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X-Linked Glucose-6-Phosphate Dehydrogenase Deficiency in *Mus musculus*

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A mouse with X-linked glucose-6-phosphate dehydrogenase (G6PD) deficiency has been recovered in offspring of 1-ethyl-1-nitrosourea-treated male mice. The activity alteration was detected in blood but can also be observed in other tissue extracts. Hemizygous, heterozygous, and homozygous mutants have, respectively, about 15, 60, and 15% G6PD remaining activity in the blood as compared to the wild type. Erythrocyte indices did not show differences between mutants and wild types. The mutation does not affect the electrophoretic migration, the isoelectric point, or the thermal stability. Kinetic properties, such as the K_m *for glucose-6-phosphate or for NADP and the relative utilization of substrate analogues, showed no differences between wild types and mutants with the exception of the relative utilization of deamino-NADP which was significantly lower in mutants. This is presently the only animal model for X-linked G6PD deficiency in humans.*

KEY WORDS: glucose-6-phosphate dehydrogenase; mouse; enzyme activity mutant; erythrocyte; X chromosome; animal model.

INTRODUCTION

Glucose-6-phosphate dehydrogenase (G6PD) (EC 1.1.1.49) catalyzes the oxidation of glucose-6-phosphate to 6-phosphogluconate, with a simultaneous

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reduction of NADP. The enzyme is thereby of great importance in regulating the intracellular concentration of NADPH, which is essential for cell function. That G6PD deficiency in humans is very heterogeneous was first demonstrated by Marks and Gross (1959). According to Luzatto and Battistuzzi (1985), 279 different variants of G6PD had been described by 1983.

Based upon their clinical effect and G6PD activity in red blood cells (RBC) the variants can be divided into five classes (Luzzatto and Battistuzzi, 1985): (1) those with severe enzyme deficiency regularly associated with chronic hemolytic anemia, (2) those with erythrocyte enzyme activity <10% of the normal value but not associated with chronic hemolysis, (3) those with enzyme activity 10–60% of the normal value and not associated with chronic hemolysis, (4) those with enzyme activity within the normal range (60–130%), and (5) those which have increased enzyme activity. Those belonging to classes 4 and 5 are of no clinical importance.

In a mutation experiment, after the administration of 250 mg 1-ethyl-1-nitrosourea/kg body weight hybrid male mice were mated to untreated T-stock females. In a total of 505 offspring derived from spermatogonia, four mutants with altered erythrocyte enzyme activity were detected (Ehling *et al.,* 1985; Charles and Pretsch, 1987). One of the mutants was a female with approximately 60% G6PD activity compared to the wild type (Charles and Pretsch, 1984a). At present this is the only mutant for the X-chromosomal G6PD locus recovered among experimental mammals and may serve as a model animal for G6PD deficiency in humans. This paper describes the genetical, biochemical, and physiological analysis of the mouse mutant.

MATERIALS AND METHODS

Animals were weighed and blood was taken with heparinized glass capillaries from the retroorbital sinus. The determinations of blood glucose, blood glucose consumption, hematocrit, red blood-cell (RBC) count, and osmotic fragility of erythrocytes were done immediately. Subsequently mice were sacrificed by prolonged ether anesthesia and dissected. Liver, lung, kidneys, spleen, and heart were removed and weighed for determining organ–somato indices. Tissue samples were excised and prepared in ice-cold 0.15 M KCl for the determination of enzyme activities. To minimize the effects of diurnal rhythm these procedures were done between 8 and 10 a.m. Blood and other tissues were prepared as described earlier (Charles and Pretsch, 1981, 1984b) with the modification that the blood extraction medium contained 10 μM NADP.

