Biochemical Heterogeneity in Glutathione Synthetase Deficiency

STEPHEN P. SPIELBERG, MICHAEL D. GARRICK, LAURENCE M. CORASH, JEAN DEB. BUTLER, FRANK TIETZE, LAVERNE ROGERS, and JOSEPH D. SCHULMAN, *Section on Human Biochemical and Developmental Genetics, National Institute of Child Health and Human Development, Bethesda, Maryland 20014; Departments of Pediatrics and Biochemistry, State University of New York, Buffalo, New York 14207; Hematology Service, Clinical Pathology Department, Clinical Center, National Institutes of Health, Bethesda, Maryland 20014; and Section on Intermediary Metabolism, National Institute of Arthritis, Metabolism, and Digestive Diseases, Bethesda, Maryland 20014*

ABSTRACT Two different clinical syndromes are associated with glutathione synthetase deficiency, one presenting with hemolytic anemia and 5-oxoprolinuria, the other with isolated hemolysis. We have differentiated these disorders on an enzymatic basis. In 5-oxoprolinuria, all cell types examined have grossly deficient enzyme activity and glutathione content. In contrast, in the nonoxoprolinuric variant, erythrocytes have decreased enzyme activity and glutathione content, whereas nucleated cells maintain substantial levels of both. The enzyme in this disorder is unstable in vitro and has shortened survival in intact erythrocytes. Nucleated cells appear able to maintain sufficient enzyme activity and concentrations of glutathione to suppress overproduction of 5-oxoproline.

INTRODUCTION

Two different human syndromes are associated with decreased activity of erythrocyte glutathione synthetase (EC 6.3.2.3), one presenting in the newborn period with massive 5-oxoprolinuria (pyroglutamic aciduria), metabolic acidosis, and hemolytic anemia (1–6), and the other at varying ages with isolated hemolytic anemia (7–9). In oxoprolinuria, erythrocytes, leukocytes, and cultured skin fibroblasts all have been shown to have diminished enzyme activity and glutathione content (4, 6). The low intracellular glutathione concentration may reduce feedback inhibition of γ-glutamyl-cysteine synthetase (EC 6.3.2.2) with overproduction of 5-oxoproline from γ-glutamyl-cysteine (3, 10, 11). γ-Glutamyl-cysteine is a substrate for both glutathione synthetase and γ-glutamylcyclotransferase (EC 2.3.2.4). The decreased activity of glutathione synthetase may make γ-glutamyl-cysteine more available as a substrate for γ-glutamylcyclotransferase, also leading to increased production of 5-oxoproline (12). Hemolytic anemic in the absence of oxoprolinuria might result if the deficit in glutathione synthetase were restricted primarily to the erythrocyte (4).

We report here that in a patient with glutathione synthetase deficiency without oxoprolinuria, the mutant enzyme is unstable in both erythrocytes and nucleated cells. Glutathione synthetase activity and glutathione content are markedly decreased in erythrocytes, whereas nucleated cells are able to maintain substantial enzyme activity and near normal glutathione levels, thus preventing accumulation of excess 5-oxoproline.

METHODS

Cells. Skin fibroblasts were grown in Eagle's minimal essential medium (National Institutes of Health, Bethesda, Md.) with 10% fetal calf serum, 2 mM glutamine, nonessential amino acids, tetracycline, and neomycin (Grand Island Biological Co., Grand Island, N. Y.). Cells were obtained from an oxoprolinuric patient reported by Spielberg et al. (6) and from a nonoxoprolinuric patient studied by Mohler et al. (9). Assays were performed on confluent cells. For enzyme assays, fibroblasts were harvested after trypsinization and then washed three times with phosphate-buffered saline, pH 7.4. For glutathione determination, monolayers

Presented, in part, at the Fifth International Congress of Human Genetics, October 1976, Mexico City. 1976. *Excerpta Med. Int. Congr. Ser.* 387: 119. (Abstr.)

Dr. Spielberg's present address is Departments of Pediatrics and Pharmacology, Johns Hopkins University, Baltimore, Md. 21205.

Received for publication 11 August 1977 and in revised form 27 December 1977.

were washed three times with phosphate-buffered saline and harvested directly into 0.02 N HCl with a rubber policeman.

