of this data is that the Hurler's β-galactosidase is deficient in a thermolabile component (or components).

This suggestion was substantiated by starch-gel electrophoresis studies. Control livers contained three separable β-galactosidase components with activity at *p*H 4 to 5—a fast-moving component and two slow-moving ones. After the preliminary incubation either of homogenates or supernatant fractions from controls, for 30 minutes at 42°C, both slow-moving β-galactosidase components were rendered inactive, whereas the fast component remained active.

A marked deficiency of the slow-moving β-galactosidase components was found in liver tissue from all patients with the Hurler's syndrome (types 1 to 3) (Fig. 3). Repeated freezing and thawing of the normal or Hurler's liver sucrose homogenates, or of the supernatant fractions in distilled water, caused no alterations of β-galactosidase patterns. No deficiency of the slow-moving components was found in liver tissue from a patient with Niemann-Pick disease, an indication that lysosomal storage in itself (in this case of sphingomyelin) does not produce the alteration. In generalized gangliosidosis, a β-galactosidase deficiency disease (*8*), both fast-moving and slow-moving components were nearly absent.

The degree of deficiency of the major slow-moving β-galactosidase in the Hurler's liver tissue was estimated from visual inspection of the gels. This gave only a very rough approximation of the degree of deficiency due to the inaccuracies inherent in the method of estimation. We estimated that, in the Hurler's tissues, the activity of the major slower-moving component was one-tenth to one-twentieth that of normal (*10*).

Examination of the separable β-galactosidase components in the kidney (Fig. 3) demonstrated the presence of four components, two of which were fast moving and two slow moving. In the Hurler's patients (types 1 to 3) both slow-moving components in kidney were markedly deficient but the degree of deficiency was not as great as that in liver, except in a patient with type 3 disease (Fig. 3).

Incubation of the gels with 4-methylumbelliferylglucoside revealed β-glucosidase activity which coincided exactly with β-galactosidase activity of the fastest-moving component in both controls and the patients' liver and kidney tissues. The slow-moving components did not possess β-glucosidase activity. No alteration of β-glucosidase activity was noted in the tissues of patients with Hurler's syndrome.

The mutation in Hurler's syndrome thus may result in preferential loss of specific β-galactosidase isoenzymes. Studies of the fast- and slow-moving β-galactosidases indicate that they are structurally different proteins, probably under separate genetic control, and can be thought of as isoenzymes (*11*). The fact that total β-galactosidase activity in some patients with the Hurler's syndrome is normal or only slightly reduced (*5, 6*) can be explained by a shift in isoenzyme pattern; increases in the fast-moving isoenzyme could give this result. Different isoenzyme patterns in different tissue could also explain the finding of varying degrees of β-galactosidase deficiency in different organs (*5, 6*). For example, the deficiency of β-galactosidase in Hurler's kidney is less than that in liver (*5, 6*); we demonstrate here that kidney normally contains smaller proportions of the slow-moving isoenzymes than liver.

Although the β-galactosidase isoenzyme deficiency could provide an explanation for the ganglioside (and other glycolipid) accumulations, before accepting it as pathogenetically significant it must be demonstrated that (i) the deficient β-galactosidase isoenzyme or enzymes participate in mucopolysaccharide turnover, (ii) the deficiency is genetically transmitted in either autosomal (types 1 and 3) or X-linked (type 2) fashion, and (iii) the nature of the mutation differs in types 1, 2, and 3.

MAE WAN HO
JOHN S. O'BRIEN
Department of Neurosciences, School of Medicine, University of California at San Diego, La Jolla 92037

