THE NORMAL HUMAN FEMALE AS A MOSAIC OF X-CHROMOSOME ACTIVITY: STUDIES USING THE GENE FOR G-6-PD-DEFICIENCY AS A MARKER*

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The question why human females do not synthesize twice as much of those enzymes controlled by a locus on the X-chromosome as do males has never been answered satisfactorily. In human autosomal mutations in which the product of gene action can be quantitated, the heterozygote appears to produce approximately one-half of the normal quantity of the protein synthesized. Examples include acatalasemia,[1] congenital methemoglobinemia due to diaphorase deficiency,[2] PTA deficiency,[3] non-spherocytic congenital hemolytic anemia due to a deficiency of pyruvic kinase,[4] and the hemoglobinopathies.[5] This so-called "dosage effect" would lead one to believe that each gene governs the synthesis of a discrete amount of enzyme. Yet, when the gene is on the X-chromosome, such as the gene governing the synthesis of antihemophilic globulin or of glucose-6-phosphate dehydrogenase (g-6-pd), the quantity of protein produced is not twice as great in females as it is in males, in spite of the fact that females have two X-chromosomes, while males only have one.

On the basis of Drosophila genetics, attempts have been made to explain this in terms of hypothetic "dosage compensator" genes.[6, 7] However, the work of Ohno and his collaborators,[8] at this institution, has suggested to us an alternative explanation which at the same time explains the markedly variable penetrance observed in the g-6-pd–deficient heterozygote.

Ohno has shown that in somatic cells of human females, the two X-chromosomes are not alike. One behaves in exactly the same manner as the autosomes, remaining in an extended state during interphase and prophase, while the other assumes a heavily condensed state, forming the Barr sex chromatin body. In male somatic cells, on the other hand, the single X never manifests positive heteropycnosis; it always appears fine and elongated. It seemed to us entirely possible that the female X which condenses during interphase is genetically inactive. The active chromosome could not always be derived either from the mother or from the father, since this would give a pattern of inheritance of sex-linked mutations quite different from the pattern as we know it to exist. It seems necessary, therefore, to assume that there is, at least for some period of time during development, randomization of the active and inactive chromosome among the dividing cells of the body. Women would then be composed of a mosaic of cells, some with a functional paternal X-chromosome, others with a functional maternal X-chromosome.

Glucose-6-phosphate dehydrogenase (g-6-pd) deficiency, an inborn error of metabolism of humans,[9] represents an ideal test system in which to examine this hypothesis. Many heterozygous females with this disorder have red cell g-6-pd levels which are approximately 50 per cent of normal. If the precursors of a heterozygous female's erythrocytes were, as our hypothesis suggests, a mosaic consisting of some cells with the maternal X-chromosome active, others with the paternal X-

chromosome active, then the erythrocytes produced should also represent a mosaic consisting of cells with normal enzyme activity and of cells with grossly deficient enzyme activity. While we have previously suggested this possibility,[10] we have only now been able to obtain experimental verification.

While our studies were under way, Lyon[11] independently formed the same hypothesis as we had, from Ohno and Hauschka's[12] observation of X-chromosome dissimilarity in mice. Based on the mosaicism of phenotypic expression in female mice heterozygous for X-linked recessive genes affecting coat color, her elegant observations strongly suggests that the same mechanism may be operative in mice.

Experimental.—Our studies have been carried out on the blood of three females with approximately 50 per cent of normal enzyme activity in their red cells, several normal subjects, and two hemizygous g-6-pd–deficient males. Two of the three intermediate reacting females were known to be heterozygotes by analysis of their pedigrees. Both of these were of Sephardic Jewish origin. The other "intermediate" female was an American Negro and can be assumed to be a heterozygote, although no pedigree analysis has been possible. Blood samples drawn into acid-citrate dextrose solutions have been used in all of the studies.

In our first studies, the fact that glutathione (GSH) stability is related to g-6-pd activity was employed to examine the hypothesis that the blood of heterozygotes consisted of two distinct populations. Aliquots of blood from normal and severely enzyme-deficient subjects were subjected to the glutathione (GSH) stability test, as described by one of us,[13] but modified in two different ways to best demonstrate the presence of separate cell populations in mixtures. In the first modification used, a number of 1 cc aliquots of the blood to be tested were incubated at 37°C in tubes containing 5 mg of acetylphenylhydrazine (APH). The tubes were mixed and oxygenated hourly by vigorous agitation against the palm of the hand. Duplicate tubes were removed for repeated determinations over a period of five hr. One-hundredths ml of 30 gm per cent glucose was added to each tube remaining at three hr to assure continued adequate glucose levels. Figure 1 demonstrates that there was a gradual decrease in the GSH content of normal blood under these conditions, but a very rapid disappearance of GSH from blood obtained from a g-6-pd–deficient male. A mixture of compatible blood from a normal and a g-6-pd–deficient male gave rise to a two-component curve (Fig. 1). This is the curve which would be predicted if the behavior of GSH in the two red cell populations were independent: a rapid component representing the disappearance of the sensitive cells in the mixture, followed by a slow component representing the remaining normal cells. This test system has been used on the red cells of the three "intermediate" females available to us for study. In each case, the disappearance of GSH occurred in two components, just as in the artificial mixture (Fig. 2). Extrapolation of the second component to the beginning of the experiment gives an estimate of the percentage of normal cells in the mixture which agrees fairly well with the percentage of normal enzyme activity in the mixed red cells. While there is no model which may be used to predict the behavior of a single population of red cells with intermediate enzyme activity, a single curve of intermediate slope would be expected rather than a two-component curve.