G6PD activity was determined at 25°C in an Eppendorf ACP 5040 automatic enzyme analyzer (Hamburg, Germany, F.R.). The reaction mixture contained 0.1 M triethanolamine/1 mM EDTA buffer, *p*H 7.5 (hereafter

designated TRAB), 1.1 mM NADP, 3 mM D-glucose-6-phosphate (G6P) (Na_2 salt), and 22.1 mM $MgCl_2$. To minimize the contribution of 6-phosphogluconate dehydrogenase to G6PD activity, all determinations were performed within 2 min after starting the reaction. For the activity determinations with the substrate analogues 2-deoxyglucose-6-phosphate, galactose-6-phosphate, and deamino-NADP, the same final molar concentration was used as with G6P and NADP, respectively. The determinations of the specific activities of nine additional enzymes [glucose phosphate isomerase (GPI), triose phosphate isomerase (TPI), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 3-phosphoglycerate kinase (PGK), phosphoglyceromutase (PGAM), pyruvate kinase (PK), lactate dehydrogenase (LDH), glutathione reductase (GR), malate dehydrogenase (MDH)] and the hemoglobin content of red blood cells were performed as described elsewhere (Charles and Pretsch, 1987).

The protein concentration of the different tissue extracts was determined either in an UVIKON 710 spectrophotometer (Kontron Instruments, Zürich, Switzerland) following the procedure of Lowry *et al.* (1951) or in the ACP 5040 with the Biuret method described by Bergmeyer *et al.* (1974).

For the determination of the steady-state kinetic parameters, the UVIKON 710 spectrophotometer was utilized. Michaelis constants (K_m) for G6P and NADP were determined in hemolysates at 25°C. So that the G6PD activity was similar in the assays, hemolysates of wild types and heterozygotes were diluted 1:6 and 1:3, respectively, and hemolysates of hemizygous and homozygous mutants were not diluted. Five concentrations of G6P (0.01, 0.02, 0.04, 0.1, and 2.0 mM) and of NADP (8, 16, 32, 64, and 128 μM) were used. The apparent K_m was calculated by the least-squares fit.

Heat-stability experiments were performed with hemolysates in a circulating constant-temperature water bath at a temperature of 46°C. Blood was diluted 1:11 in TRAB, containing 5 mM dithiothreitol (DTT), 0.05% saponin, and crystalline bovine serum albumin (0.55 mg/ml). At 5-min time intervals, aliquots were removed from the samples, quickly mixed with ice-cold TRAB containing 5 mM DTT, and chilled in an ice bath. The residual G6PD activity was assayed shortly thereafter.

Plasma glucose concentration was determined by the glucose oxidase–peroxidase method (Werner *et al.,* 1970) after centrifugation of freshly drawn blood. For glucose consumption determinations, blood was preincubated at 37°C for 10 min, four aliquots were taken at intervals of 15 min, and the glucose concentration was measured. The decrease in glucose concentration was linear during the chosen time.

Red blood cells (RBC) were counted using Thoma's blood diluting pipette and an improved Neubauer's counting chamber (Brand, Wertheim, Germany, F.R.). For the hematocrit determination, blood was centrifuged in

Table I. Distribution of Progeny in Different Crosses Between Wild-Type Males (a/−), Hemizygous Males (a-m1Neu/−), Wild-Type Females (a/a), Heterozygous Females (a/a-m1Neu), and Homozygous Females (a-m1Neu/a-m1Neu)

Cross *G6pd* genotypes	No. of matings	No. of litters	No. of offspring (mean litter size)	*G6pd* genotype				
				a/−	a-m1Neu/−	a/a	a/a-m1Neu	a-m1Neu/a-m1Neu
(a/−) × (a/a)	7	15	80 (5.3)	37	—	43	—	—
(a-m1Neu/−) × (a/a)	12	47	287 (6.2)	142	—	—	145	—
(a/−) × (a/a-m1Neu)	21	68	445 (6.4)	119	108	100	118	—
(a-m1Neu/−) × (a/a-m1Neu)	17	56	346 (6.5)	90	88	—	78	90
(a/−) × (a-m1Neu/a-m1Neu)	4	19	120 (6.5)	—	53	—	67	—
(a-m1Neu/−) × (a-m1Neu/a-m1Neu)	11	40	232 (5.5)	—	115	—	—	117

hematocrit capillaries at 8000 rpm for 10 min at room temperature in a Microfuge (Heraeus-Christ, Osterode, Germany, F.R.).