Erythrocytes were prepared from heparinized blood by centrifugation at 1,000 *g* for 10 min. Cells were washed three times with normal saline. Erythrocytes used in phthalate ester experiments were prepared from blood that was defibrinated by swirling with glass beads; leukocytes were removed by filtration through Whatman no. 2 filter paper (13) (Whatman, Inc., Clifton, N. J.).

Leukocytes were isolated from heparinized blood by sedimenting erythrocytes with an equal volume of 6% dextran (mol wt 254,000) in normal saline for 30 min. The supernate was decanted and cells were pelleted at 800 *g* for 10 min. Remaining erythrocytes were removed by hypotonic lysis in 0.2% NaCl three times (14).

Determination of glutathione concentration and glutathione synthetase activity. For enzyme assays, fibroblasts, erythrocytes, and leukocytes were suspended in 0.05 M sodium phosphate, pH 7.6, and sonicated with a Branson Sonifier (Branson Sonic Power Co., Danbury, Conn.) for 20 s. Sonicates were centrifuged at 30,000 *g* for 30 min at 4°C, and supernates were used in the assay. Glutathione synthetase activity was assayed by measuring [1-^{14}C]glycine incorporation into ophthalmic acid with the same reaction mixture as Orlowski and Wilk (15). After incubation at 37°C for 2 h, reactions were stopped by heating in boiling water for 1 min. The precipitate was removed by centrifugation. A 100-μl aliquot (total volume 250 μl) of the supernate was fractionated on a 0.6 × 4-cm Dowex-1 column (Bio-Rad Laboratories, Richmond, Calif.; AG 1-X4, acetate, 100–200 mesh). Glycine was removed with two 5-ml washes of 0.005 N acetic acid and then ophthalmic acid was eluted with two 5-ml washes of 1 N acetic acid. Aliquots of the latter fractions were counted by liquid scintillation in Aquasol (New England Nuclear, Boston, Mass.). The glutathione content of cells was determined by the method of Tietze (16). Proteins were assayed by the method of Lowry et al. (17).

Preparation of light and dense erythyrocytes. Erythyrocytes were layered over a mixture of phthalate esters with a density of 1.097 (18), and spun in Caraway tubes at 15,500 *g* for 30 min in a microhematocrit centrifuge (Clay-Adams, Div. Becton, Dickinson & Co., Parsippany, N. J.). Cells above (25%) and below (75%) the phthalate ester layer were assayed for glutathione, glutathione synthetase activity, hemoglobin, and reticulocyte count.

Incorporation of glycine into glutathione by intact erythrocytes. 10–100 μCi of [2-^{3}H]-glycine (43 Ci/mmole, Schwarz-Mann, Orangeburg, N. Y.) were dissolved in plasma. Erythrocytes were incubated with the [^{3}H]glycine for 4 h at 37°C. Cells were then subjected to fractionation through phthalate esters as above. Cells were hemolyzed in 4 vol of distilled water, proteins were precipitated with TCA, and incorporation of [^{3}H]glycine into glutathione was determined after precipitating with $CdCl_2$ (19).

Measurement of plasma and urine 5-oxoproline. Freshly obtained plasma or urine was acidified with 0.4 N perchloric acid and passed through a Dowex-50 (H+) column (5). The effluent was lyophilized and then the material was dissolved in distilled water and fractionated on Dowex-1 (formate) according to Wilk and Orlowski (20). The oxoproline-containing fraction was lyophilized and repurified by paper chromatography (21). The 5-oxoproline was then converted to L-glutamic acid and assayed with glutamic dehydrogenase (EC 1.4.1.3) (5).

RESULTS

The two forms of glutathione synthetase deficiency are readily distinguished by comparing 5-oxoproline levels in plasma and urine. Thus, our patient without oxoprolinuria had normal plasma and urine 5-oxoproline concentrations: 0.04 mM urine (normal 0.17 ±0.05 mM;[1] oxoprolinuria 25–200 mM [1–5]); 0.03 mM plasma (normal 0.035±0.005 mM; oxoprolinuric 0.7–5 mM [1–5]).

The glutathione content and glutathione synthetase activity of various cell types in the two forms of glutathione synthetase deficiency are presented in Table I. Glutathione concentration and enzyme activity were markedly decreased in erythrocytes in both syndromes. While the oxoprolinuric leukocytes and fibroblasts showed similar large decreases in glutathione and enzyme, nucleated cells from the nonoxoprolinuric patient maintained substantial enzyme activity; gluta-

[1] Mean of five normals±SEM.