References and Notes

1. V. A. McKusick, *Heritable Disorders of Connective Tissue* (Mosby, St. Louis, ed. 3, 1966), p. 325.
2. R. Ledeen, K. Salsman, J. Gonatas, A. Taghavy, *J. Neuropathol. Exp. Neurol.* **24**, 341 (1965).
3. F. Van Hoof and H. G. Hers, *C. R. Acad. Sci. (Paris)* **259**, 1281 (1964); D. Lagunoff and T. L. Gritzka, *Lab. Invest.* **15**, 1578 (1966).
4. J. C. Frantantoni, C. W. Hall, E. F. Neufeld, *Proc. Nat. Acad. Sci. U.S.* **60**, 699 (1968).
5. P. A. Ockerman, *Clin. Chim. Acta* **20**, 1 (1968); F. Van Hoof and H. G. Hers, *Eur. J. Biochem.* **7**, 34 (1968).
6. M. C. MacBrinn, S. Okada, M. Woollacott, V. Patel, A. L. Tappel, J. S. O'Brien, *N. Engl. J. Med.*, in press.
7. We thank B. H. Landing, J. Woodard, D. Tatter, D. Lagunoff, A. Crocker, and V. A. McKusick for these frozen tissues.
8. S. Okada and J. S. O'Brien, *Science* **160**, 1002 (1968).
9. O. Smithies, *Biochem. J.* **71**, 585 (1958).
10. We have fractionated the three β-galactosidase isoenzymes from liver by Sephadex column chromatography and demonstrated that the peak which corresponds to the major slow-moving isoenzyme is reduced to one-tenth that of normal in one patient with the Hurler's syndrome (type 1). P. A. Ockerman and B. Hultberg [*Scand. J. Clin. Lab. Invest.* **22**, 199 (1968)] have published a similar finding after Sephadex chromatography of β-galactosidase from the liver of patients with the Hurler's syndrome.
11. D. Robinson, R. G. Price, N. Dance [*Biochem. J.* **102**, 525 (1967)] have demonstrated that the purified fast-moving and slow-moving β-galactosidases from rat kidney also have different *p*H optimums and molecular weights.
12. Supported in part by NIH grants NB 06576 and HE 08429.

28 April 1969

Glutathione Reductase: Stimulation in Normal Subjects by Riboflavin Supplementation

Abstract. *Glutathione reductase of hemolyzates from clinically normal subjects is activated by the addition of flavin-adenine dinucleotide. One-half maximum stimulation could be achieved by approximately 0.02 micromolar flavin-adenine dinucleotide; prior addition of adenosine triphosphate, adenosine diphosphate, or adenosine monophosphate prevented activation. Stimulation of glutathione reductase activity of red cells of normal subjects occurred when they were given 5 milligrams of riboflavin daily for 8 days. The degree of stimulation in vitro by flavin-adenine dinucleotide and in vivo by riboflavin was inversely proportional to dietary intake of riboflavin. The variety of clinical disorders which have been associated with glutathione reductase deficiency may have, as a common denominator, abnormalities in flavin-adenine dinucleotide formation.*

Most vitamins serve as substrates for the synthesis of coenzymes. When frank vitamin deficiencies occur, insufficient quantities of coenzyme may be synthesized, and the rates of the corresponding enzymatic reactions are slowed. For example, thiamine deficiency causes a decrease in the activity of transketolase (*1*), an enzyme which has thiamine pyrophosphate as a cofactor. Similarly, pyridoxine deficiency results in a decrease in the activity of

aminotransferases which have pyridoxyl phosphate as a coenzyme (*2*). In normal subjects, however, it is generally assumed that tissues are "saturated" with vitamins and that, therefore, the availability of vitamins is not a limiting factor in the rates of enzyme reactions.

Red cell glutathione reductase (E.C. 1.6.4.2) contains flavin-adenine dinucleotide (FAD) (*3*) and requires this riboflavin-containing coenzyme for its activity. We have found that the activity of red cell glutathione reductase from apparently normal subjects is strongly stimulated in vitro by minute quantities of FAD and in vivo by the administration of physiological amounts of riboflavin.

Normal hospital personnel or their family members served as volunteers. Seven females and two males, aged 11 to 43 years, participated. No attempt was made to regulate the dietary intake of the volunteers during the course of investigation. A 5- to 7-day dietary history was taken during the initial part of the study, and the average intake of riboflavin was calculated according to standard tables of food composition (*4, 5*). Riboflavin was administered as 5 mg tablets. Blood was collected in 1 mg of neutralized ethylenediaminetetraacetic acid (EDTA) per milliliter of blood. Part of each blood sample was centrifuged, the plasma and buffy coat were removed, and packed cells were frozen at −20°C. The FAD, flavin, mononucleotide (FMN), and riboflavin content of the packed red cells was measured fluorometrically (*6*) within 7 days of their collection. A sample of red cells was washed three times in isotonic sodium chloride solution, lysed in 19 volumes of distilled water, frozen in a mixture of dry ice and acetone, and thawed; the stroma were removed by centrifugation at 5000*g* for 10 minutes.