Osmotic fragility of erythrocytes (Dacie and Lewis, 1984) was tested by mixing 5 μl blood with 1 ml NaCl solution and incubating the sample for 2 hr at 37°C. After centrifugation in a Centrifuge 5413 (Eppendorf, Hamburg, Germany, F.R.) at 11500 rpm for 3 min, the hemoglobin content was measured in the supernatant spectrophotometrically at 546 nm.

Polyacrylamide gel electrophoresis (Fehrnström and Moberg, 1977) was performed using the Tris–HCl–EDTA buffer method (Kirkman and Hendrickson, 1963) or the Tris–EDTA–borate system (Boyer *et al.*, 1963). Isoelectric focusing was carried out on ultrathin-layer polyacrylamide gels with a final *p*H range of 4.5–7 (Winter *et al.*, 1977) prepared according to the procedure of Radola (1980). The staining for G6PD was done in the dark at 37°C as described by the WHO Scientific Group (1967).

RESULTS

Genetic Characterization

The G6PD activity in blood of the original female mutant was 60% of the wild type. Breeding tests showed that the mutant was heterozygous for an allele determining wild-type activity ($G6pd^{a}$) and an allele determining low activity ($G6pd^{a\text{-}m1Neu}$). In the offspring of crosses with C3H/El males ($G6pd^{a}$/Y), besides wild types and heterozygous females, ($G6pd^{a\text{-}m1Neu}$/Y) males with only 15% G6PD activity in blood were found.

Additional breeding experiments have been conducted. Mating of G6PD hypoactive males with wild-type females results in wild-type males and heterozygous females. After crossing G6PD hypoactive males with heterozygous females, homozygous ($G6pd^{a\text{-}m1Neu}/G6pd^{a\text{-}m1Neu}$) mutant females with approximately 15% G6PD activity in blood were recovered. The results of all the matings performed so far are summarized in Table I.

Enzyme Activity Measurements

In Table II the G6PD specific activities are given after measurement in blood and several other tissues. By comparing the mutant genotypes with the wild types, the G6PD hypoactivity is clearly demonstrated in all investigated tissues. However, the relative reduction of activity in mutants varies depending upon the tissue examined.

The activity of additional enzymes was determined in whole-blood extracts. Seven enzymes required for glycolysis (GPI, TPI, GAPDH, PGK, PGAM, PK, and LDH) and one enzyme of the pentose phosphate pathway

Table II. Glucose-6-Phosphate Dehydrogenase Specific Activities[a] in Several Tissues of Different G6PD Genotypes[b]

Organ	*G6pd* genotype				
	a/–	a-m1Neu/–	a/a	a/a-m1Neu	a-m1Neu/a-m1Neu
Blood	13.6 ± 0.7	2.0 ± 0.2 (15%)	12.0 ± 0.6	6.9 ± 0.5 (58%)	1.5 ± 0.2 (13%)
Liver	4.6 ± 0.4	1.4 ± 0.2 (30%)	6.6 ± 0.7	4.2 ± 0.4 (64%)	1.9 ± 0.2 (29%)
Kidney	17.5 ± 1.0	7.4 ± 0.8 (43%)	15.5 ± 1.4	11.0 ± 0.6 (71%)	5.9 ± 0.4 (38%)
Lung	25.4 ± 1.5	5.5 ± 0.5 (22%)	25.1 ± 1.2	15.5 ± 1.3 (62%)	5.0 ± 0.3 (20%)
Spleen	42.5 ± 3.0	12.8 ± 0.9 (30%)	44.3 ± 1.4	30.1 ± 2.4 (68%)	13.0 ± 1.1 (29%)
Brain	21.7 ± 1.1	4.3 ± 0.3 (20%)	20.5 ± 0.9	11.9 ± 1.0 (58%)	3.2 ± 0.3 (16%)
Heart	3.9 ± 0.2	0.8 ± 0.05 (21%)	3.8 ± 0.4	2.1 ± 0.2 (55%)	0.8 ± 0.08 (21%)
Adrenal gland	71.5 ± 5.8	14.9 ± 1.2 (21%)	83.1 ± 4.2	51.1 ± 4.8 (62%)	20.8 ± 0.9 (25%)

[a] Means ± SE of 10 animals for each genotype. Activities expressed as U/g Hb in the blood and as U/g protein in the other tissues. The Hb and the protein content, respectively, were comparable in both sexes and all genotypes. The percentages of wild-type activity are given in parentheses.
[b] Same symbols are used as in Table I.

