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D-Glycerate kinase deficiency as a cause of D-glyceric aciduria

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D-Glycerate kinase was measured in human livers thanks to a new, sensitive radiochemical assay. The enzyme was extremely unstable in extracts prepared in water, but was partly stabilized in a homogenization mixture containing inorganic phosphate, D-glycerate and EGTA. When extracted in such a stabilizing mixture, glycerate kinase activity amounted to 0.86 ± 0.21 U/g in control livers and to 0.03 U/g in the liver of a patient with D-glyceric aciduria. In contrast, D-glycerate dehydrogenase (glyoxylate reductase) and triokinase activities were not deficient in the liver of the same patient. It is concluded that D-glycerate kinase deficiency is a cause of D-glyceric aciduria.

Glycerate, D-; Glycerate kinase, D-; Glyceric aciduria, D-; Inborn metabolic error; Serine

1. INTRODUCTION

D-Glycerate, an intermediate of one of the pathways of serine degradation and of a minor pathway of fructose metabolism is normally not detectable in urine [1,2]. It is, however, excreted in large amounts by a few patients with D-glyceric aciduria, a rare inborn error of metabolism associated with delayed psychomotor growth, mental retardation and seizures [1–4]. The enzymatic defect responsible for this condition has up to now not been described, partly because of the unavailability of liver samples from patients with this disorder, and partly because of the lack of knowledge about the fate of D-glycerate in humans. Although it now seems clear that D-glycerate is an intermediate in the degradation, not in the synthesis of L-serine [5], D-glycerate kinase has been reported to be poorly active in human liver and, hence, was suspected of being devoid of a physiological role [6].

It is shown here that the activity of D-glycerate kinase in normal human liver has been underestimated by previous investigators, presumably because of its great instability in extracts, and that this enzyme is profoundly deficient in the liver of a new case of D-glyceric aciduria.

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2. MATERIALS AND METHODS

2.1. *Materials*

Coenzymes and auxiliary enzymes were from Boehringer (Mannheim, FRG). The hemicalcium salt of D-glycerate, lithium hydroxypyruvate, sodium glyoxylate and sodium EGTA were from Sigma (St. Louis, MO). Chemicals were from Merck (Darmstadt, FRG). Dowex AG 1-X8 was from BioRad laboratories (Richmond, CA).

2.2. *Synthesis of D-[^{14}C]glycerate*

10 μCi (40 nmol) of [U-^{14}C]glucose (The Radiochemical Centre, Amersham, Bucks) were incubated at 25°C, in a final volume of 1 ml, in the presence of 50 mM Tris, pH 8, 5 mM Mg acetate, 200 μM ATP, 1 μM fructose 2,6-bisphosphate, 50 μM AMP, 5 mM sodium arsenate, 1 mM NAD and the following mixture of enzymes that had been freed from most of their ammonium sulfate by centrifugation and resuspension in a 10 mM Tris buffer, pH 8, containing 1 μM fructose 2,6-bisphosphate, 10 μg yeast hexokinase, 10 μg yeast phosphoglucose isomerase, 50 μg rabbit muscle 6-phosphofructo-1-kinase, 10 μg rabbit muscle aldolase, 10 μg rabbit muscle triose-phosphate isomerase and 50 μg rabbit muscle glyceraldehyde 3-P-dehydrogenase. The reaction was followed by measuring A_{340} and was arrested after 90 min by heating the mixture for 5 min at 80°C. The sample was centrifuged and the resulting supernatant applied on a small (0.5 × 4 cm) AG1-X8 (200–400 mesh,

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Cl form) column. The column was washed successively with 3 ml water, 3 ml of 0.15 M NaCl and, finally, with 3 × 1 ml of 0.4 M NaCl in order to elute glycerate 3-phosphate. The latter was converted to D-glycerate by a 60 min incubation at 30°C in the presence of 20 mM Tris, pH 8, 5 mM Mg acetate and 10 μg calf intestine alkaline phosphatase in a final volume of 4 ml. The reaction was stopped by a 5 min incubation at 80°C. After cooling, the mixture was diluted 4-fold with water and applied to a 5 cm^3 AG1-X8 column which was washed with 10 ml water. D-Glycerate was then eluted with 10 ml of 0.2 M NaCl. The overall yield of the conversion of [^{14}C]glucose to D-[^{14}C]glycerate was about 70%. The purity of the final product was indicated by the fact that it could be completely phosphorylated by a partially (approx. 200-fold) purified preparation of rat liver D-glycerate kinase.