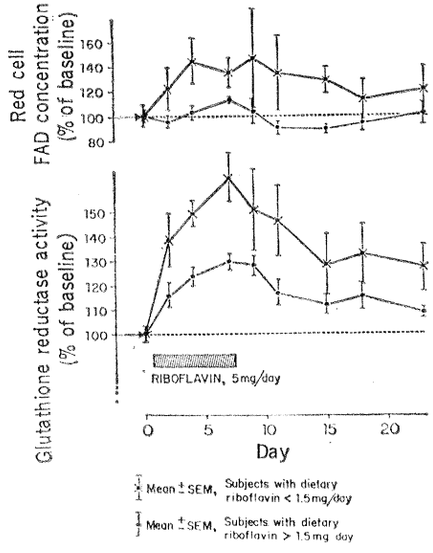

Fig. 1. Effect of riboflavin administration on glutathione reductase and red cell FAD of nine normal individuals. The value for each subject was expressed as a percentage of that individual's base line value. Base line glutathione reductase and FAD values (mean ± 1 S.E.) for the subjects with riboflavin intake greater than 1.5 mg/day were: glutathione reductase activity without FAD stimulation, 7.11 ± 0.36 international units (I.U.) per gram of hemoglobin (Hb); glutathione reductase activity with 1 μM FAD, 9.18 ± 0.51 I.U. per gram of Hb; red cell FAD concentration, 0.547 ± 0.020 μM. Average base line activities and standard errors of the mean for the four subjects with riboflavin intake of less than 1.5 mg/day were: glutathione reductase without FAD stimulation, 5.09 ± 0.86 I.U. per gram of Hb; glutathione reductase activity with 1 μM FAD, 9.26 ± 0.96 I.U. per gram of Hb; red cell FAD concentration, 0.398 ± 0.042 μM.

The activity of red cell glutathione reductase from apparently normal subjects is strongly stimulated in vitro by minute quantities of FAD and in vivo by the administration of physiological amounts of riboflavin.

Glutathione reductase activity was assayed spectrophotometrically in a 1-ml system containing the following: 0.05 ml of 1*M* tris-HCl buffer *p*H 8.0 (25°C); 0.01 ml of 0.2*M* neutralized EDTA; 0.010 ml of hemolyzate; 0.1 ml of 0.033*M* neutralized oxidized glutathione (GSSG); 0.1 ml of 1.0 m*M* reduced nicotinamide-adenine dinucleotide phosphate (NADPH); FAD, as indicated; and water to make 1.00 ml. Oxidized glutathione was omitted from the blank. The reaction was followed for 20 to 30 minutes at 340 nm in a Gilford model 2000 or model 2400 recording spectrophotometer at 37°C. The order in which reagents were added to the assay system influenced the rate of the glutathione reductase reaction. Maximum rates were obtained when the order of addition was: (i) FAD, (ii) GSSG, and (iii) NADPH. Therefore we first incubated the reaction mixture at 37°C and then added FAD (or water), GSSG, and NADPH, in that order, at 10-minute intervals. Results were expressed as the number of micromoles of NADPH oxidized per gram of hemoglobin per minute.

The addition of as little FAD to the assay system as to give a final concentration of 0.01 μmole/liter resulted in substantial stimulation of glutathione reductase activity of normal hemolyzates. Addition of 1 μM FAD (final concentration) to hemolyzates from 21 normal subjects caused 27 to 157 percent stimulation of glutathione reductase activity. Riboflavin and FMN were without effect.

Glutathione reductase in hemolyzates was "stripped" of FAD by treatment with acid and ammonium sulfate (*3*). Since the stripped enzyme had some activity without added FAD, and since, at low FAD concentrations, reactivation was time-dependent a classical kinetic analysis of the relation between enzyme and FAD could not be made. However, one-half maximum reactivation of preparations from the red cells of an individual with unusually low glutathione reductase activity and from individuals with a normal glutathione reductase activity was achieved with approximately 0.02 μM FAD.

The activation of stripped glutathione reductase or of normal hemolyzate was markedly inhibited by the addition of adenosine triphosphate (ATP), adenosine diphosphate, and, to a lesser extent, adenosine monophosphate. This effect resembled competitive inhibition in that it was most apparent at low FAD concentrations. It could be demonstrated easily with nucleotide concentrations as low as 0.1 m*M*, less than 10 percent of the normal level of ATP in red cells. The order of addition of FAD and adenine nucleotide was of great importance. If, for example, 1 m*M* ATP (final concentration) was added to stripped enzyme and followed after 10 minutes incubation by the addition of 0.020 μM FAD, reactiva-