(GR) were determined. MDH was also quantitatively measured. Wild-type values were in the range of those reported by Charles and Pretsch (1987). No differences were observed in the activity of the nine enzymes obtained for the diverse G6PD genotypes.

Biochemical Characterization

In Fig. 1 the results of the thermal inactivation studies are shown. No significant differences were detected in the characteristics of G6PD in hemolysates from the different genotypes when the experiments were performed at a temperature of 46°C.

To test whether there is a difference in the relative rates at which wild-type animals and mutants can utilize substrate analogues of G6P or NADP, the G6PD activities for 2-deoxyglucose-6-phosphate, galactose-6-phosphate, and deamino-NADP have been determined (Table III). The results indicate a significant difference between the various genotypes only in the case of the relative utilization of deamino-NADP. The relative utilization of deamino-NADP was about 90% in wild types, nearly 70% in heterozygous mutants, and approximately 40% in hemizygous and homozygous mutants.

The K_m of mouse G6PD was determined for all genotypes to be approximately 40 μM for G6P and 6 μM for NADP (Table III). There was no significant difference between wild types and mutants. The values observed here are similar to the values reported in the literature for wild-type animals (Hutton, 1971).

Polyacrylamide gel electrophoresis as well as isoelectric focusing was performed with hemolysates and kidney, liver, or spleen extracts. Due to the diminished enzyme activity, there was a decreased staining intensity of the mutant G6PD bands as compared to the wild type. No change in the electrophoretic mobility of samples of different genotypes could be observed.

Physiological Characterization

Hematological properties of the mouse G6PD mutant have been investigated (Table IV): hematocrit values, red blood-cell count, hemoglobin content, and subsequently the erythrocyte indices exhibit no significant differences between the different genotypes. This is in accordance with human G6PD variants. In most subjects the hematological phenotype is practically normal, and acute hemolysis, which can be severe and even life-threatening, is seen only in response to an exogenous trigger.

The osmotic-fragility test, i.e., the addition of blood to a series of salt solutions of rising tonicity and the spectrophotometrical assessment of lysis, measures the ability of red cells to swell in hypotonic media. It provides, for