A second modification of the glutathione stability test was also devised. In this system, the degree of oxygenation and the period of observation were longer, but

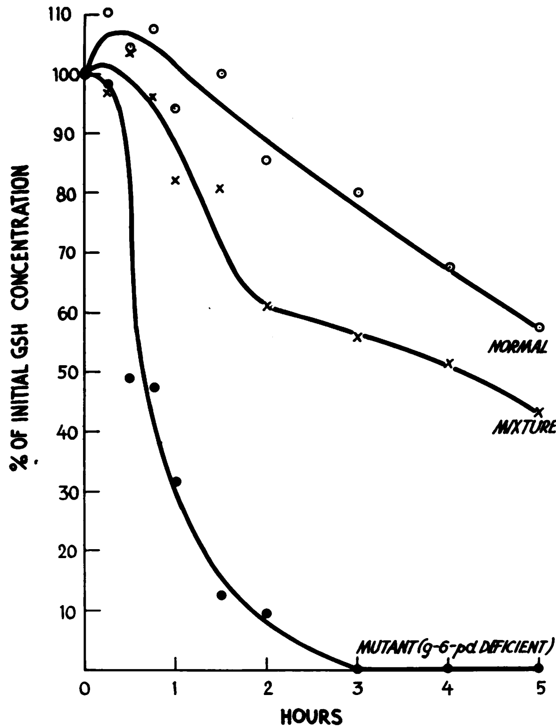

FIG. 1.—The rate of disappearance of GSH from the blood of a g-6-pd–deficient male, a normal subject, and an artificial mixture. One-cc samples of whole blood were incubated with 5 mg acetylphenylhydrazine. Conditions of the study are described fully in the text. Each point represents the average of duplicate determinations.

the concentration of APH was reduced. Oxygenation of blood samples was carried out continually by shaking a 40 ml blood sample in a 125 ml Erlenmeyer flask in a 37°C Dubnoff metabolic incubator at 110 cycles per min. Two or 3 mg APH was added per ml of blood. Two-tenths ml of a 30 gram per cent glucose solution was added to the mixture every two hr to make certain that adequate glucose was present throughout the entire 9 hr period of the study. Because both the blood filtrates and the nitroprusside solution are somewhat unstable, it is difficult to achieve a high degree of precision in studies of this long duration. When the GSH level of normal and g-6-pd–deficient blood samples were plotted against time, sigmoid curves were obtained. In the case of the g-6-pd–deficient male hemizygote, the maximum slope was observed between $^1/_2$ and $1^1/_2$ hr; in normal cells, the maximum slope occurred at between 6 and 8 hr. Artificial mixtures of g-6-pd–deficient and normal cells behaved as the sum of their parts (Fig. 3). There were two inflections in the curve; one at 1 to 2 hr, the other at 6 to 8 hr. If the red cell population of a female heterozygote with this disorder were to consist of cells with little or no enzyme activity and cells with normal activity, a curve with two slope maxima would be observed upon incubation with APH and periodic determination of glutathione levels. On the other hand, if the female heterozygote were to have a red cell population consisting of red cells with enzyme activity intermediate between that of normal and enzyme-deficient blood, a single maximum slope would be ex-

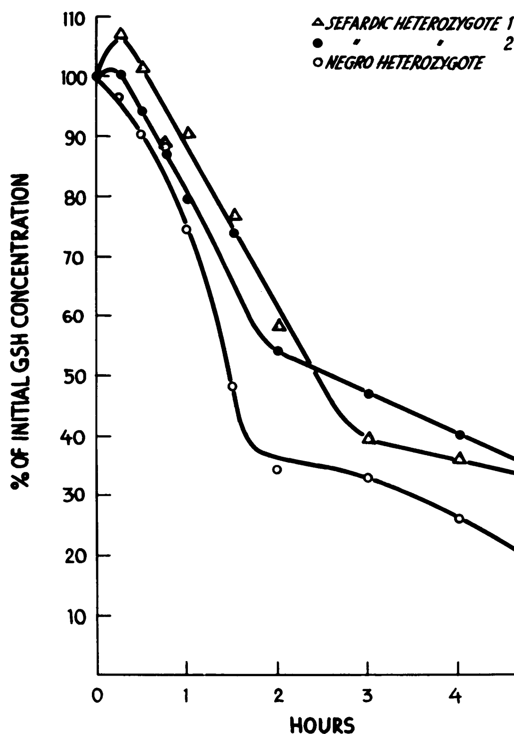

FIG. 2.—The rate of disappearance of GSH from the blood of three females with "intermediate" blood g-6-pd levels. Conditions of the test as in Figure 1.

pected. Figure 4 illustrates the effect of incubation with APH upon blood samples obtained from the one intermediate female studied by this technique. When either 2 or 3 mg of APH per cc of whole blood was used, two maxima were found in the slope of the curve. As in the artificial mixture, these occurred at one-half to 2 hours' and 6 to 8 hours' incubation.

