RAPID DIAGNOSIS OF MAPLE SYRUP URINE DISEASE (BRANCHED CHAIN KETOACIDURIA) BY MICRO-ENZYME ASSAY IN LEUKOCYTES AND FIBROBLASTS*

Institut für Humangenetik, Universität Hamburg** (W. Germany)
(Received January 22, 1973)

SUMMARY

Two micro-enzyme assays are described for measuring directly the level of the branched chain α-keto acid decarboxylase in leukocytes and cultured fibroblasts, utilizing the conversion of [α-1-14C]keto acids as substrates. The cell material per assay is greatly reduced by using microtiter plates: 50 µl of whole venous blood or about 10000 fibroblasts, tested in monolayer, are needed per assay, and yet, accurate and reproducible results are obtained. The blood test improves and facilitates the early specific diagnosis of maple syrup urine disease in newborn infants, the test in fibroblasts assures early prenatal diagnosis.

INTRODUCTION

Maple syrup urine disease (MSUD) is biochemically characterized by a deficiency in the oxidative decarboxylation of the α-keto acids deriving from the branched chain amino acids leucine, valine, and isoleucine. These amino acids as well as their corresponding α-keto acids (KIC = α-keto isocaproic acid, KIVA = α-keto isovaleric acid, and MEVA = α-keto-β-methyl valeric acid) are elevated in the plasma of untreated patients; branched chain α-keto acid decarboxylase activity is markedly reduced in leukocytes and fibroblasts.

Recently it has become clear that MSUD is heterogenous. Aside from the classic type with very severe clinical course and nearly absent oxidase activity, variant forms have been described which show reduced levels of oxidase activity, but somewhat higher than in classic MSUD. Clinically they are more benign, sometimes with intermittent symptoms or delayed onset. Scriver et al. reported a form that can be cured by thiamine. In the different forms of the disease, the high molecular branched chain α-keto acid oxidase complex, with its three specific enzyme proteins...
(decarboxylase, transacylase, and lipoamide oxidoreductase) might be altered in different ways.

Effective and direct enzyme assays are required (i) for an accurate classification of the clinically different types and a reasonable assessment of the prognosis, (ii) for a reliable differentiation of MSUD from other forms of hyperleucinemia or hypervalinemia, (iii) for prenatal diagnosis and genetic counseling in high risk families (heterozygote test).

Enzyme assays for MSUD currently available are complicated and time consuming.

We here describe two rapid methods of direct enzyme assay which require only little cell material. One method provides a rapid and definitive diagnostic test for MSUD from 50 μl of venous blood per assay, the other measures the enzyme level in fibroblasts in monolayer, including an exact determination of residual enzyme activities. This assay appears suitable for biochemical characterization of MSUD. Both assays determine the liberation of 14CO2 from branched chain [α-L-14C]keto acids (KIC, KIVA, MEVA) as substrates, primarily a function of the cellular decarboxylase. On a microtiter plate, as many as 44 single assays can be processed within 1 h of working time.

MATERIALS

DL-[L-14C]leucine and DL-[I-14C]valine were obtained from The Radiochemical Centre, Amersham. Sodium salts of the unlabelled α-keto acids were bought from Sigma, St. Louis. Fetal calf serum and growth media (DME = Dulbecco’s Modified Eagle Medium) were purchased from Gibco, New York; HEPES (N-2 hydroxyethylpiperazine sulfonic acid) and PBS (Dulbecco’s phosphate buffered saline, pH 7.2) from Scrv;a, Heidelberg; microtiter plates, Type Cook, 96 wells of 7.5 mm diameter, from Greiner, Nütingen, Germany, and glass fibre platelets, Ø 10 mm, from Millipore, Neu Isenburg, Germany.

The cocktail for scintillation counting contained 3.2 l toluol, 1.6 l ethanol, 19.2 g 2,5-diphenyloxazole (POPOP), and 0.48 g 1,4-bis-(5-phenyloxazolyl-2)-benzene (POPOP).

