*DEFICIENCY OF 2-OXO-GLUTARATE: GLYOXYLATE CARBOLIGASE ACTIVITY IN PRIMARY HYPEROXALURIA**

By Jürgen Koch, E. L. Robert Stokstad, Hibbard E. Williams, and Lloyd H. Smith, Jr.

DEPARTMENT OF NUTRITIONAL SCIENCES, UNIVERSITY OF CALIFORNIA, BERKELEY, AND DEPARTMENT OF MEDICINE, UNIVERSITY OF CALIFORNIA MEDICAL CENTER, SAN FRANCISCO

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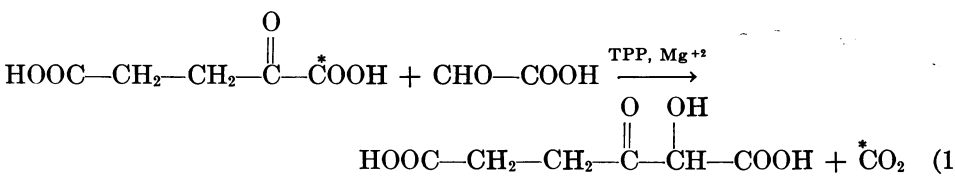
Primary hyperoxaluria is a genetic disorder of glyoxylate metabolism characterized by increased urinary excretion of oxalate, glycolate, and glyoxylate.[1-3] Calcium oxalate crystals may be deposited within the urinary tract, within the renal parenchyma (as nephrocalcinosis), and more widely throughout the body (a condition termed oxalosis). The clinical syndrome is usually manifested by kidney stones occurring in early childhood followed by chronic renal failure and death before the age of 20. Some adult patients with less severe forms of the disease have been described.

Hyperoxaluria may be produced in man or in experimental animals by pyridoxine deficiency.[4, 5] Previous attempts to demonstrate the presumed enzyme defect of the genetic disorder by *in vitro* techniques have not been successful. Certain *in vivo* studies summarized elsewhere[2, 3] suggested that the defect might lie in the conversion of glyoxylate to glycine, i.e., the apoenzyme equivalent of pyridoxine deficiency. More recent studies have failed to demonstrate reduced activity of glutamate:glyoxylate or alanine:glyoxylate aminotransferase in liver or kidney specimens from patients with this disease.[6] It was concluded that the enzyme defect must occur in some other pathway of glyoxylate metabolism.

Recently Koch and Stokstad[7] characterized in rat liver mitochondria an enzyme—2-oxo-glutarate:glyoxylate carboligase—which might be of quantitative importance in mammalian metabolism of glyoxylate:



(1)

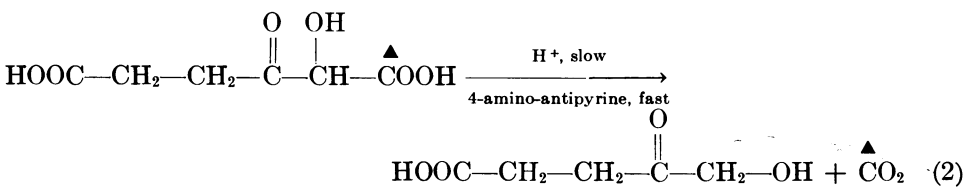
The activity of this enzyme in cytoplasmic and mitochondrial fractions from liver, spleen, and kidney obtained from patients undergoing renal homotransplantation was compared with that found in five patients with primary hyperoxaluria. The activity of the cytoplasmic carboligase, but not the mitochondrial enzyme, was found to be reduced in the genetic disorder.

Tissue Samples.—Liver, kidney, and spleen were obtained from patients undergoing bilateral nephrectomy and splenectomy prior to renal homotransplantation for chronic renal insufficiency secondary to either chronic pyelonephritis or chronic glomerulonephritis. These patients, age 20–42, were not receiving immunosuppressive or corticosteroid therapy at the time of surgery. Tissues were placed immediately in iced 0.85 per cent saline and transported to the laboratory within two to four minutes where homogenization and fractionation were carried out.

Five patients with primary hyperoxaluria were studied. In all patients urinary oxalate and glycolate levels were elevated on several occasions.[2] Liver, kidney, and spleen were obtained from patient DR at surgery performed eight weeks after cadaver kidney transplantation.[8] During this period the patient was receiving large doses of corticosteroids. Kidney tissue was obtained from patient TE during a left pyeloplasty. Liver tissue from patients JM and TS was obtained by needle biopsy. All three patients had normal creatinine clearances and were receiving no drugs at the time of biopsy. Kidney tissue from patient SI was obtained at the time of postmortem examination performed approximately six hours after death due to chronic renal failure. The kidney was frozen for approximately one month before homogenization and fractionation.