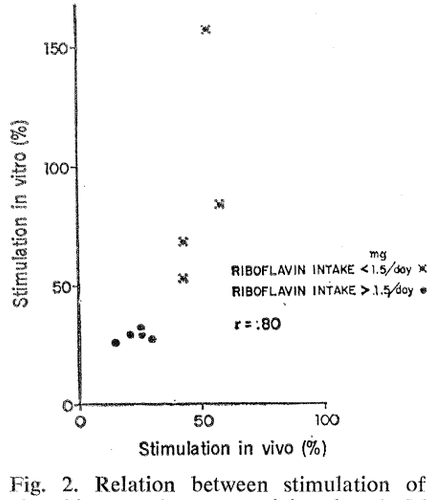

Fig. 2. Relation between stimulation of glutathione reductase activity by 1 μM FAD in vitro and stimulation of glutathione reductase by riboflavin administration in vivo. The degree of stimulation in vitro by FAD is plotted as the average of all assays before administration of riboflavin. The degree of stimulation in vivo is plotted from the ratio of the average glutathione reductase activities during 8 days of administration of 5 mg of riboflavin per day to the average base line enzyme activity.

tion of the enzyme was inhibited by 92 percent. If the order of addition of FAD and ATP was reversed, however, glutathione reductase activity was only inhibited 28 percent.

To determine whether FAD catalyzed the glutathione reductase reaction in the free form or whether the FAD became bound to protein, we added FAD in a final concentration of 0.125 μM to an incomplete reaction system, comprised of the tris buffer, EDTA, water and hemolyzate. Dialysis against medium without FAD failed to cause any loss of enzyme activity. It appeared that once the enzyme had been incubated with FAD firm binding of the FAD to enzyme occurred. Thus, although very small amounts of FAD activate the dilute enzyme in vitro, these quantities cannot be compared directly to the effect of FAD in intact red cells, since much of red cell FAD is undoubtedly protein bound and not readily available to glutathione reductase, and, in addition, the high concentrations of ATP within red cells may interfere with binding.

To determine the effect of administration of riboflavin on red cell glutathione reductase, nine normal subjects were studied before, during, and after administration of riboflavin. Five subjects had a dietary intake of riboflavin in excess of the recommended daily allowance (*4*) of 1.5 mg. They were estimated to be taking an average of 1.58, 1.77, 2.74, 2.79, and 3.70 mg/day. The other subjects had an intake less than the recommended daily allowance, averaging 0.67, 0.78, 1.05, and 1.09 mg of riboflavin daily. In all cases the intake was greater than that which causes frank riboflavin deficiency (*7*).

Two to six base line estimations of red cell FAD and glutathione reductase were carried out over 3 to 20 days on each of the nine subjects. Each volunteer was then given 5 mg of riboflavin daily for 8 days. Red cell FAD, FMN, and riboflavin were determined, and glutathione reductase was estimated at appropriate intervals. In each case the activity of red cell glutathione reductase was increased. These changes were most striking in the subjects with an average daily riboflavin intake of less than 1.5 mg, but they could also be observed in each of the other subjects, even when the dietary riboflavin intake was greater than 3 mg/day. An increase in red cell FAD concentrations could also be detected in most cases, especially when amounts of riboflavin in the diet were less than 1.5 mg/day. Only traces of free riboflavin and FMN were detected in the red cells and showed little change when riboflavin was administered (Fig. 1). None of the subjects reported any marked changes in feeling of well-being or other symptoms during riboflavin supplementation. The degree of increase of glutathione reductase activity during administration of riboflavin was closely related to the degree of stimulation in vitro of glutathione reductase by FAD and to the base line dietary level of riboflavin (Fig. 2).

Staal *et al.* (*8*) studied the partially purified glutathione reductase of a patient with α-thalassemia whose red cell glutathione reductase activity was 50 percent of normal. They considered their subject to have a genetically abnormal enzyme with an increased Michaelis constant for GSSG and found that enzyme activity was normal after administration of FMN for several weeks. Glatzle *et al.* (*9*) found that glutathione reductase of frankly riboflavin-deficient individuals was slightly stimulated in vitro by the addition of FAD; the enzyme from normal red cells was essentially unaffected by FAD. They suggested that Staal's patient might have been deficient in riboflavin. No experimental details were given in either study.

In our assay, the glutathione reductase activity of all hemolyzates was stimulated by FAD; the administration of 5 mg of riboflavin daily uniformly caused an increase in red cell glutathione reductase activity. These studies indicate that the intake of riboflavin, even in individuals taking a normal diet, is apparently inadequate to maintain saturation of red cell glutathione reductase with FAD.