[I-14C]-labelled branched chain α-keto acids, α-keto isocaproic acid (KIC) and α-keto isovaleric acid (KIVA) were prepared from the corresponding radioactive DL-amino acids by oxidative deamination according to Rüdiger et al. and handled as described there. The labelled α-keto acids (specific radioactivity for KIC 55 mCi/m mole and 37.5 mCi/m mole for KIVA) were adjusted to 106 counts/min/ml and the solution was stored at −20°. Immediately prior to use, quantities needed for a series of tests were thawed and adjusted to pH 4–5 with 1 N NaOH.

ASSAY PROCEDURE

Blood cells

0.2 ml of venous blood is mixed with 0.2 ml of ACD, pH 6.5 and 0.05 ml of a solution of [α-L-14C]keto acid (KIC or KIVA) to give a final pH of 6.9. 0.1 ml of this incubation mixture is pipetted into the wells of a microtiter plate. The arrangement of a plate is shown in Fig. 1. Each well represents a single assay. Usually 4 parallel assays with one substrate are performed per blood sample. The actual amount of sub-
Fig. 1. Arrangements of 44 decarboxylase assays and four blanks on a microtiter plate.

strate per test depends on the content of the stock solution and is here 9.3 nmoles of \([1-^14C]KIC\) (9.2 \(\times 10^5\) counts/min) and 22.7 nmoles of \([1-^14C]KIVA\) (1.52 \(\times 10^6\) counts/min). A blank is used for each blood sample with about 1000-fold excess of the respective unlabelled substrate.

Each well is tightly covered with a glass fibre platelet soaked with 3.5 N NaOH. The whole plate is tightly closed with a cover plate and incubated at 37° in a moist atmosphere. During incubation, the \(^{14}CO_2\) liberated from the substrate is trapped by the alkaline platelets. After 3 h of incubation, the platelets are lifted and 1 drop of 10% H\(_2\)SO\(_4\) is added carefully to the incubation mixture with a tuberculine syringe. The wells are immediately reclosed by the alkaline platelet and left for 10 min at room temperature. This procedure substantially increases the yield of the trapped \(^{14}CO_2\). Finally the platelets are removed with forceps and transferred into vials with 12 ml scintillation cocktail each, and counted in a Packard Tricarb scintillation spectrometer. Identical counts for each assay during different counting cycles were obtained after storing the vials at 4° for 6 to 8 h.

The leukocyte count, according to routine methods, ranged from 5000–10000/\(\text{mm}^3\) in all samples.

**Fibroblasts**

Fibroblasts were cultured by standard methods from explants of skin biopsies from individuals who were homozygous for MSUD, obligate heterozygotes, or normal controls of unknown state with respect to heterozygosity for MSUD. The growth medium was DME medium, buffered with 20 mM HEPES to pH 7.6 at air equilibrium, containing 16% fetal calf serum and antibiotics.

Twenty-three cell strains were assayed as normal controls, five being obligate
heterozygote for MSUD and four derived from affected patients. Two patients (H.G. and K.Q.) showed a moderate clinical course, and two (K.C. and A.R.) the clinical picture of classical MSUD. The five obligate heterozygotes belonged to A.R. (1), K.Q. (2), H.G. (1) and to a patient suffering from intermittent type of MSUD (1).

One day before the test, the fibroblasts are trypsinized (0.25% trypsin in physiological saline) and about 50,000 to 100,000 cells suspended per ml of DME medium. 10,000–20,000 cells (0.2 ml, about 5–10 µg of cell protein) are seeded into each well of a microtiter plate and incubated at 37°C overnight. A confluent layer usually forms after 12–24 h. Then the medium is sucked off and replaced by 0.1 ml/well of a freshly prepared reaction mixture, composed of 3.5 ml PBS, 1.0 ml fetal calf serum, and 0.5 ml of 0.5 mM labelled α-keto acid (KIC: 2.475 x 10^7 ± 10% counts/min, KIVA: 1.6875 x 10^7 ± 10% counts/min corresponding to 5–6 nmoles per test.