Cell Fractionation.—The procedure of Hogeboom[9] was followed. Liver was homogenized in a Potter-Elvehjem homogenizer, kidney and spleen in a low-speed Waring Blendor in 0.25 *M* sucrose–0.005 *M* K-Na-phosphate pH 7.0. The supernatant, centrifuged at 27,000 × *g* for 30 minutes (Sorvall RC-2), is designated cytoplasmic enzyme. In one case high-speed supernatants were prepared (144,000 × *g*, 30 minutes, Beckman L-2). The mitochondria were resuspended in 0.25 *M* sucrose–0.005 *M* K-Na-phosphate pH 7.0. At this stage the samples were frozen in a dry ice/acetone mixture and transported in dry ice for assay. The thawed mitochondrial suspension was sonicated with a Branson S-110 for one minute. The supernatant obtained after centrifugation (27,000 × *g*, 30 minutes) is designated mitochondrial enzyme.

Assay.—The enzyme was assayed as previously described.[7] This assay measured the nonenzymatic decarboxylation of α-hydroxy-β-keto-adipate (reaction 2), the product which is formed in enzymatic reaction (reaction 1):



(2)

Warburg vessels (H_2-atmosphere) contained in the main compartment: enzyme, 100 μmoles K-Na-phosphate pH 7.0, 5 μmoles $MgCl_2$, 0.2 μmole TPP, and 2.5 μmoles 2-oxo-glutarate. The reaction was started by tipping 2.0 μmoles U-C^{14}-glyoxylate (100,000 dpm × $\mu mole^{-1}$) from one side arm. The final volume was 2.5 ml. Incubation time was one hour at 30°C. The reaction was stopped by adding a mixture of 0.1 ml 0.2 *M* 4-amino-antipyrine and 0.05 ml 25 per cent trichloroacetic acid from the other side arm. $C^{14}O_2$ was equilibrated with alkali-soaked filter paper in the center well for another hour. The contents of the center well were counted in a scintillation counter (Bray solution, Packard 3000) with internal standardization.

Isolation of the Reaction Products as 2,4-Dinitrophenylhydrazones.—The standard assay mixture was incubated with either 17.7 mg mitochondrial or 24 mg cytoplasmic enzyme from kidney for one hour at 30°C. Both enzyme preparations were dialyzed overnight against 0.05 *M* K-Na-phosphate pH 7.0, 10^{-3} *M* mercaptoethanol, and in 3 ml volume. Mitochondrial enzyme, heated for ten minutes in

TABLE 1

2-OXO-GLUTARATE:GLYOXYLATE CARBOLIGASE ACTIVITY IN HUMAN TISSUES

Tissue	Fraction	Enzyme Activity (nmoles × mg protein^{-1} × hr^{-1}) Control subjects†	Hyperoxaluric Patients DR	JM	TS	TE	SI‡
Liver	Mito.* (5)	86 ± 12 (61–125)	64	60	54	—	—
	Cyto. (5)	45 ± 7 (17–58)	7	8	6	—	—
Spleen	Mito. (6)	67 ± 6 (50–90)	49	—	—	—	—
	Cyto. (6)	65 ± 3 (55–74)	8	—	—	—	—
Kidney	Mito. (8)	115 ± 9 (80–150)	30	—	—	90	(121)
	Cyto. (8)	93 ± 6 (66–110)	10	—	—	25	(34)

* Mito. = mitochondrial; Cyto. = cytoplasmic; no. in parentheses = no. of control subjects.
† Mean ± standard error followed by range of values.
‡ Kidney obtained at autopsy and frozen prior to cell fractionation.

boiling water, was also incubated as a control. The reaction was stopped with 2 ml 2,4-dinitrophenylhydrazine solution (10^{-2} *M* in 2 *N* HCl). The mixtures were kept at room temperature overnight and then extracted with ether in Kutscher-Steudel extractors. The ether was evaporated, and the residues were dissolved in ethyl acetate and subjected to paper chromatography (butanol-2 *N* NH_3-ethanol 7:2:1 v/v, ascending Whatman no. 1). The papers were scanned in a Packard 385/7200. The bis-2,4-dinitrophenylhydrazones of α-hydroxy-β-keto-adipate were eluted with 2 *N* NH_3 and aliquots were taken for counting in the scintillation counter and for scanning a spectrum in the visible region in 0.1 *N* NaOH (Cary 11).