TABLE I

Glutathione (GSH) Content and Glutathione Synthetase (GSH-S) Activity of Cells in GSH-S Deficiency with and without 5-Oxoprolinuria

	Erythrocytes		Leukocytes		Fibroblasts	
	GSH*	GSH-S‡	GSH§	GSH-S‖	GSH§	GSH-S‖
Normal	1.19±0.04¶ (5)	18.2±0.2 (4)	4.23±0.15 (6)	79±13 (3)	6.9±0.7 (11)	136±3 (9)
Oxoprolinuria	0.11	0.3	1.05	2	1.1	6
Without oxoprolinuria	0.18	0.3	2.95	16	7.7	69

* Milligrams per milliliter packed erythrocytes.

‡ Nanomoles per milligram hemoglobin per hour.

§ Micrograms per milligram protein.

‖ Nanomoles per milligram protein per hour.

¶ Normal expressed as mean±SEM. Numbers in parentheses refer to the number of subjects or cell lines examined. Patient values are the mean of at least two determinations.

thione content was 70% of normal mean in leukocytes and was normal in fibroblasts.

The differences between the two disorders suggested mutation to an inactive enzyme in oxoprolinuria vs. an unstable enzyme in the nonoxoprolinuric variant. As shown in Table II, normal and oxoprolinuric glutathione synthetase activities were not significantly affected by overnight dialysis at 4°C. However, the nonoxoprolinuric fibroblast and leukocyte enzyme activity was greatly reduced by dialysis. The nonoxoprolinuric nucleated cell enzyme activity also showed greater than normal instability on storage at 4°C for 16 h as well as on dialysis. Dialysis was used to remove small molecular weight compounds from both normal and mutant homogenates which might interact with the enzyme.

Erythrocytes were fractionated according to buoyant density to examine if instability of the nonoxoprolinuric enzyme in vitro reflected its behavior in vivo (Table III). There was an enrichment of reticulocytes in the normal and patient's lighter fractions, although the patient had a higher reticulocyte count in both fractions. The denser cell fraction (older cells) from a normal individual had values for glutathione content, glutathione synthetase activity, and incorporation of [^{3}H]glycine into glutathione by intact cells that were all approximately 25% decreased compared to the lighter (younger) fraction. Each of these parameters was decreased by a much greater extent in the patient's more dense cells compared with his lighter cells.

DISCUSSION

Assignment of the enzyme defect in 5-oxoprolinuria to glutathione synthetase resulted in large measure from the elucidation of the sequence of reactions in the γ-glutamyl cycle by Meister and Tate (22). Studies on the regulation of the enzymes of the cycle (3, 10, 11) led to the hypothesis that decreased intracellular glutathione in glutathione synthetase deficiency may lead to decreased feedback inhibition of γ-glutamyl-cysteine synthetase with overproduction of γ-glutamyl-cysteine which, in turn, is converted to 5-oxoproline by γ-glutamylcyclotransferase and(or) γ-glutamyltranspeptidase. 5-Oxoproline would then accumulate in quantities that exceed the capacity of 5-oxoprolinase to convert it to glutamate. Utilization of γ-glutamyl-cysteine to form glutathione by glutathione synthetase is normally greater than its conversion to 5-oxoproline by γ-glutamylcyclotransferase (12). Deficiency of glutathione synthetase may make γ-glutamyl-cysteine more available to γ-glutamyl-cyclotransferase, exacerbating 5-oxoproline overproduction.

Our present studies provide evidence for an enzymatic mechanism for the occurrence of oxoprolinuric and nonoxoprolinuric forms of glutathione synthetase deficiency. The mutant enzyme in a patient with the nonoxoprolinuric variant is unstable on dialysis and exhibits a greater than normal decrease in enzyme activity in older vs. younger erythrocytes. Instability of both the mutant erythrocyte and nucleated cell enzyme suggests a mutation involving a common element of the enzyme in both cell types, rather than an isolated effect on an erythrocyte isozyme. Decreased enzyme activity in the patient's older cells was detected despite his hemolytic anemia with shortened erythrocyte life-span, as reflected by the higher reticulocyte count even in his more dense cell fraction. Glutathione normally protects hemoglobin and eryth-