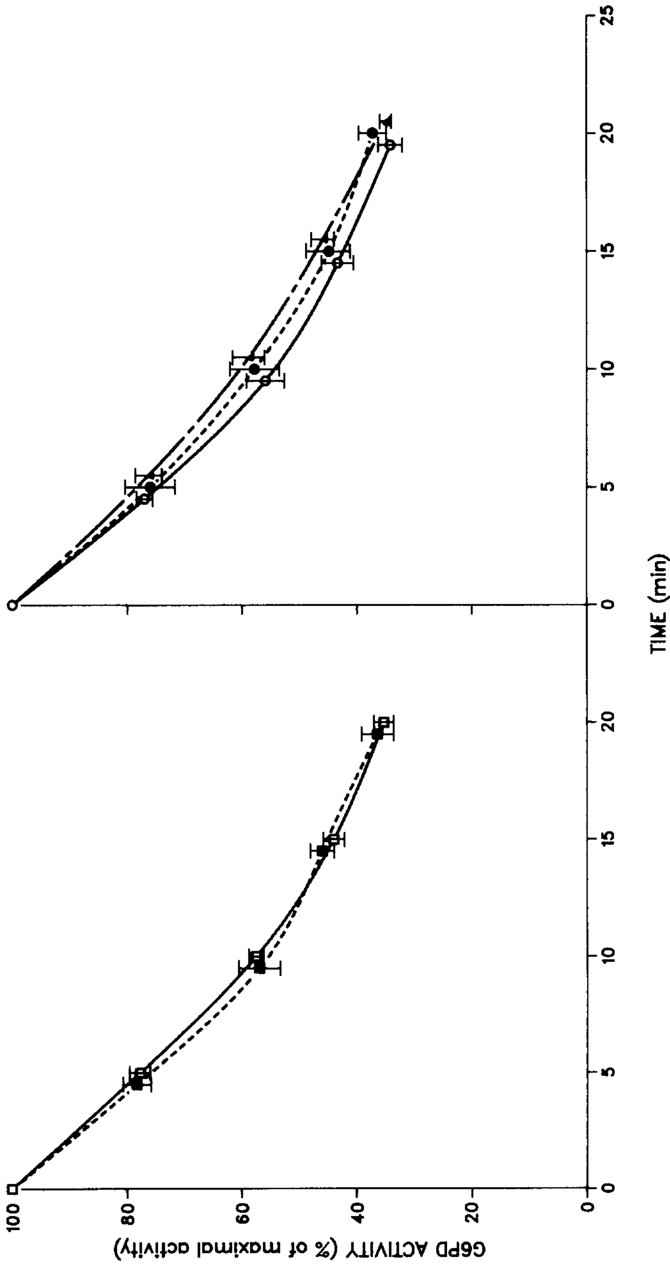

Fig. 1. Heat-denaturation properties of whole-blood G6PD after incubation at 46°C. Means ± SD of four animals per genotype. (□) Wild-type male; (■) hemizygous male; (○) wild-type female; (●) heterozygous mutant female; (▲) homozygous mutant female.

Table III. Utilization of 2-Deoxyglucose-6-Phosphate (2dG6P), Galactose-6-Phosphate (Gal6P), and Deamino-NADP as Substrate Analogues for Glucose-6-Phosphate and NADP[a] and Michaelis Constants[b] (K_m) for the Substrates G6P and NADP

	Percentage			K_m (μM)	
G6pd genotype[c]	2dG6P	Gal6P	Deamino-NADP	G6P	NADP
a/–	15 ± 1	12 ± 1	90 ± 2	40 ± 2	6.3 ± 0.9
a-mlNeu/–	16 ± 3	14 ± 1	35 ± 6*	37 ± 3	6.0 ± 0.5
a/a	16 ± 1	11 ± 1	89 ± 1	40 ± 3	6.8 ± 1.3
a/a-mlNeu	17 ± 3	13 ± 1	70 ± 6*	40 ± 3	6.4 ± 0.8
a-mlNeu/a-mlNeu	16 ± 3	15 ± 1	44 ± 8*	38 ± 4	5.6 ± 1.1

[a]Expressed as a percentage of the activity (means ± SD) measured with the same final concentrations of glucose-6-phosphate or NADP for four to six animals.
[b]Means ± SE for 10 animals.
[c]Same symbols are used as in Table I.
*Significant differences ($P < 0.01$) between wild types and mutants (Student t test).

example, a measure of spherocytosis or leptocytosis and demonstrates the relationship between surface area and volume. In Fig. 2 the osmotic-fragility curves for wild-type males and females are shown. A sex-dependent difference can be demonstrated; however, no differences between wild-type and hemizygous males or among wild-type, heterozygous, and homozygous females were observed.

The measurement of the glucose concentration in plasma and the glucose consumption in blood as well as the determination of body weight and organ-somato indices yielded no significant differences between wild-type and mutant animals.

DISCUSSION

G6PD Deficiency in Related Species

It is known in humans that the structural gene for G6PD is located on the X chromosome. The phenotypic consequences of an abnormal gene leading to the production of a defective G6PD enzyme are dependent upon genotype. The defect is fully expressed in the male hemizygote and female homozygote and only partially expressed in the female heterozygote. During differentation one X chromosome in females is inactivated, resulting in heterozygotes having two populations of red cells, those with normal enzyme activity and those with deficient enzyme activity (Sansone *et al.,* 1964).