Miscellaneous.—Protein was determined according to Lowry *et al.*[10] with bovine serum albumin as a standard (crystallized and lyophilized, Sigma Chem. Co.). U-C^{14}-glyoxylate was purchased from Calbiochem; 2-oxo-glutarate, glyoxylate, and thiamine pyrophosphate were obtained from General Biochemicals; 4-amino-antipyrine was a product of Mann Research Lab.; and C^{14}-benzoic acid from Packard Instrument Co. was used for internal standardization.

Results.—Properties of the normal enzymes: The intracellular distribution of the human 2-oxo-glutarate:glyoxylate carboligase in liver, spleen, and kidney is given in Table 1. Extracts from kidney have the highest specific activity. The fraction solubilized from mitochondria from liver and kidney had a somewhat higher specific activity than the fraction which is designated as cytoplasmic enzyme (27,000 × *g* supernatant). This fraction still contains a considerable amount of subcellular particles, but high-speed centrifugation raises the specific activity of this "cytoplasmic fraction" by about 15 per cent (144,000 × *g* supernatant). This indicates that the enzyme activity is not associated to a large extent with the remaining particles in the 27,000 × *g* supernatant. Enzyme activity also was detected in mitochondria and cytoplasm of skeletal muscle and in leucocytes, especially in the granular fraction, but no activity was found in erythrocytes.

The requirements of the mitochondrial and cytoplasmic kidney enzymes are shown in Table 2. The cofactor requirement of the mitochondrial enzyme is more apparent after the same conditions of dialysis. Glycolate, oxalate, and glycine, which are the metabolites most closely related to glyoxylate, had no influence on the activity of the cytoplasmic enzyme at levels of 10^{-2} *M*.

The shape of the pH-activity curve, with an optimum of 6.5, is similar for the cytoplasmic and mitochondrial enzyme and very similar to that of the rat liver enzyme.[7] The rate of $C^{14}O_2$ release is the same as reported for the purified enzyme from rat liver mitochondria,[7] when incubations with mitochondrial or cytoplasmic enzyme from human kidney are stopped with the mixture of TCA and 4-amino-

TABLE 2

REQUIREMENTS OF THE HUMAN 2-OXO-GLUTARATE:GLYOXYLATE CARBOLIGASE FROM KIDNEY

		Cytoplasmic enzyme (nmoles $C^{14}O_2$)	Mitochondrial enzyme (nmoles $C^{14}O_2$)
Complete		290	295
TPP	omitted	259	231
$MgCl_2$	"	252	219
2-Oxo-glutarate	"	9	8
Heated enzyme		10	11

Standard assay with indicated changes. The assays contained 2.2 mg protein of a mitochondrial extract or 3.4 mg protein of the cytoplasmic fraction of a control kidney. Both protein solutions were dialyzed for 15 hr against 0.01 *M* K-Na-phosphate pH 7.0, 10^{-3} *M* mercaptoethanol.

antipyrine as a catalyst for the decarboxylation of the β-keto acid,[11] or with TCA alone (reaction 2).

The isolation of the reaction products as 2,4-dinitrophenylhydrazones also indicates that the mitochondrial and the cytoplasmic fractions catalyze the same reaction (Fig. 1). Two new 2,4-dinitrophenylhydrazones of red-brown color appear in the chromatograms with *Rf* 0.19 and *Rf* 0.40 (glyoxylate *Rf* 0.52, 0.37; 2-oxo-glutarate *Rf* 0.14). Both new compounds contain radioactivity and both turn blue after being sprayed with a saturated solution of KOH in ethanol. The compound with *Rf* 0.19 was identified as the bis-2,4-dinitrophenylhydrazone of α-hydroxy-β-keto-adipate by comparison with the bis-2,4-dinitrophenylhydrazone obtained from synthesized α-hydroxy-β-keto-propionate.[12] Both compounds have the same chromophore and similar spectral properties (absorption band near 550 nm in 0.1 *N* NaOH).[7, 12] Assuming the same molecular extinction coefficient,[7, 12] the specific radioactivity of the α-hydroxy-β-keto-adipate was calculated to be 109,000 dpm × $\mu mole^{-1}$.

This fact indicates that both C-atoms of glyoxylate are retained in the product of the enzymatic reaction. The second "blue" compound (*Rf* 0.40) is very likely the bis-2,4-dinitrophenylhydrazone of 5-hydroxy-levulinic acid, the decarboxylation product of α-hydroxy-β-keto-adipate. This compound should contain only one C-atom of the glyoxylate. Because of the poor separation of the more highly labeled glyoxylate, the specific radioactivity could not be accurately determined.

