repeated twice using different preparations with essentially identical results.

apparent subunits. Mutations affecting different regions of any of the structural genes coding for the BCKDH complex may lead to impairment of the function of the entire complex. The reported variations in the BCKDH activity and the clinical expression in patients with the disease support the proposal of a possible mutation of different regions of the complex. Chuang et al. studied the activities of the E_1, E_2, and E_3 components of the enzyme, separately, in a disrupted preparation of cultured skin fibroblasts obtained from two classical MSUD patients (13). They found that both the overall BCKDH complex and the E_1 component activity exhibited sigmoidal kinetics, as a function of the substrate concentration. They speculated that the activity of the high affinity component of E_1 is deficient in classical MSUD. The E_1 component of the enzyme complex is known

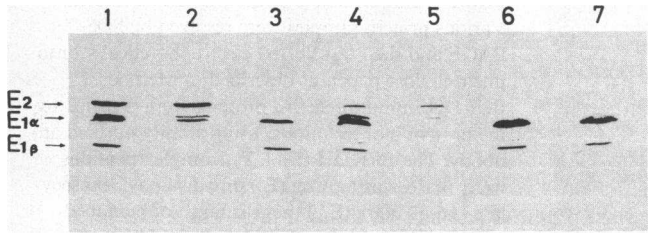

Figure 7. Comparison of the BCKDH ($E_1 + E_2$) complex in lymphoblastoid cells and in skin fibroblasts derived from the same patients with MSUD. The procedures were the same as for Fig. 6 except that skin fibroblast extracts containing 250 μg protein were applied. Lanes *1–3* and lanes *4–7* are lymphoblastoid cells and skin fibroblasts, respectively. (Lane *1*) A disease-free cell line; (lane *2*) GM 1655; (lane *3*) GM 1366; (lane *4*) a disease-free cell; (lane *5*) GM 1654; (lane *6*) GM 1364; (lane *7*) GM 612. GM 1655 and GM 1366 correspond to GM 1654 and GM 1364, respectively. A lymphoblastoid cell line corresponding to GM 612 was not available.

to be composed of $E_{1\alpha}$ and $E_{1\beta}$ subunits, however, the subunit involved has remained obscure. The results of our kinetic studies on three lymphoblastoid cell lines derived from classical MSUD patients are in accord with Chuang's observations, and an immunoblot study indicated that $E_{1\beta}$ is involved in the mutant enzymes. Although the antibodies used are for the bovine BCKDH complex, we found that these antibodies clearly reacted with the $E_{1\alpha}$, $E_{1\beta}$, and E_2 subunits of human liver, kidney, and lymphoblastoid cells (Fig. 5). The possibility that the antibody prepared against BCKDH components, (except for E_3) partly reacted with PDH components was not supported by the results of our study (Fig. 5). It is likely that the absence of the $E_{1\beta}$ subunit found in all these classical MSUD cell lines thus indicates a lack of the subunit protein or loss of immunogenic sites of the protein in these mutant enzymes. A low titer or low affinity of the antibodies for the mutant $E_{1\beta}$ subunit may be another explanation. We did not detect the $E_{1\beta}$ subunit in a lymphoblastoid cell preparation until we used affinity purified antibodies against the bovine BCKDH complex. The E_3 component of these cell lines were stained normally and exhibited normal functions. Thus, the present study suggests that the structural region might involve the $E_{1\beta}$ subunit of these mutant BCKDH complexes, and if so, would provide an explanation for the altered kinetics observed in our classical MSUD cell lines. We have no adequate explanation for the weak staining of the $E_{1\alpha}$ subunit of these classical MSUD cell lines. As speculated in case of the PDH complex (45), a defect in one of the two peptides of E_1 might inhibit transport of the mutated precursor peptide into the mitochondria, or assembly of the heterotetramer ($\alpha_2\beta_2$), leading to rapid degradation of one or both subunits. Studies using specific cDNA probes and in vitro translation should elucidate the nature of the defect of E_1.