Wells containing reaction medium without cells serve as reagent blanks in order to estimate the spontaneous decomposition of the branched chain α-keto acids at the adjusted pH of 6.8–6.9 of the mixture. Blanks with freshly prepared substrates typically exhibit 375 counts/min (3.7 pmoles of substrate) for KIC and 750 counts/min (11 pmoles of substrate) for KIVA.

The plates are handled further according to our assay with leukocytes, except that incubation is extended to 4 h and final acidification is omitted. After removing the glass fibre platelets, the monolayer is rinsed several times with prewarmed PBS and then dissolved in 2.5 N NaOH (0.05 ml/well). The mixture is dried overnight at 80°C. The alkaline residue is neutralized with 0.2 ml of an exactly adjusted HCl and cell protein is determined according to Lowry et al. Results are expressed in pmoles 14CO2 evolved per 106 µg cell protein and 4 h.

RESULTS

The decarboxylase reaction was linear for at least 6 h in fibroblasts and for 3 h in leukocytes (Fig. 2). During the incubation time used in the present investigation (4 h for fibroblasts and 3 h for leukocytes), less than 3% of the substrate were converted.

In contrast to the assay in fibroblasts, a substantial amount of 14CO2 was retained as bicarbonate in the incubation mixture in the assay using heparinized whole blood, probably caused by erythrocyte carbonic anhydrase and reduced hemoglobin (Fig. 2). The radioactivity retained could be expelled by final acidification of the incubation mixture which resulted in a linear reaction during the first 3 h.

Furthermore, Fig. 2 shows that substrate conversion correlated quite well with the number of intact leukocytes in the test system and was independent from erythrocytes.

The decarboxylase activities of 17 different blood samples from normal controls, two patients suspected to have MSUD, and members of their family are shown in Fig. 3. They were assayed under standardized conditions 1 to 5 h after the blood had been drawn. The amount of 14CO2 evolved per 106 leukocytes within 3 h averaged for controls at 12.1 ± 4.6 pmoles for KIC and 34.8 ± 8.3 for KIVA as substrates. KIVA was constantly converted faster than KIC, but to determine the ratio KIVA/KIC did not seem to be appropriate (for the present results) because we did not supply equal amounts of both substrates per test.
DIAGNOSIS OF KETOACIDURIA

Fig. 2. $^{14}$CO$_2$ trapped by the NaOH-soaked glass fibre platelets in decarboxylase assays with
blood of a normal control starting 1 h after drawing blood. The incubation mixture (0.1 ml)
contained 133,300 leukocytes and 11.4 nmoles of [1-$^{14}$C]KIC per test. Incubation mixture with final
acidification - - - - - - ; without acidification - - - - - - - - blank. The system contained 11.4
nmoles [1-$^{14}$C]KIC and 11 μmoles non-radioactive KIC. - - - - Assay with the same blood sample
after reduction of the leukocytes to 4,440 per test by a cotton wool column according to Busch
and Pele.\textsuperscript{17}

Fig. 3. Branched chain α-keto acid decarboxylase activity in leukocytes. Enzyme activities are
expressed in amounts of $^{14}$CO$_2$ evolved per $10^6$ leukocytes and 3 h (means of 4 parallel tests) from
9.3 nmoles [1-$^{14}$C]KIC and 22.7 nmoles [1-$^{14}$C]KIVA as substrates. Whole blood was assayed
1–3 h after drawing. Symbols: o normal controls; △ patients; □ obligate heterozygotes; × sibs
of the patients.

