References and Notes

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2. The glow modulator lamps were loaned by Dr. F. C. Campbell, and the amplifier to power them was designed by Dr. J. F. Robson, both of the Physiological Laboratory, Cambridge. This work was supervised by R. L. Gregory and supported by a D.S.I.R. (S.R.C.) research studentship.

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Glutathione Reductase in Red Blood Cells: Variant Associated with Gout

Abstract. *A variant of red cell glutathione reductase, characterized by greater electrophoretic mobility, increased enzyme activity per unit of hemoglobin, and an autosomal mode of inheritance, has been found in Negro populations. There appears to be an association of this variant with primary gout.*

During a survey of 1473 Negro male outpatients for variants of red cell glucose-6-phosphate dehydrogenase (G-6-PD) (*1*) by starch-gel electrophoresis (*2*), the extra slabs of most gels were stained for glutathione reductase by a two-stage procedure (*3*). This procedure results in a dark stain, whereas the reduced nicotinamide-adenine dinucleotide phosphate (NADPH) applied to the gel remains unaltered. The three light bands which appear for each samples (Figs. 1 and 2) are assumed to represent regions where NADPH has been oxidized. The light band nearest the origin of the sample on the gel is the location of glutathione reductase since this band does not appear unless oxidized glutathione is present in the staining mixture. The middle band, with migration identical to that of hemoglobin A, and the distal band appear with or without oxidized glutathione in the staining mixture. Presumably these two bands do not represent glutathione reductase. That they do not reflect some action of hemoglobin on NADPH is suggested by the presence of apparently identical bands on electrophoresis of serum or plasma.

For the G-6-PD study (*1*), hemolyzates were adjusted to a hemoglobin concentration of 0.2 g per 100 ml of hemolyzate before application to gels, and electrophoresis was carried out for 12 hours in the cold room. Under these conditions glutathione reductase in some samples migrated definitely faster and in other samples questionably faster than in most samples. When electrophoresis of the 196 samples most recently obtained from individuals included in the G-6-PD study (all from Negro male medical outpatients) was repeated and conducted for 14 to 15 hours instead of the usual 12 hours, the variant moved farther from the usual, more slowly migrating form (Fig. 1); most of the bands previously recorded as questionably fast spread into a wide band suggestive of the fast and slow bands merged together (Fig. 2).

Samples from 6 of these 196 individuals showed the electrophoretically fast band; 40 showed the wide band thought to represent the heterozygous state; and 150 showed the usual more slowly migrating band of glutathione reductase. If the fast and slow bands represent homozygotes and the wide band the heterozygote, gene frequency for the variant is .133, and for the usual form of the enzyme it is .867 with a standard error of .017. From this data, 3.5 individuals homozygous for the variant, 45.2 heterozygous for the variant and the usual form, and 147.3 homozygous for the usual form would theoretically be expected, a distribution which does not differ significantly from the observed distribution. Chi-square is 2.4334; P is less than .2 and greater than .1, with one degree of freedom. Since gene frequencies calculated for hospital patients do not necessarily reflect the frequencies in the population from which the patients are drawn, electrophoresis was subsequently performed on blood samples from 125 presumably healthy Negroes, 79 males and 46 females, randomly selected in Austin, Texas. In this group gene frequencies of .132 for the glutathione reductase variant and .868 for the usual form of the reductase, with standard error of .021, are almost identical with those found for the 196 outpatients.

Electrophoresis of red cell glutathione reductase from both parents and all children of nine Negro families (*4*) is consistent with the assumption that the wide band represents the heterozygous state and that there is an autosomal mode of inheritance for the enzyme. Among these families are three unrelated individuals who are homozygous for the variant enzyme and whose parents are each heterozygous. A fourth individual homozygous for the variant has a father homozygous for the variant and a heterozygous mother. For family studies, enzyme activity was determined as described (*5*), and each sample was then adjusted to the same enzyme activity per unit volume for application to the gels.