Deficiencies in activity of G6PD occur in 3 to 20% of males from many parts of the world where falciparum malaria has been endemic (Kirkman,

Table IV. Hematological Properties of Different G6PD Genotypes[a]

	G6pd genotype				
	a/–	a-mlNeu/–	a/a	a/a-mlNeu	a-mlNeu/a-mlNeu
Hematology					
Hematocrit (%)	47.5 ± 0.6	46.6 ± 0.8	47.0 ± 0.7	47.2 ± 0.8	46.7 ± 1.0
RBC ($\times 10^{12}$/liter)	8.46 ± 0.18	8.35 ± 0.18	8.38 ± 0.15	8.17 ± 0.14	8.44 ± 0.16
Hemoglobin (g/liter)	154 ± 6	151 ± 4	155 ± 5	155 ± 12	153 ± 4
Erythrocyte indices					
Mean corpuscular hemoglobin (pg)	18.2 ± 0.6	18.1 ± 0.7	18.5 ± 0.7	19.0 ± 1.2	18.1 ± 0.9
Mean corpuscular volume (fl)	56.1 ± 0.9	55.8 ± 1.9	56.1 ± 2.0	57.8 ± 2.5	55.4 ± 1.8
Mean corpuscular hemoglobin concentration (g/L)	324 ± 10	324 ± 18	329 ± 9	338 ± 28	328 ± 15

[a]Same symbols are used as in Table I. Values are expressed as means ± SE of 12 animals for each genotype.

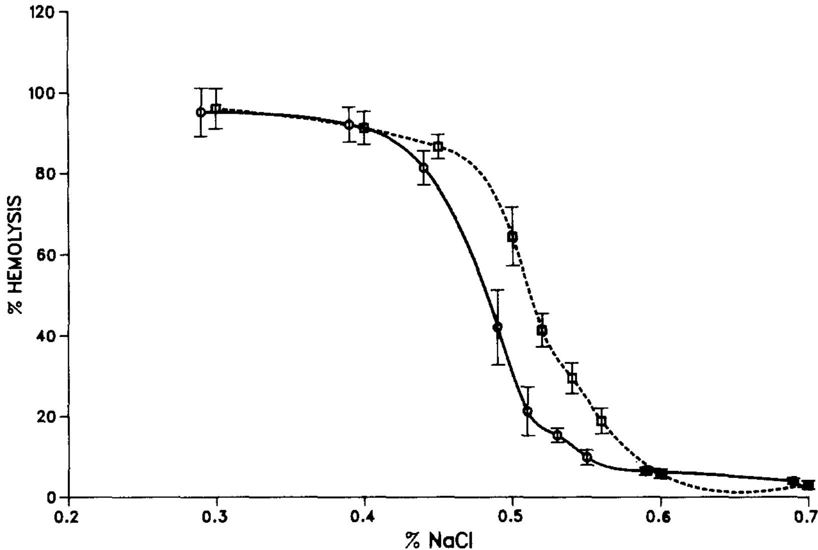

Fig. 2. Erythrocyte osmotic-fragility curves of wild-type male (□) and female (O) mice.

1971). In consideration of this high frequency, the WHO Scientific Group (1967) emphasized the need for laboratory animal models of this human hereditary disorder.

G6PD deficiency was reported in the literature in a colony of rats and in one dog. Werth and Müller (1967) observed a heritable deficiency of G6PD in erythrocytes in the rat. The biochemical evaluation of the animals and the analysis of the mode of inheritance were impossible due to the remarkably high rate of mortality among newborns and young rats as well as to the frequency of sterility in the affected family. It is not clear whether the increased mortality and decreased fertility observed in the lineages of crosses of the presumed G6PD mutation were a direct result of the presumed mutation. In any event, the potential animal model for G6PD deficiency in the rat has been lost.

After screening 3300 dogs, Smith *et al.* (1976) detected one animal with a mild deficiency of erythrocyte G6PD. The activity was approximately 44% of normal when assayed by conventional spectrophotometric techniques. Although the dog had several clinical problems for 2 months, no abnormality could be directly attributable to the reduced enzymatic activity. Biochemically the enzyme variant was electrophoretically slower but within the normal range for kinetic parameters, utilization of substrate analogues, *p*H optimum, and heat stability. Offspring from the deficient dog, littermates, or other related animals could not be located, which excluded pedigree analysis.