Fig. 4. Branched chain α-keto acid decarboxylase activity in fibroblasts. Activities are expressed
as amounts of $^{14}$CO$_2$ evolved per 10 μg cell protein and 4 h (means of 4 parallel assays) from
5 nmoles of [1-$^{14}$C]KIC and [1-$^{14}$C]KIVA respectively. Fibroblasts of normals (○), obligate hetero-
zygotes (□) and affected individuals (△) were tested.
TABLE I

STATISTICAL EVALUATION OF THE RESULTS SHOWN IN FIG. 4

<table>
<thead>
<tr>
<th>Activity [pmoles (^{14}\text{CO}_2)] against</th>
<th>KIC</th>
<th>KIVA</th>
<th>KIVA/KIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normals</td>
<td>$\bar{x} \pm s$</td>
<td>$s \pm s$</td>
<td>V</td>
</tr>
<tr>
<td></td>
<td>Activity</td>
<td>Residual activity</td>
<td>pmoles</td>
</tr>
<tr>
<td>--------</td>
<td>----------</td>
<td>-------------------</td>
<td>------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>47.0 \pm 12.6</td>
<td>86.2 \pm 28.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.26</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>Obligate</td>
<td>24.4 \pm 5.7</td>
<td>49.7 \pm 15.2</td>
</tr>
<tr>
<td>heterozygotes</td>
<td>0.23</td>
<td>0.39</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

Symbols

<table>
<thead>
<tr>
<th>Symbols</th>
<th>pmoles (^{14}\text{CO}_2), KIC in % of normals</th>
<th>pmoles (^{14}\text{CO}_2), KIVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.R.</td>
<td>3.6</td>
<td>8</td>
</tr>
<tr>
<td>K.C.</td>
<td>1.4</td>
<td>3</td>
</tr>
<tr>
<td>K.O.</td>
<td>3.4</td>
<td>7</td>
</tr>
<tr>
<td>H.G.</td>
<td>1.9</td>
<td>4</td>
</tr>
</tbody>
</table>

V = Variation coefficient ($s/\bar{x}$); N = number of determinations.

In spite of relatively high residual enzyme activities, especially using KIVA as substrate, both patients S.S. and H.G. were clearly identified. The activities of normal controls as well as obligate heterozygotes did not overlap with those of patients. Evidently this method does not discriminate heterozygotes and normal homozygotes.

Fibroblasts of different ages in culture were tested when confluent. The activities obtained from normals, obligate heterozygotes, and affected individuals with interindividual variations of the enzyme level are shown in Fig. 4 and Table I. Small variations in age and density of the cellular monolayer did not influence the rates of substrate conversion per 10 \(\mu\text{g}\) protein.

The intraindividual variation coefficient ($s/\bar{x}$) of the enzyme activity measured against both substrates in a normal cell strain (6 independent estimations) was found to be 0.15, whereas the interindividual variation was 0.26 for KIC and 0.33 for KIVA. Fibroblasts of the obligate heterozygote subjects showed for KIC 52% and for KIVA 58% of normal activity.

Four patients with MSUD had residual enzyme activities in the range of 3–8% of normal controls, KIVA was constantly converted at a higher rate than KIC. The KIVA/KIC ratio was determined 1.89 \pm 0.58 and was identical in heterozygotes and affected individuals, except one patient (H.G.). In this patient the residual enzyme activity with KIVA was 4 times higher than with KIC in both fibroblasts and leukocytes.

DISCUSSION

Our assay for the branched chain \(\alpha\)-keto acid decarboxylase in white blood cells utilizes whole blood and obviates the previous leucocyte separation. A similar procedure used more than 10-times the amount of blood per test and radioactive amino acid as substrate. Since branched chain \(\alpha\)-keto acid oxidase is a mitochondrial enzyme complex, erythrocytes do not influence the substrate conversion specifically. Final acidification of the reaction medium was sufficient to overcome the loss of labelled.
bicarbonate in the reaction medium which followed the hydration of liberated $^{14}\text{CO}_2$
by erythrocyte carbonic anhydrase.

The range of enzyme activities obtained in relation to the total leukocyte count
was wide. Differences in time elapsed between drawing blood and test, and actual
composition of blood samples under study may have accounted for this fact in the
following way: leukocytes represent a mixed population with different vitality of
each cell type; neutrophile granulocytes are especially vulnerable and their real
functional state might be difficult to assess, because morphologically intact cells may
yet be disturbed functionally. Furthermore, it can be expected that lymphocytes and
granulocytes differ in decarboxylase activity. The method used thus far for quanti-
tative comparison of decarboxylase activity in both cell types are not absolutely
conclusive, because decreased viability of neutrophile granulocytes may mimic
preferential location of the decarboxylase in lymphocytes. No attempt was made to
eliminate the contribution of enzyme activity of the blood platelets separately.