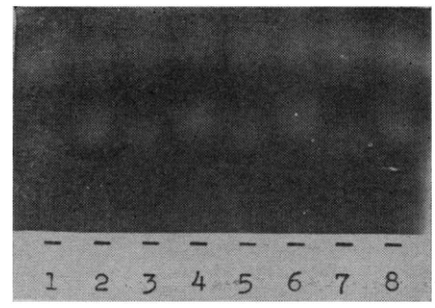

Fig. 1. Row of light bands nearest origin (bottom) indicates location of glutathione reductase on starch gel after electrophoresis with samples adjusted to the same enzyme activity. The same sample of the usual form of glutathione reductase in the odd-numbered positions is alternated with the same sample of the faster moving variant form in the even-numbered positions.

There was no association between the common G-6-PD variants and the glutathione reductase variant among the 196 Negro male outpatients. Twenty-five with G-6-PD of type A, 19 with type A(−), and 105 with type B were homozygous for the usual form of glutathione reductase. Seven with G-6-PD of type A, 5 with type A(−), and 34 with type B were heterozygous or homozygous for the glutathione reductase variant. Chi-square was 2.041 and P was about .9 (two degrees of freedom).

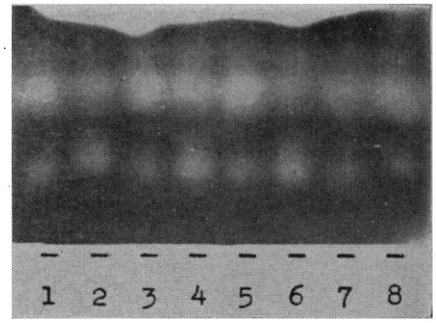

Fig. 2. The usual form of glutathione reductase is in positions 1, 3, 5, and 7. The electrophoreticaly fast variant is in positions 2 and 8. The broad band thought to represent the heterozygote for the usual and variant forms is in position 4 and 6. Samples are adjusted to the same enzyme activity.

Mean activity for red cell glutathione reductase (moles of NADPH oxidized per milliliter per minute) of 11 individuals homozygous for the variant is 9.50×10^{-8} with S.D. 1.71×10^{-8}; for 17 homozygous for the usual form, mean activity is 7.44×10^{-8} with S.D. 1.31×10^{-8}, referred to a hemoglobin concentration of 1 g per 100 ml of hemolyzate. Enzyme assays were performed as described (*5*) except that final hemoglobin concentration of reaction mixtures was 0.048 instead of 0.105 g per 100 ml of hemolyzate. The increased mean activity of the enzyme variant compared to the activity of enzyme from normal individuals is statistically significant by analysis of variance; F is 11.3793, and P is smaller than .01 (one degree of freedom).

Because of our previous finding of elevated activity of red cell glutathione reductase in a group of Caucasians with untreated primary gout (*6*) and because a majority of the 28 Negro males with primary gout in the G-6-PD study (*1*) appeared to have the glutathione reductase variant on initial electrophoresis, electrophoresis of the stored samples from the gout patients was repeated for a 14-hour period instead of the former 12-hour period. After assay, the samples were adjusted to the same enzyme activity for application to gels, where they were alternated with samples from homozygotes with the usual form of the enzyme. All samples had been stored in the cold room with acid-citrate-dextrose solution for a similar period of time. Each sample was run in duplicate on separate gels. There was a strong association between the glutathione reductase variant and primary gout (Table 1). Chi-square with Yates' correction was 42.6126, and P was smaller than .001 (two degrees of freedom).

Potentially, increased glutathione reductase activity, which appears to be characteristic of the variant enzyme, could result in greater availability of NADP, stimulation of glucose metabolism over the NADP-dependent pentose phosphate pathway, increased ribose production via that pathway, and eventually increased uric acid production from ribose. Excess ribose production via this pathway has been postulated as a possible mechanism for increased uric acid production in persons with gout (*7*).