Stepp and Reed reported that the thiamine pyrophosphate binding site of E_1 is located on the $E_{1\alpha}$ subunit in the PDH complex (46). Both the PDH and BCKDH complexes contain small amounts of a specific kinase and a specific phosphatase that modulate the activity of the E_1 by phosphorylation and dephosphorylation of the $E_{1\alpha}$ subunit, respectively (2). Although it has been proposed that the $E_{1\beta}$ subunit catalyzes the reductive acetylation of the lipoyl moiety of the E_2 component in the case of PDH complex, attempts thus far to separate the $E_{1\alpha}$ and $E_{1\beta}$ subunits with retention of catalytic activity have not been fruitful (46).

The integrity of both the $E_{1\alpha}$ and $E_{1\beta}$ subunits may favor a conformation with a high substrate affinity. This speculation is given support by observations of the intermittent MSUD cell lines (R.F., K.F.) and another cell line (GM 1366). Similar to disease-free cell lines, these lines showed hyperbolic kinetic patterns for both the overall BCKDH complex and E_1 component activity. The overall enzyme activities of these cell lines were ~ 10% of the control level, whereas the E_1 component activity was 30–40% of the control level in the R.F. and GM 1366 and 60–65% in the K.F. cell line. The blotting study showed normal staining of $E_{1\alpha}$, $E_{1\beta}$, and E_3, but weak staining and absence of E_2 in the R.F. and GM 1366 cell line, respectively. These components were all normally stained in the K.F. cell line. Our observations suggest that enzyme-related disorders in these cell lines most likely involve the E_2 rather than the E_1 component, although E_2 cross-reacting material was normal in the K.F. cell line.

Danner et al. reported data on a patient with MSUD and an E_2 component deficiency, as determined by the immunoblot

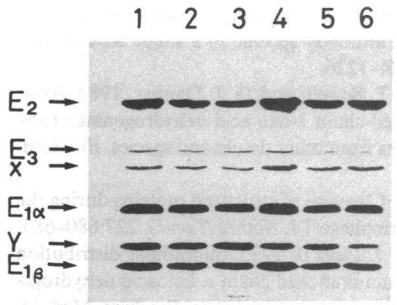

Figure 8. Immunoblots of the PDH complex in lymphoblastoid cell lines from MSUD subjects. The procedures were the same as for Fig. 6 except that antiserum raised against the bovine PDH complex was used for the immunoblotting. The cell lines used were the same as for Fig. 6, except that lane 7 (GM 1366) was omitted. The PDH complex peptides were, in decreasing size; (E_2) dihydrolipoyl transacetylase, (E_3) dihydrolipoyl dehydrogenase, X, ($E_{1\alpha}$) the α-subunit of pyruvate dehydrogenase, Y, and ($E_{1\beta}$) the β-subunit of pyruvate dehydrogenase. X was consistently found in our purified preparation of the PDH complex. The origin of Y is unknown.

method (14). They also reported that 23 of 24 cell lines derived from MSUD patients possessed all the immunoreactive BCKDH proteins. In our study, five of the six cell lines showed abnormal staining of $E_{1\alpha}$, $E_{1\beta}$, or E_2 components. This discrepancy may be related to the specificity of the antibodies used, or there may be differences between Caucasians and Japanese.

The genetic heterogeneity of MSUD was observed in studies involving genetic complementation analysis, in which the presence of two complementation groups was demonstrated among patients with classical MSUD (15, 16). Our present observations support the results of complementation analysis, on a molecular basis, since three classical cell lines showed abnormalities of the $E_{1\beta}$ subunit and one cell line derived from a patient who had "classical symptoms" (as cited from "The Human Genetic Mutant Cell Repository, List of Genetic Variants Chromosomal Aberrations and Normal Cell Cultures Submitted to the Repository," U.S. Department of Health and Human Service, National Institutes of Health publication No. 81-2011) did not contain the E_2 component.