2.3. *Human liver specimens*

Two needle biopsies taken from a young female patient with D-glyceric aciduria at the age of 9 and 12 months were kindly provided by Dr C. Largillière (Lille). This patient showed delayed psychomotor development, axial hypotonia and spastic tetraparesia. The presence of high concentrations (typically 20–50 mM) of D-glyceric acid was disclosed by gas chromatography and by enzymatic analysis (Largillière, personal communication). The full clinical history and the biological findings concerning this patient will be reported elsewhere (Largillière et al., in preparation). The control samples used in this study were either biopsy specimens from patients suspected of having another metabolic disorder, or pieces of donor tissue obtained in the course of liver transplantations (kindly provided by Professor J.B. Otte, Brussels). The liver samples were homogenized in a Kontes glass homogenizer with 50 vols of water or of a mixture containing 10 mM P_i, pH 7.1, 50 μM D-glycerate and 1 mM EGTA. These homogenates were used as such for the radiochemical assay of D-glycerate kinase; the supernatant resulting from a 5 min centrifugation at 10000 × *g* was used for the other enzymatic assays.

2.4. *Enzyme assays*

In preliminary experiments, D-glycerate kinase was assayed by a spectrophotometric procedure [7]. The mixture of this assay contained 50 mM Hepes, pH 7.1, 0.15 mM NADH, 20 mM KCl, 7 mM Mg acetate, 0.1% Triton X-100, 5 mM EGTA, 2.5 mM ATP, 2.5 mM ADP, 1 mM D-glycerate and 2 μg/ml of rabbit muscle enolase and pyruvate kinase as well as 10 μg/ml rabbit muscle lactate dehydrogenase. In other experiments, a radiochemical assay was used. This assay was initiated as soon as possible after preparation of the homogenate (< 10 min). 50 μl homogenate were incubated at 30°C in an assay mixture containing 50 mM Hepes, pH 7.1, 20 mM KCl, 7 mM Mg acetate, 0.1% Triton X-100, 5 mM EGTA, 0.1 mM D-[^{14}C]glycerate (50000 cpm) and 5 mM ATP in a final volume of 0.2 ml. EGTA was included in the assay because Ca^{2+}, which is present in the commercial preparation of D-glycerate, was found to inhibit glycerate kinase. 50 μl portions were removed at various times from the reaction mixture and mixed with 200 μl of 95% ethanol. These samples were subsequently diluted with 1 ml water and applied on small (0.4 × 4 cm) Dowex AG1-X8 (Cl form) columns. The columns were washed with 6 ml of 10 mM Tris, pH 8, containing 0.125 mM NaCl in order to elute D-glycerate. Phosphoglycerates were eluted with 3 ml of 400 mM NaCl in 10 mM Tris, pH 8. 2 ml of the latter fraction were mixed with 15 ml of Pico Fluor™ 15 (Packard Instrument Company) and counted for radioactivity.

Triokinase was assayed spectrophotometrically at 30°C in an assay mixture containing 50 mM Hepes, pH 7.1, 2.5 mM Mg acetate and 0.075 mM NADH as well as 2 μg glycerol-3-phosphate dehydrogenase and 0.2 μg triose phosphate isomerase in a total volume of 1 ml. The assay of D-glycerate dehydrogenase (glyoxylate reductase [8,9]) was performed at 25°C in a mixture containing 50 mM Hepes, pH 7.1, 100 mM KCl, 0.125 mM NADPH and 200 μM hydroxypyruvate or glyoxylate. Care was taken to consider only initial rates, i.e., before 10% of the substrates had been consumed. Fructose-1,6-bisphosphatase was assayed spectrophotometrically at 25°C in an assay mixture (1 ml) containing 50 mM imidazole, pH 8, 50 mM KCl, 20 mM Mg acetate, 0.5 mM NADP, 0.2 mM EDTA, 5 μg yeast glucose-6-phosphate dehydrogenase and 50 μg yeast phosphoglucose isomerase. One unit of enzyme is the activity corresponding to the formation of 1 μmol of product/min.