The physiologic effects of moderately severe glutathione reductase deficiency are not well understood. Although deficiency of this red cell enzyme has been associated with a variety of abnormal clinical states (*10*), including drug-induced pancytopenia, nonspherocytic hemolytic anemia, oligophrenia, catatonia, and spasticity, a cause-and-effect relation between enzyme lack and disease is by no means certain. Glutathione reductase deficiency has been observed in some normal subjects (*11*). Furthermore, severe deficiency of glutathione reductase has been associated with homozygous hemoglobin C disease (*12*), a disorder which would be very difficult to ascribe to a defect in glutathione reductase activity, since it is clearly due to an amino acid substitution in the beta chain of hemoglobin. However, many of the clinical states which have been associated with glutathione reductase deficiency may have as a common denominator abnormalities in riboflavin nutrition or riboflavin metabolism. In fact, dietary riboflavin deficiency (*13*), feeding of a riboflavin antagonist (*14*), and glutathione reductase deficiency (*10*) have been reported to be associated with hypoplastic anemia. Thus, a deficiency of one of the enzymes required for the synthesis of FAD from riboflavin, flavokinase or FAD pyrophosphorylase, might result in diminution of glutathione reductase activity of red cells as only one of a number of disturbances of activity of other flavin enzymes.

ERNEST BEUTLER
Division of Medicine, City of Hope Medical Center, Duarte, California

References and Notes

1. M. Brin, S. S. Shohet, C. S. Davidson, *J. Biol. Chem.* **230**, 319 (1958); S. J. Wolfe, M. Brin, C. S. Davidson, *J. Clin. Invest.* **37**, 1476 (1958).
2. N. Raica, Jr., and H. E. Sauberlich, *Amer. J. Clin. Nutr.* **15**, 67 (1964); A. Jacobs, I. A. J. Cavill, J. N. P. Hughes, *ibid.* **21**, 502 (1968).
3. E. M. Scott, I. W. Duncan, V. Ekstrand, *J. Biol. Chem.* **238**, 3928 (1963). A. Icen, *Scand. J. Clin. Lab. Invest. Suppl.* **96**, 1 (1967).
4. *Food and Nutrition Board of National Research Council, Recommended Dietary Allowances* (National Academy of Science, Washington, D.C., 1968).
5. C. F. Bowes and H. N. Church, *Food Values of Portions Commonly Used* (Lippincott, Philadelphia, ed. 10, 1966); D. Turner, *Handbook of Diet Therapy* (Univ. of Chicago Press, Chicago, ed. 4, 1965); *Agriculture Handbook No. 8, Composition of Foods*, (Agricultural Research Service, U.S. Department of Agriculture, 1963).
6. O. A. Bessey, O. H. Lowry, R. H. Love, *J. Biol. Chem.* **180**, 755 (1949); H. B. Burch, O. A. Bessey, O. H. Lowry, *J. Biol. Chem.* **175**, (1948).
7. A. Keys, A. F. Henschel, O. Michelsen, J. M. Brozek, J. H. Crawford, *J. Nutr.* **27**, 165 (1944); R. D. Williams, H. L. Mason, P. L. Cusick, R. M. Wilder, *ibid.* **25**, 361 (1943).
8. G. E. J. Staal, P. W. Helleman, P. W. van Milligen-Boersma, M. C. Verloop, *Ned. Tijdschr. Geneesk.* **112**, 1008 (1968).
9. D. Glatzle, F. Weber, O. Wiss, *Experientia* **24**, 1122 (1968).
10. H. D. Waller, G. W. Löhr, E. Zysno, W. Gerok, D. Voss, G. Strauss, *Klin. Wochenschr.* **43**, 413 (1965); H. D. Waller, in *City of Hope Symposium Series*, E. Beutler, Ed. (Grune & Stratton, New York, 1968), vol. 1, p. 185; G. W. Löhr and H. D. Waller, *Med. Klin.* **57**, 1521 (1962); P. E. Carson, G. J. Brewer, C. Ickes, *J. Lab. Clin. Med.* **58**, 804 (1961).
11. E. Beutler, *J. Clin. Invest.* **48**, 7a (1969).
12. H. Waller, J. Bremer, H. Schönthal, W. Dorow, *Klin. Wochenschr.* **45**, 824 (1967).
13. H. Foy and A. Kondi, *J. Pathol. Bacteriol.* **65**, 559 (1953); *Brit. Med. J.* **1**, 937 (1961).
14. M. Lane and C. P. Alfrey, Jr., *Blood* **25**, 432 (1965).
15. Supported by HE 07449. I thank A. Meul, C. West, and M. Mitchell for technical assistance, and B. Berman for estimating dietary riboflavin intakes.

21 May 1969