Acknowledgments

We are indebted to the physicians of the following medical care units for provision of the peripheral lymphocytes from the patients with MSUD; Dr. Y. Wada, Department of Pediatrics, Nagoya City University Medical School; Dr. Y. Ichiba, Children's Medical Center, National Okayama Hospital; Dr. S. Nagaki, Department of Pediatrics, Tokyo Women's Medical College; and Dr. M. Yoshino, Department of Pediatrics, Kurume University School of Medicine. We are grateful to Professor J. E. Seegmillar (Department of Medicine, University of California, San Diego) for the suggestion to use lymphoblastoid cell lines when studying MSUD and to M. Ohara (Kyushu University) for pertinent comments on the manuscript.

Supported by a research grant from the the Ministry of Health and Welfare of Japan (Monitoring of Congenital Defects) and a grant in aid for scientific research from the Ministry of Education, Science and Culture of Japan (56480191).

References

1. Tanaka, K., and L. E. Rosenberg. 1983. Disorders of branched chain amino acid and organic acid metabolism. *In* The Metabolic Basis of Inherited Disease. J. B. Stanbury, J. B. Wyngaarden, D. S. Fredrickson, J. L. Goldstein, and M. S. Brown, editors. McGraw-Hill Book Co., New York. 440–473.

2. Reed, L. J., Z. Damuni, and M. L. Merryfield. 1985. Regulation of mammalian pyruvate and branched-chain α-keto acid dehydrogenase complexes by phosphorylation-dephosphorylation. *Curr. Top. Cell. Regul.* 27:41–49.

3. Pettit, F. H., S. J. Yeaman, and L. J. Reed. 1978. Purification and characterization of branched chain α-keto acid dehydrogenase complex of bovine kidney. *Proc. Natl. Acad. Sci. USA.* 75:4881–4885.

4. Heffelfinger, S. C., E. T. Sewell, and D. J. Danner. 1983. Identification of specific subunits of highly purified bovine liver branched-chain ketoacid dehydrogenase. *Biochemistry.* 22:5519–5522.

5. Reed, L. J., F. H. Pettit, S. J. Yeaman, W. M. Teague, and D. M. Bleile. 1980. Structure, function and regulation of the mammalian pyruvate dehydrogenase complex. *In* Enzyme Regulation and Mechanism of Action. P. Mildner and B. Ries, editors. Pergamon Press, Oxford. 47–56.

6. Menkes, J. H., P. L. Hurst, and J. M. Craig. 1954. A new syndrome: progressive familial infantile cerebral dysfunction associated with an unusual urinary substance. *Pediatrics.* 14:462–466.

7. Dancis, J., M. Levitz, S. Miller, and R. G. Westall. 1959. Maple syrup urine disease. *Br. Med. J.* 1:91–93.

8. Morris, M. D., B. D. Lewis, P. D. Doolan, and H. A. Harper. 1961. Clinical and biochemical observations on an apparently nonfatal variant of branched-chain ketoaciduria (maple syrup urine disease). *Pediatrics.* 28:918–923.

9. Dancis, J., J. Hutzler, and T. Rokkones. 1967. Intermittent branched-chain ketonuria. Variant of maple-syrup-urine disease. *N. Engl. J. Med.* 276:84–89.

10. Schulman, J., T. J. Lustberg, J. L. Kennedy, M. Museles, and J. E. Seegmiller. 1970. A new variant of maple syrup urine disease (branched chain ketoaciduria). *Am. J. Med.* 49:118–124.

11. Scriver, C. R., S. Mackenzie, C. L. Clow, and E. Delvin. 1971. Thiamine-responsive maple-syrup-urine disease. *Lancet.* i:310–312.

12. Dancis, J., J. Hutzler, S. E. Snyderman, and R. P. Cox. 1972. Enzyme activity in classical and variant forms of maple syrup urine disease. *J. Pediatr.* 81:312–320.