TABLE II

*Effect of Dialysis on Glutathione Synthetase (GSH-S) Activity in Glutathione Synthetase Deficiency**

	Fibroblasts (GSH-S)‡		Leukocytes (GSH-S)‡	
	Fresh	Dialyzed	Fresh	Dialyzed
Normal	130	116	81	80
Oxoprolinuria	6	5	2	2
Without oxoprolinuria	69	9	16	5

* 30,000-*g* supernates of cell sonicates were assayed for GSH-S activity fresh or after 16 h dialysis against 0.05 M sodium phosphate, pH 7.6, at 4°C. Assay conditions are as in text.

‡ Nanomoles per milligram protein per hour; mean of two determinations.

TABLE III

*Glutathione (GSH) Content, Glutathione Synthetase (GSH-S) Activity, and Incorporation of [^{3}H]Glycine into GSH in Lighter and More Dense Erythrocytes Partitioned by Centrifugation with a Mixture of Phthalate Esters**

		GSH	GSH-S	[^{3}H]Glycine incorporation into GSH by intact erythrocytes
	% reticulocytes	μg/g Hb	nmol/mg Hb/h	dpm/mg Hb
Normal				
Lighter	5.4	1,054	28.9	1,230
More dense	0.5	730	22.6	780
GSH-S deficient Without oxoprolinuria				
Lighter	12.9	111	1.0	44
More dense	6.4	23	0.3	15

* Erythrocyte separations and GSH-S assays are as in text. Values are means of two determinations.

rocyte cell membranes from oxidative damage (23). Shortened erythrocyte survival in this disorder may result from the accelerated decline in cell glutathione content with cell age, comparable to the decrease in reduced glutathione seen in glucose-6-phosphate dehydrogenase deficiency (24). The data on fibroblasts and leukocytes from this patient show that nucleated cells are able to maintain substantial enzyme activity; the cells thus contain glutathione levels sufficient to prevent overproduction of 5-oxoproline. In contrast, in oxoprolinuria, the gross deficiency of glutathione synthetase activity and glutathione content in nucleated cells as well as in erythrocytes results both in hemolytic anemia and overproduction of 5-oxoproline.

There appears to be considerable genetic heterogeneity among the mutations leading to glutathione synthetase deficiency. In addition to oxoprolinuric and nonoxoprolinuric forms of the disorder, we previously reported quantitatively different abnormal apparent enzyme affinities for glycine in two patients with oxoprolinuria (6). There are data which suggest the possibility of isozymic forms of glutathione synthetase in erythrocytes and nucleated cells.[2] It is possible that other patients may present with abnormalities of one isozymic form of the enzyme leading to further heterogeneity among the oxoprolinuric and nonoxoprolinuric forms of glutathione synthetase deficiency.

ACKNOWLEDGMENTS

We thank Barbara Foley for expert technical assistance and Dr. D. N. Mohler of the University of Virginia School of Medicine for helping to arrange studies on the nonoxoprolinuric patient.

This investigation was supported, in part, by grant HD-03967 from the National Institutes of Health.