Enzyme activity in patients with primary hyperoxaluria: Activity of 2-oxo-glutarate:glyoxylate carboligase in cytoplasmic extracts of liver, kidney, and spleen from hyperoxaluric subjects was considerably lower than that in control tissues (Table 1). The activity of this enzyme was approximately 15 per cent of the mean activity in control liver. In four of the five control subjects the activity of hepatic cytoplasmic carboligase exceeded 47 nmoles × mg $protein^{-1}$ × hr^{-1}. No clear explanation can be given for the single low control value of 17, obtained from a patient addicted to narcotics. In the three patients from whom liver biopsies were obtained (one uremic and two with normal renal function) activity of cytoplasmic carboligase was 7, 8, and 6 nmoles × mg $protein^{-1}$ × hr^{-1}, respectively. The single determination of splenic cytoplasmic carboligase in primary hyperoxaluria was 12 per cent of the control value. The activity of kidney cytoplasmic carboligase from three patients with primary hyperoxaluria was 23 per cent of control value. In all three tissues no overlap was observed between the values in hyperoxaluria and in control tissues. In contrast enzyme activity in mitochondrial extracts from liver and spleen of patients with primary hyperoxaluria was within the range of

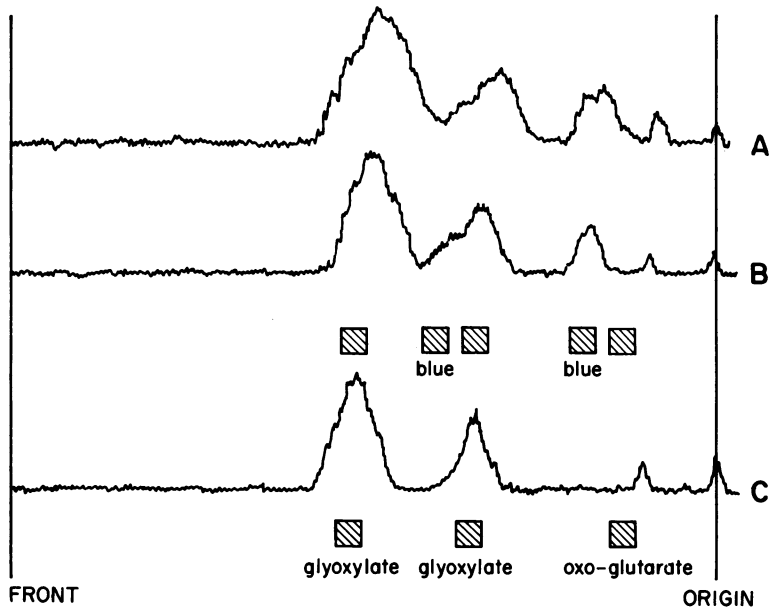


FIG. 1.—Distribution of the radioactivity of the 2,4-dinitrophenylhydrazones on paper chromatograms. (*A*) Incubation with cytoplasmic kidney enzyme; (*B*) incubation with mitochondrial enzyme; (*C*) control with heated enzyme. Conditions in the text.

values in control tissues. The activity in kidney mitochondria from patient DR was somewhat decreased but was within the normal range in patients TE and SI.

The reduced enzyme activity in cytoplasm from hyperoxaluric tissues cannot be the result of thiamine deficiency, because TPP is routinely added to the assay incubations. The activity of the mitochondrial enzyme from one patient with hyperoxaluria (DR) was compared with and without addition of TPP. When TPP was omitted from the standard reaction mixture, the mitochondrial extracts showed only a 4 per cent decrease in enzyme activity, which suggests further that the patient did not suffer from thiamine deficiency. In extracts of liver mitochondria from rats fed a thiamine-deficient diet for 25 days, the activity of the enzyme was decreased to 50 per cent when TPP was omitted from the incubation mixture.[13]

Addition of the kidney cytoplasmic fraction from a patient with hyperoxaluria (DR) did not decrease enzyme activity in a similar preparation from control kidney.