13. Chuang, D. T., W.-L. Niu, and R. P. Cox. 1981. Activities of branched-chain 2-oxo acid dehydrogenase and its components in skin fibroblasts from normal and classical-maple-syrup-urine disease subjects. *Biochem. J.* 200:59–67.

14. Danner, D. J., N. Armstrong, S. C. Heffelfinger, E. T. Sewell, J. H. Priest, and L. J. Elsas. 1985. Absence of branched chain acyltransferase as a cause of maple syrup urine disease. *J. Clin. Invest.* 75: 858–860.

15. Lyons, L. B., R. P. Cox, and J. Dancis. 1973. Complementation analysis of maple syrup urine disease in heterokaryons derived from cultured human fibroblasts. *Nature (Lond.).* 243:533–535.

16. Singh, S., I. Willers, and H. W. Goedde. 1977. Heterogeneity in maple syrup urine disease: aspects of cofactor requirement and complementation in cultured fibroblasts. *Clin. Genet.* 11:277–284.

17. Jinno, Y., I. Akaboshi, and I. Matsuda. 1984. Complementation analysis in lymphoid cells from five patients with different forms of maple syrup urine disease. *Hum. Genet.* 68:54–56.

18. Robinson, B. H., J. Taylor, and W. G. Sherwood. 1977. Deficiency of dihydrolipoyl dehydrogenase (a component of the pyruvate and α-ketoglutarate dehydrogenase complexes): a cause of congenital chronic lactic acidosis in infancy. *Pediat. Res.* 11:1198–1202.

19. Taylor, J., B. H. Robinson, and W. G. Sherwood. 1978. A defect in branched-chain amino acid metabolism in a patient with congenital lactic acidosis due to dihydrolipoyl dehydrogenase deficiency. *Pediat. Res.* 12:60–62.

20. Matsuda, I., J. Yamamoto, N. Nagata, N. Ninomiya, I. Akaboshi, H. Ohtsuka, and T. Katsuki. 1977. Lysosomal enzyme activities in cultured lymphoid cell lines. *Clin. Chim. Acta.* 80:483–486.

21. Jinno, Y., I. Akaboshi, T. Katsuki, and I. Matsuda. 1984. Study on established lymphoid cells in maple syrup urine disease. Correlation with clinical heterogeneity. *Hum. Genet.* 65:358–361.

22. Skaper, S. D., D. P. Molden, and J. E. Seegmiller. 1976. Maple syrup urine disease: branched-chain amino acid concentrations and metabolism in cultured human lymphoblasts. *Biochem. Genet.* 14:527–539.

23. Seegmiller, J. E. 1978. Collection and uses of lymphocyte cultures. *Monogr. Hum. Genet.* 9:244–247.

24. Wilson, J. M., B. W. Baugher, P. M. Mattes, P. E. Daddona, and W. N. Kelley. 1982. Human hypoxanthine-guanine phosphoribosyltransferase. Demonstration of structural variants in lymphoblastoid cells derived from patients with a deficiency of the enzyme. *J. Clin. Invest.* 69:706–715.

25. Shigematsu, Y., K. Kikuchi, T. Momoi, M. Sudo, Y. Kikawa, K. Nosaka, M. Kuriyama, S. Haruki, K. Sanada, and N. Hamano. 1983. Organic acids and branched-chain amino acids in body fluids before and after multiple exchange transfusions in maple syrup urine disease. *J. Inherited Metab. Dis.* 6:183–189.

26. Nagaki, S., H. Oguni, H. Sugie, R. Umezu, M. Osawa, M. Hara, and Y. Fukuyama. 1981. A case of variant type maple syrup urine disease. *J. Jpn Pediatr. Soc.* 85:1531–1539.