3. RESULTS

3.1. *D-Glycerate kinase assay*

In previous work, D-glycerate kinase was measured by a spectrophotometric assay, in which the 2-phosphoglycerate formed was converted enzymatically to lactate with concomitant oxidation of NADH. In the present work, it was found that the blank, corresponding to the oxidation of NADH in the absence of D-glycerate, amounted to about 1/5th of the D-glycerate kinase activity present in rat liver (~3 U/g fresh wt). It was, however, approximately equal to the D-glycerate kinase activity present in human livers (0.13 U/g as a mean according to [6]), preventing an accurate determination of this activity in human biopsy specimens.

It was, therefore, necessary to set up a more sensitive assay. The method developed in this study is based on the ATP-dependent conversion of D-[^{14}C]glycerate into [^{14}C]phosphoglycerates, which can then be separated by a stepwise salt gradient on small AG 1-X8 columns. Due to the low K_m value of human liver D-glycerate kinase (approx. 15 μM, see below) the activity of D-glycerate kinase could be measured under near V_{max} conditions with 100 μM D-glycerate. As illustrated in fig.1, the formation of [^{14}C]phosphoglycerates was readily detectable with an amount of homogenate corresponding to 0.2 mg liver. This formation was dependent on the presence of ATP and was linear with respect to time and to the amount of extract

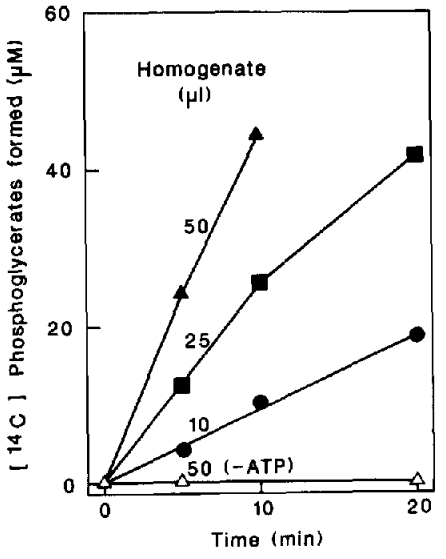

Fig.1. Time-course of the ATP-dependent conversion of [^{14}C]glycerate into phosphoglycerates by a human liver homogenate. A sample of human liver homogenate was homogenised in 50 vols of 10 mM KP_i, pH 7.1, 50 μM D-glycerate and 1 mM EGTA. The indicated amounts of resulting homogenate were incubated in the D-glycerate kinase assay mixture (closed symbols) or in the same mixture except ATP was omitted (△).

added, at least until 25% of the radioactive substrate had been consumed. Under the conditions of the assay, a doubling of the blank value (approx. 0.5% of the total radioactivity) in 20 min corresponded to 5 μU of enzyme, a value which was considered to be the detection limit of the assay.

3.2. *D-Glycerate kinase assay in human liver*

The activity of D-glycerate kinase present in human liver extracts prepared in water (table 1) was in close agreement with the mean value of 0.13 U reported by Heinz et al. [6]. The enzyme was, however, quite unstable in such extracts. As shown in fig.2, the loss of activity at 0°C was most rapid during the first 10 min following homogenization and then became progressively slower. Because of the rapid initial loss, the activity measured as soon as possible (~1 min) after homogenization could, nonetheless, be an underestimate of the actual value in the liver. It was, therefore, of interest to find extraction conditions that would stabilize the human liver enzyme.