Variation of G6PD Activity and G6PD Deficiency in Mice

Hutton (1971) demonstrated that in mice the erythrocyte G6PD activity characteristic of each of 16 mouse strains falls into one of three distinct classes. There is no indication that structural variation is responsible for the variation in G6PD activity among strains, since partially purified enzyme from each class has the same thermal stability, *p*H–activity profile, Michaelis constants for G6P and NADP, electrophoretic mobility, and activity using substrate analogues. The quantitative differences in red-cell G6PD activity are regulated not by X-linked genes but by alleles at two or more autosomal loci.

Epstein (1969) demonstrated that G6PD enzyme activity in oocytes of the mouse was directly related to the number of X chromosomes in X/O and X/X females. Based on these observations the *G6pd* locus, controlling the major isozyme of G6PD, was concluded to be localized on the X chromosome of the mouse. X linkage of the G6PD deficiency described in this paper is shown very clearly by the results of the breeding experiments (Table I). Linkage tests with the X-linked markers *Hq, Ta,* and *Xcat* have been performed recently (Peters *et al.,* in preparation; Favor and Pretsch, in preparation).

The G6PD deficiency does not affect litter size in matings of the different genotypes. The genetic segregation data show no disadvantage for a definite genotype and no sterile mouse has been found among the tested animals. In accordance with this, no significant differences could be observed in the tested physiological traits of the various G6PD genotypes. The previously described elevated spleen weights in hemizygotes (Charles and Pretsch, 1984a) could not be verified. However, the population of hemizygous male mutants appears to be heterogeneous. Most individuals have a normal spleen weight, while a small portion displays an increased spleen weight. Perhaps an as yet unidentified environmental stress affects some individuals, although standard husbandry measures have been taken to assure similar conditions for all animals.

The hematological findings exclude the possibility that the decreased G6PD activity in blood extracts is influenced by a distinct red-cell age distribution of the various genotypes. Therefore, in comparison with the human G6PD deficiency variants, the mouse mutant falls into class 3 with regard to its biochemical and hematological characteristics, with moderate to mild enzyme deficiency (10–60% of normal activity) and no chronic hemolysis. However, it is remarkable that a strong correlation between clinical severity and level of G6PD activity in humans does not exist (Kirkman, 1971).

A clear sex difference found in osmotic fragility of erythrocytes was observed, although there was no significant difference in the other hematolog-

ical parameters between males and females. It may be possible that a sex difference in the plasma is responsible for this fact. It is known that plasma compounds, i.e., sterol derivatives, may influence osmotic fragility (Streuli *et al.,* 1981).

The mechanism by which a mutation which leads to a deficiency in enzyme activity might be due to decreased synthesis, accelerated breakdown, decreased catalytic activity, or any combination thereof. There is strong evidence that the mouse mutation has exclusively diminished the catalytic activity of the mutated enzyme and the underlying defect is a structural gene mutation. This is as follows.

(1) The deficiency is expressed to a similar extent in all tissues studied. Small differences of the expression may be due to tissue-to-tissue differences in proteases (Beutler, 1983).

(2) There are significant differences in the relative utilization of deamino-NADP between G6PD of wild types and G6PD of mutants. Such differences in the physicochemical properties have been shown to detect structural variants of human G6PD (Beutler *et al.,* 1968).

(3) As shown above, the deficiency is transmitted X linked. Today for mice, only two autosomal regulatory genes, *Gdr-1* and *Gdr-2,* have been described, which are postulated to govern the level of G6PD activity in erythrocytes of the mouse (Hutton, 1971).

The importance of the mouse mutant described consists in its possible use as an animal model for human G6PD deficiency. There are an estimated 100 million individuals in the world who are G6PD deficient (WHO Working Group, 1982). Human defects of this red-cell enzyme, in various forms, are the basis of favism, primaquine sensitivity and other drug-sensitive hemolytic anemia, anemia and jaundice in newborn, and chronic nonspherocytic hemolytic anemia (Beutler *et al.,* 1968). Different variants of the enzyme are found at a high frequency in African, Mediterranean, and Asiatic populations (Porter *et al.,* 1964). The question of an eventual selection advantage of human heterozygotes to malaria (Luzzatto *et al.,* 1969; Bienzle *et al.,* 1972) might be answered using the G6PD mouse mutant. This fact has been held responsible for the high frequency of the particular alleles in human populations from malarial regions.

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