The plasma portion added to the test system contributes to the inaccuracy. The
amount of blood per assay contains some variable amount of branched chain amino
acids: less than 3 nmoles of free leucine and less than 4.5 nmoles of free valine. Pro-
vided the transamination step is not rate limiting, the amino acids will reduce the
specific radioactivity of the substrate added. Especially in untreated MSUD without
restriction in diet, when the plasma level of amino acids and $\alpha$-keto acids are increased,
but also in isolated hyperleucinemia or hypervalinemia, the results from the assay
with whole blood will be relatively too low.

In view of the differences of the blood samples we did not attempt to evaluate
the normal range of leukocyte decarboxylation. Although heterozygotes for MSUD
cannot be diagnosed with the assay in whole blood, it provides a rapid and specific
diagnostic test for MSUD as long as appropriate controls are run along with each
suspected patient.

Results that are more precise and reproducible, including exact estimations of
the residual level of enzyme activity in affected patients, are obtained with the assay
in fibroblasts. As a fairly homogeneous cell population, their enzyme level of branched
chain $\alpha$-keto acid decarboxylation varies by 20%.

Although, as a group, the obligate heterozygotes had only approximately 50% of
the normal enzyme level, the overlap between both groups does not allow a reliable
differentiation of a single case.

However, this micromethod clearly and consistently identifies patients with
MSUD and separates them from normals and heterozygotes. Residual enzyme activi-
ties from four patients with MSUD varied for both substrates from 3-9% of the
mean normal level, similar residual enzyme activities were obtained with 5 nmoles of
$[1-^{14}\text{C}]$leucine as substrate. The residual activity of K.C. and A.R., patients presenting
the clinical picture of classic MSUD, is higher when compared to residual activity of
corresponding patients reported by Dancis et al. 11.

The pH of the test medium seems to be rather critical, especially for the deter-
mination of the residual activity. Our blanks were probably lower than others, because of the low initial pH of 6.8-6.9 in our assay. This apparently prevents a
further drop of the pH owing to cell metabolism as compared to blanks which remain
at the original pH of about 7.2 and consequently could lead to a higher degree of
unspecific alkaline decomposition of the substrate.
Fetal calf serum added to the test system for nutritional requirements of the fibroblasts also contains branched chain amino acids, which dilutes the labelled substrates as a constant proportion. Consequently, we observed an increase in $^{14}$CO$_2$ liberation when we used dialysed fetal calf serum.

We could not test MEVA as substrate because [I-$^{14}$C]isoleucine, which is needed for substrate preparation, was not available. Both tests gave lower counts/min when L-[I-$^{14}$C]leucine was measured as substrate and were reproducible.

Our test requires about 10000 cells per test; this will improve the prospects for an early prenatal diagnosis of MSUD. We successfully tested cultured amniotic cells equivalent to 5–6 µg of cell protein and found their decarboxylase activity within the range of normal fibroblasts. The sensitivity of the assay allows to reduce the cell number per test further by at least a factor of 5. In the course of prenatal diagnosis, repeated assays are possible 14 days after amniocentesis$^{14}$ even from cells of the primary culture.

ACKNOWLEDGEMENTS

We thank Miss Sabine Buss and Mrs Henriette Westermann for technical assistance. For supply of the fibroblasts of the patients K.C., A.R., H.E., K.Q. we are indebted to Dr. L. Elsas, Atlanta, Dr. V. A. McKusick, Baltimore, Dr. H.-P. Koepp, Hamburg and Dr. L. Schuchmann, Freiburg.

REFERENCES

15 J. L. Tullies, Blood, 8 (1953) 593.