Bovine serum albumin at a concentration of 1 mg/ml, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 mM EGTA or 10 mM P_i did not appreciably stabilize the enzyme (not shown). In contrast, the use of a homogenization medium containing 10 mM KP_i, 50 μM D-glycerate and 1 mM EGTA not only slowed down the loss of D-glycerate kinase activity (fig.2), but also resulted in a severalfold higher initial enzymic activity (fig.2, table 1). Extraction of the livers with water followed immediately by the addition of the stabilization mixture was found not to be satisfactory, because it yielded an activity amounting to 30% of the value obtained in homogenates prepared directly in the stabilizing buffer (table 1).

Table 1 also shows that, whatever the homogenization procedure used, the activity of D-glycerate kinase was at least 10-fold lower in the liver of the patient with D-glyceric aciduria. Mixing experiments yielded values that were the arithmetic means of the activities found in the homogenates tested separately, indicating that the low activity found in the liver of the patient was not due to the presence of an inhibitor (not shown). The K_m of the patient's enzyme for D-

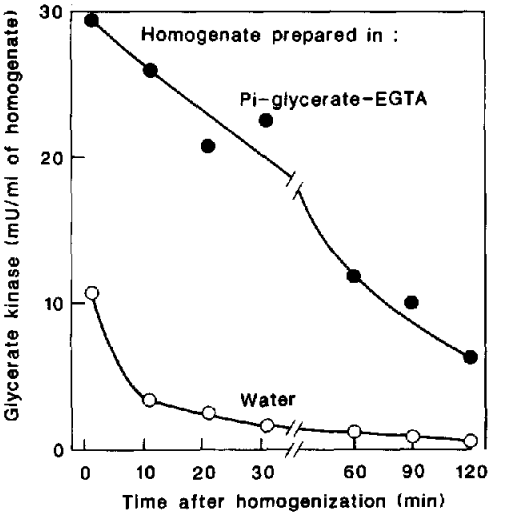

Fig.2. Loss of D-glycerate kinase activity in human liver homogenates. Samples from a human liver were homogenised in 50 vols of water (○) or of a homogenising mixture containing 10 mM KP_i, 50 μM D-glycerate and 1 mM EGTA (●), and the resulting homogenates were maintained at 0°C. D-Glycerate kinase activity was determined at the indicated times by the radiochemical assay.

Table 1

Activity of glycerate kinase and of other enzymes in human liver

Enzyme	Control	Range	Patient
Glycerate kinase			
Extraction in water	0.15 ± 0.03 (10)	0.04– 0.32	<0.005
P_i/D-glycerate/EGTA	0.86 ± 0.21 (5)	0.26– 1.60	0.03
Water, then supplemented with P_i/D-glycerate/EGTA	0.28 ± 0.08 (4)	0.12– 0.48	0.03
Glycerate dehydrogenase			
Substrate: Hydroxypyruvate	19.1 ± 1.6 (6)	13.9 –23.1	30.9
Glyoxylate	11.9 ± 0.9 (6)	8.8 –14.5	14.8
Triokinase	1.71 ± 0.31 (8)	0.61– 2.89	1.16
Fructose-1,6-bisphosphatase	3.7 ± 0.6 (6)	2.1 – 6.0	5.7

Enzymatic activities (U/g liver) ± SE

glycerate was not detectably different from the K_m of controls (15 μM). In contrast with D-glycerate kinase, the activities of fructose-1,6-bisphosphatase and of triokinase were in the normal range. The latter enzyme displayed with 200 μM D-glyceraldehyde about 60% of the activity found with 100 μM dihydroxyacetone both in the patient and in controls. It is noticeable that the activity of D-glycerate dehydrogenase measured with NADPH and either hydroxypyruvate or glyoxylate as substrates was slightly above the range of the control values. These activities were not attributable to lactate dehydrogenase since the pyruvate dependent oxidation of NADPH in human liver extracts amounted to less than 0.5 U/g.