In the United States, gout in Negroes and in Caucasians appears to occur with similar frequency (*9*). Differences of method between this study, where primary gout was found in 1.9 percent of 1473 Negro male general medical and medical specialty clinic outpatients, and other surveys of gout among Negroes (*9*) make it impossible to determine whether the frequency of gout in this outpatient group is similar to that found by others in Negro populations.

Table 1. Frequency of glutathione reductase (GSSG-R) phenotypes among gout patients and general medical patients. Chi-square with Yates' correction is 42.6126; P is less than .001 (two degrees of freedom).

GSSG-R phenotype*	Patients	
	Gout (No.)	General medical (No.)
S	5	150
FS	15	40
F	8	6

*S indicates the usual electrophoretically slower type; F indicates the electrophoretically fast variant.

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3. Staining procedure for glutathione reductase. For the first stage, 3.5 g of hydrolyzed starch was melted in 35 ml of water by heating. At 62°C, 12 mg of NADPH and 30 mg of oxidized glutathione in 1 ml of water; 1 ml of phosphate buffer, 1*M*, *p*H 7.0; and 1 ml of EDTA (ethylenediaminetetraacetic acid), 0.2*M*, *p*H 7.0, were mixed with the melted starch which was then poured over the surface of two gel slabs. After 10 minutes in the cold room, the slabs were incubated at 37°C for 2 hours. The starch overlay was then wiped from the gels with tissue, and the gels were rinsed with about 10 ml of de-ionized water and dried with tissue. For the second stage 3.5 g of starch was melted in 30 ml of water 4 ml of tris, 1*M*, *p*H 8.8. Immediately after the starch melted, 18 mg of nitro blue tetrazolium (or for a darker background 12 mg of tetranitro blue tetrazolium) in 5 ml of water and 0.4 ml of 0.5 percent methylene blue were mixed with the melted starch which was then poured over the gel slabs at 60°C. After the prepared slabs were incubated for 2 hours at 37°C, the starch overlay was removed as before. Gels were stored in tap water in the cold room until photographed. Use of the staining constituents in liquid overlays resulted in less distinct enzyme bands than when the stain was used in melted starch overlays. No glutathione reductase bands appeared on the gel if phenazine methosulfate was substituted for methylene blue in the second stage of the staining procedure with a liquid overlay, despite complete protection of the staining solution from light.
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10. Supported by USPHS grant HE 04516-07.

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Metabolism of Rotenone in vitro by Tissue Homogenates from Mammals and Insects

Abstract. *Hydroxylation of rotenone in vitro in the enzyme system composed of microsomes and reduced nicotinamide-adenine dinucleotide phosphate, and in living mice and houseflies, yields products tentatively identified as rotenolone I; rotenolone II; 8′-hydroxyrotenone; 6′,7′-dihydro-6′,7′-dihydroxyrotenone; two rotenolones of each of the last-mentioned two compounds; and uncharacterized polar materials. The toxicity of certain of these rotenoids to mice is of the same order as that of rotenone.*

Rotenone, in the form of ground derris roots, is used as an insect poison for the control of pests on plants and animals; as a fish poison to manage fish populations in reservoirs, lakes, and streams; and to eliminate undesirable species. As an insecticide chemical, rotenone has a short residual action and is exempt from the requirement of a residue tolerance when applied to growing crops in accordance with good agricultural practice. The fact that the metabolic fate of rotenone in insects and mammals is incompletely understood, although it has been rated as moderately to highly toxic to mammals, caused us to undertake the work reported here.

The enzyme system involved in the coupled oxidation of reduced nicotinamide-adenine dinucleotide ($NADH_2$) and reduction of cytochrome *b* is inhibited by very low concentrations of rotenone, whether or not this enzyme system is derived from species that are highly susceptible or resistant to rotenone poisoning (*1–3*). Thus, selective toxicity probably results from species