REFERENCES

1. Jellum, E., T. Kluge, H. C. Borresen, O. Stokke, and L. Eldjarn. 1970. Pyroglutamic aciduria: A new inborn error of metabolism. *Scand. J. Clin. Lab. Invest.* **26:** 327–335.
2. Hagenfeldt, L., A. Larsson, and R. Zetterstrom. 1974. Pyroglutamic aciduria: Studies of an infant with chronic metabolic acidosis. *Acta Paediatr. Scand.* **63:** 1–8.
3. Larsson, A., R. Zetterstrom, L. Hagenfeldt, R. Andersson, S. Dreborg, and H. Hornell. 1974. Pyroglutamic aciduria (5-oxoprolinuria), an inborn error of glutathione metabolism. *Pediatr. Res.* **8:** 852–856.
4. Wellner, V. P., R. Sekura, A. Meister, and A. Larsson. 1974. Glutathione synthetase deficiency, an inborn error of metabolism involving the γ-glutamyl cycle in patients with 5-oxoprolinuria (pyroglutamic aciduria). *Proc. Natl. Acad. Sci. U. S. A.* **71:** 2505–2509.
5. Marstein, S., E. Jellum, B. Halpern, L. Eldjarn, and T. L. Perry. 1976. Biochemical studies of erythrocytes in a patient with pyroglutamic acidemia (5-oxoprolinemia). *N. Engl. J. Med.* **295:** 406–412.
6. Spielberg, S. P., L. I. Kramer, S. I. Goodman, J. Butler, F. Tietze, P. Quinn, and J. D. Schulman. 1977. 5-Oxoprolinuria: Biochemical observations and case report. *J. Pediatr.* **91:** 237.
7. Prins, H. K., M. Oort, J. A. Loos, C. Zurcher, and T. Beckers. 1966. Congenital nonspherocytic hemolytic anemia associated with glutathione deficiency of the erythrocytes. *J. Hematol.* **27:** 145–166.
8. Boivin, P., C. Galand, R. Andre, and J. Debray. 1966. Anemes hemolytique congenitales avec deficit isole en glutathion reduit par deficit en glutathione synthetase. *Nouv. Rev. Fr. Hematol.* **6:** 859–866.
9. Mohler, D. N., P. W. Majerus, V. Minnich, C. E. Hess, and M. D. Garrick. 1970. Glutathione synthetase deficiency as a cause of hereditary hemolytic disease. *N. Engl. J. Med.* **283:** 1253–1257.
10. Richman, P. G., and A. Meister. 1975. Regulation of γ-glutamylcysteine synthetase by nonallosteric feedback inhibition by glutathione. *J. Biol. Chem.* **250:** 1422–1426.
11. Larsson, A., and B. Mattsson. 1976. On the mechanism of 5-oxoproline overproduction in 5-oxoprolinuria. *Clin. Chim. Acta.* **67:** 245–253.
12. Heinle, H., and A. Wendel. 1976. Does a modified γ-glutamyl cycle exist in human erythrocytes? *Hoppe-Seyler's Z. Physiol. Chem.* **357:** 1459–1463.
13. Piomelli, S., G. Lurinsky, and L. R. Wasserman. 1967. The mechanism of red cell aging. 1. Relationship between cell age and specific gravity evaluated by ultracentrifugation in a discontinuous density gradient. *J. Lab. Clin. Med.* **69:** 659–674.
14. Schneider, J. A., K. Bradley, and J. E. Seegmiller. 1967. Increased cystine in leucocytes from individuals homozygous and heterozygous for cystinosis. *Science (Wash. D. C.).* **157:** 1321–1322.
15. Orlowski, M., and S. Wilk. 1975. Intermediates of the γ-glutamyl cycle in mouse tissues. *Eur. J. Biochem.* **53:** 581–590.
16. Tietze, F. 1969. Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione. *Anal. Biochem.* **27:** 502–522.
17. Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. F. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
18. Danon, D., and Y. Marikovsky. 1964. Determination of density distribution of red cell population. *J. Lab. Clin. Med.* **64:** 668–674.
19. Minnich, V., M. B. Smith, M. J. Brauner, and P. W. Majerus. 1971. Glutathione biosynthesis in human erythrocytes. I. Identification of the enzymes of glutathione synthesis in hemolysates. *J. Clin. Invest.* **50:** 507–513.
20. Wilk, S., and M. Orlowski. 1975. Determination of pyrrolidine carboxylate and γ-glutamyl amino acids by gas chromatography. *Anal. Biochem.* **69:** 100–113.
21. Palekar, A. G., S. S. Tate, and A. Meister. 1974. Formation of 5-oxoproline from glutathione in erythrocytes by the γ-glutamyltranspeptidase-cyclotransferase pathway. *Proc. Natl. Acad. Sci. U. S. A.* **71:** 293–297.
22. Meister, A., and S. Tate. 1976. Glutathione and related γ-glutamyl compounds: Biosynthesis and utilization. *Ann. Rev. Biochem.* **45:** 559–604.
23. Kosower, N. S., and E. M. Kosower. 1974. Protection of membranes by glutathione. *In* Glutathione. L. Flohe, H. Ch. Benohr, H. Sies, H. D. Waller, and A. Wendel, editors. Academic Press, Inc., New York. 216–227.
24. Beutler, E. 1972. Disorders due to enzyme defects in the red blood cell. *Adv. Metab. Disord.* **6:** 131–160.

[2] Spielberg, S. P., and J. D. Schulman. Unpublished observations.