Discussion.—Several human tissues were found to contain an enzyme with the properties of the 2-oxo-glutarate:glyoxylate carboligase, which in previous work was purified 85-fold from rat liver mitochondria.[7] The structure of the product formed by this enzyme was substantiated to be a α-hydroxy-β-keto-adipate by means of labeling experiments, kinetics, and optical properties of the 2,4-dinitrophenylhydrazone. The structure of a α-hydroxy-β-keto-adipate for the product formed by the human cytoplasmic and mitochondrial enzyme also seems justified on the basis of the kinetic experiments and the properties of the 2,4-dinitrophenylhydrazones.

Kawasaki *et al.* recently reported studies on glyoxylate and 2-oxo-glutarate metabolism with intact rat liver mitochondria.[14] These authors propose the struc-

ture of the α-keto-β-hydroxy-adipate for the product in the carboligase reaction. The present results, as well as those earlier reported for the rat liver enzyme,[7] cannot be explained by a α-keto-β-hydroxy acid. Steward and Quayle[15] have recently presented data on a partially purified enzyme from pig liver mitochondria, which catalyzes a synergistic decarboxylation of glyoxylate and 2-oxo-glutarate. The product of the reaction, however, was not characterized. In neither of these studies was the cytoplasmic enzyme activity specifically studied.

A number of previous studies, reviewed elsewhere in detail,[2] have been carried out in patients with primary hyperoxaluria. Dietary oxalate is not excessively absorbed in this disorder.[16] Injection of C^{14}-oxalate has demonstrated it to be metabolically inert in man.[17] It has been concluded that excessive urinary excretion of oxalate results from its increased synthesis. Several previous lines of evidence have suggested that excess oxalate synthesis in primary hyperoxaluria results from a block in the metabolism of glyoxylate: (1) Increased urinary excretion of oxalate, glycolate, and glyoxylate occurs.[3] (2) The rate of metabolism of C^{14}-glyoxylate and C^{14}-glycolate to C^{14}-CO_2 is reduced.[2] (3) C^{14}-glyoxylate administered intravenously exhibits an increased conversion to glycolate and oxalate; a similar increased conversion of C^{14}-glycolate to oxalate occurs (presumably via glyoxylate).[2] (4) The metabolism of ascorbic acid, another oxalate precursor, is normal in hyperoxaluria.[18] From these *in vivo* incorporation studies, and by analogy with pyridoxine deficiency, it was concluded that the defect probably was in the enzymatic conversion of glyoxylate to glycine.[3] In recent studies, however, no such defect of either glutamate or alanine:glyoxylate aminotransferase in liver or kidney could be found,[6] in contrast to a reported defect in glycine synthesis from glyoxylate in autopsy kidney specimens in primary hyperoxaluria reported by others.[19]

Low activity of 2-oxo-glutarate:glyoxylate carboligase activity was found in cytoplasmic preparations of liver, spleen, and kidney in five patients with primary hyperoxaluria, although not all preparations were obtained from each patient (Table 1). Mitochondrial activities of the enzyme were not uniformly reduced. No inhibition was found on mixing hyperoxaluric and normal enzyme preparations. Oxalate and glycolate, which are overproduced in primary hyperoxaluria, did not inhibit 2-oxo-glutarate:glyoxylate carboligase. Crawhall and Watts, who first described synergistic decarboxylation of glyoxylate and 2-oxo-glutarate by intact mitochondria,[20] also found this activity (presumably identical with that of 2-oxo-glutarate:glyoxylate carboligase) to be normal in hepatic mitochondria from three patients with primary hyperoxaluria.[21] These investigators did not study cytoplasmic preparations. In the studies summarized in Table 1 some of the cytoplasmic enzyme may represent leakage from mitochondria. This is the probable explanation for the relatively high cytoplasmic activity in patient SI, whose kidney had been frozen prior to cell fractionation.

These studies suggest that cytoplasmic 2-oxo-glutarate:glyoxylate carboligase, an activity selectively reduced in primary hyperoxaluria, is an isozyme of the mitochondrial enzyme. Preliminary work supports this conclusion. Enzyme preparations from human kidney cytoplasm and mitochondria show somewhat different properties during purification, and TPP is less tightly bound by the mitochondrial enzyme.[22]

Summary.—(1) Activity of 2-oxo-glutarate:glyoxylate carboligase was measured in mitochondrial and cytoplasmic preparations from human liver, spleen, and kidney. (2) The enzyme activity of the cytoplasmic but not of the mitochondrial preparations was markedly reduced in five patients with primary hyperoxaluria.

* Since the mechanism of the enzyme is not established, the trivial name for the enzyme was chosen in accordance with glyoxylate carboligase.[23] It is likely that the enzyme is a 2-oxo-glutarate:glyoxylate succinic aldehyde transferase.

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