27. Sakaguchi, Y., S. Okada, M. Yoshino, C. Yasuoka, I. Yoshida, H. Watari, Y. Koga, S. Aramaki, and F. Yamashita. 1983. A report of a patient with maple syrup urine disease and normal psychomotor development. *J. Jpn. Pediatr. Soc.* 87:2426–2435.

28. Dulbecco, R., and M. Vogt. 1954. Plaque formation and isolation of pure lines with poliomyelitis viruses. *J. Exp. Med.* 99:167–182.

29. Loewenstein, J., H. R. Scholte, and E. M. Wit-Peeters. 1970. A rapid and simple procedure to deplete rat-liver mitochondria of lysosomal activity. *Biochim. Biophys. Acta.* 223:432–436.

30. Mackall, J., M. Meredith, and M. D. Lane. 1979. A mild procedure for the rapid release of cytoplasmic enzymes from cultured animal cells. *Anal. Biochem.* 95:270–274.

31. Hogeboom, G. H. 1955. Fractionation of cell components of animal tissues. *Methods Enzymol.* 1:16–19.

32. Lawson, R., K. G. Cook, and S. J. Yeaman. 1983. Rapid purification of bovine kidney branched-chain 2-oxoacid dehydrogenase complex containing endogenous kinase activity. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 157:54–58.

33. Matuda, S., T. Shirahama, T. Saheki, S. Miura, and M. Mori. 1983. Purification and immunochemical studies of pyruvate dehydrogenase complex from rat heart, and cell-free synthesis of lipoamide dehydrogenase, a component of the complex. *Biochim. Biophys. Acta.* 741: 86–93.

34. Goudie, R. B., C. H. W. Horne, and P. C. Wilkinson. 1966. A simple method for producing antibody specific to a single selected diffusible antigen. *Lancet.* ii:1224–1226.

35. Heffelfinger, S. C., E. T. Sewell, and D. J. Danner. 1983. Antibodies to bovine liver branched-chain 2-oxo acid dehydrogenase cross-react with this enzyme complex from other tissues and species. *Biochem. J.* 213:339–344.

36. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

37. Danner, D. J., and L. J. Elsas II. 1975. Subcellular distribution and cofactor function of human branched chain α-ketoacid dehydrogenase in normal and mutant cultured skin fibroblasts. *Biochem. Med.* 13: 7–22.

38. Ichiyama, A., S. Nakamura, Y. Nishizuka, and O. Hayaishi. 1970. Enzymic studies on the biosynthesis of serotonin in mammalian brain. *J. Biol. Chem.* 245:1699–1709.

39. Ide, S., T. Hayakawa, K. Okabe, and M. Koike. 1967. Lipoamide dehydrogenase from human liver. *J. Biol. Chem.* 242:54–60.

40. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.

41. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.

42. Danner, D. J., S. K. Lemmon, J. C. Besharse, and L. J. Elsas II. 1979. Purification and characterization of branched chain α-ketoacid dehydrogenase from bovine liver mitochondria. *J. Biol. Chem.* 254:5522–5526.

43. Eisenstein, R., G. Hoganson, R. Miller, and A. Harper. 1985. Characterization of branch chain keto acid dehydrogenase (BCKAD) deficiency in maple syrup urine disease (MSUD). *Am. J. Hum. Genet.* 37:A8. (Abstr.)

44. Stanley, C. J., and R. N. Perham. 1980. Purification of 2-oxo acid dehydrogenase multienzyme complexes from ox heart by a new method. *Biochem. J.* 191:147–154.

45. Ho, L., C-W. C. Hu, S. Packman, and M. S. Patel. 1986. Deficiency of the pyruvate dehydrogenase component in pyruvate dehydrogenase complex-deficient human fibroblasts. Immunological identification. *J. Clin. Invest.* 78:844–847.

46. Stepp, L. R., and L. J. Reed. 1985. Active-site modification of mammalian pyruvate dehydrogenase by pyridoxal 5′-phosphate. *Biochemistry.* 24:7187–7191.