4. DISCUSSION

4.1. *D-Glycerate metabolism in the liver*

D-Glycerate arises in higher animals through the metabolism of serine and of fructose. Serine can indeed be converted by transamination to hydroxypyruvate, the latter being reduced to D-glycerate by D-glycerate dehydrogenase. Although it has been believed for some time that these two reactions might be involved in the biosynthesis of serine [10], there is now ample evidence that they are part of one of the serine degradation pathways [5] and the fact that D-glycerate dehydrogenase deficiency results in the accumulation of L-glycerate [11–13], which is produced from hydroxypyruvate by lactate dehydrogenase [9], is a strong argument in support of this thesis. Furthermore, liver D-glycerate dehydrogenase displays much more affinity for NADPH and NADP (Van Schaftingen, unpublished) than respectively for NADH and NAD, and, since it is located in the cytosol [14], can presumably only function as a reductase in vivo.

In addition to being converted to hydroxypyruvate, serine can be deaminated to pyruvate by serine deaminase, but the activity of this enzyme has been reported to be negligible in human liver [5].

D-Glycerate can also be produced by the oxidation of D-glyceraldehyde, a product of fructose metabolism. It appears, however, that, under normal circumstances, most of the glyceraldehyde is phosphorylated by triokinase [15], which in human liver is fairly active [6].

Since, as discussed above, D-glycerate dehydrogenase cannot utilise D-glycerate in vivo, the only known fate of this compound is to be phosphorylated by D-glycerate kinase to 2-phosphoglycerate. It is shown here that D-glycerate kinase activity has been underestimated in human liver by previous investigators [6], presumably because of its instability. When extracts were prepared in the presence of a stabilising mixture, a mean value of 0.86 U/g was recorded, which may also be an underestimation, since the homogenisation medium did not completely stabilise the enzyme.

4.2. *Enzymatic defect in D-glyceric aciduria*

The accumulation of D-glyceric acid in patients with D-glyceric aciduria can conceivably result either from a decreased utilisation or from an overproduction of this compound. Since the only fate of D-glycerate in the liver appears to be its conversion to 2-P-glycerate by D-glycerate kinase, it is concluded that the marked deficiency of this enzyme found in the liver of the patient studied here is responsible for her metabolic disorder. Because D-glycerate is an intermediate in serine metabolism and that this amino acid is continuously taken up by the liver, at least in the rat [16], this interpretation accounts for the fact that the excretion of D-glycerate was continuous in this patient and that it increased following a serine load (Largillière, personal communication). These two characteristics were also found in the patient of Wadman et al. [2], who, most likely, suffered from the same enzymatic defect. From the protein intake of this patient (2.9 g per day and per kg) and assuming that serine represents 5% of the protein, it can be calculated that the amount of D-glycerate excreted by Wadman's patient (1.4 mmol/6 h at the age of 55 days) roughly corresponded to his serine intake. Although serine intake is not the only source of this amino acid, which indeed is also synthesized de novo, this comparison indicates that the pathway of serine metabolism via hydroxypyruvate and D-glycerate is quantitatively important in humans.

In contrast, D-glycerate overproduction might be the cause of the D-glyceric aciduria in the patient described by Duran et al. [4]. The excretion of D-glyceric acid by this patient was extremely dependent on the ingestion of fructose or of dihydroxyacetone, and was not influenced by a serine load. On this basis, Duran and co-workers suggested a triokinase deficiency. It is indeed expected that, in the absence of this enzyme, a major part of the glyceraldehyde arising from fructose metabolism would be converted to D-glycerate. Since the activity of D-glycerate kinase in the liver is somewhat lower than the activities of fructokinase and of fructose-1-phosphate aldolase (respectively 1.23 and 2.08 U/g at 25°C [6]), D-glycerate would then escape the liver and be found in blood and in urine. This interpretation, however, does not account for the fact that the patient excreted some D-glycerate on a fructose-free regimen. Unfortunately, the possibility that the patient had a triokinase deficiency could not be tested directly by enzymatic assay. It is noteworthy that the triokinase activity was normal in the patient studied here.

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