We have also been able to demonstrate that the "intermediate" female behaves as if there were two red cell populations with respect to the rate of methemoglobin reduction. In the presence of dyes such as Nile blue sulfate or methylene blue, methemoglobin reduction is almost entirely TPN-linked, and, therefore, depends upon the level of g-6-pd activity. Methemoglobin estimations were carried out by the method of Evelyn and Malloy.[14] In the presence of Nile blue sulfate, normal red cells rapidly reduced methemoglobin induced by nitrite treatment of the cells. Mutant cells reduced methemoglobin very slowly. When mixtures were studied, a two-component curve was seen (Fig. 5). (It is of interest that methylene blue cannot be used in such studies. With this agent artificial mixtures do not behave in the predicted fashion. It appears that in the presence of methylene blue the enzymatic activity of normal red cells can result in the reduction of methemoglobin in mutant cells. These studies will be reported in detail elsewhere.[15]) Examination of red cells of the heterozygote shows that methemoglobin reduction occurs in two distinct components just as in an artificial mixture of mutant and normal red cells (Fig. 6). A single population of red cells with intermediate g-6-pd activity would

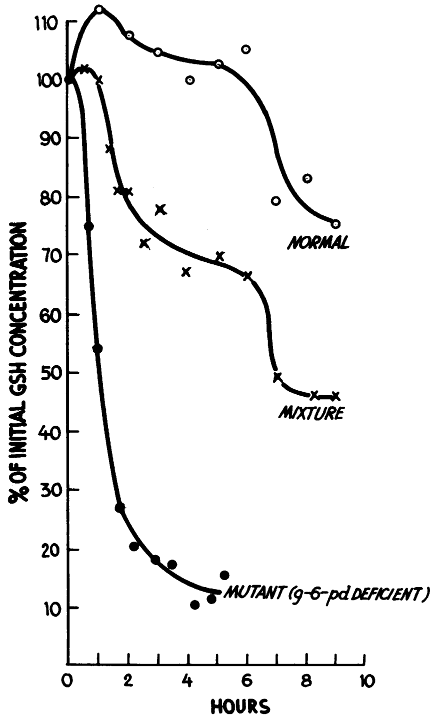

FIG. 3.—The rate of disappearance of GSH from the blood of a g-6-pd–deficient male, a normal subject, and an artificial mixture. Repeated samples were taken from a flask containing 2 mg APH/ml of blood. The samples were shaken continually and aliquots were removed for GSH determinations. Conditions of the study are fully described in the text.

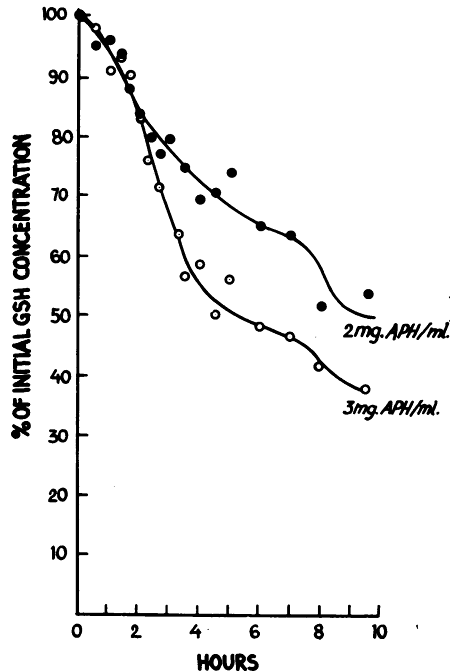

FIG. 4.—The rate of GSH disappearance from the blood of a Negro female with intermediate red cell g-6-pd levels. Conditions of the study as in Figure 3, except that both 2 mg and 3 mg APH/ml blood were used.

be expected to give a curve with a single intermediate slope until the percentage of methemoglobin was less than 20 per cent. In point of fact, we have been able to demonstrate that red cells whose methemoglobin-reducing capacity has been partially impaired with 2×10^{-4} M iodoacetate behave in this fashion.

Discussion.—These studies strongly suggest that the red cells of the heterozygote represent a mosaic of g-6-pd–deficient and normal units. It is almost certain that these units are the red cells themselves, since it seems almost inconceivable to us that each cell consists of multiple metabolically independent compartments. The presence of a mosaic of g-6-pd–deficient and normal red cells is consistent with the hypothesis that the X-chromosome with the normal gene was active in the case of one cell population, while the chromosome with the mutant gene was active in the other. While we consider the evidence for the existence of two red cell populations to be strong, it is indirect. Final proof will need to come from histochemical studies now underway in this laboratory.

We have for some time been disturbed by the fact that it is not uncommon for heterozygotes for g-6-pd deficiency to have entirely normal or absent red cell enzyme. Although some population surveys[16, 17] suggest that the percentage of

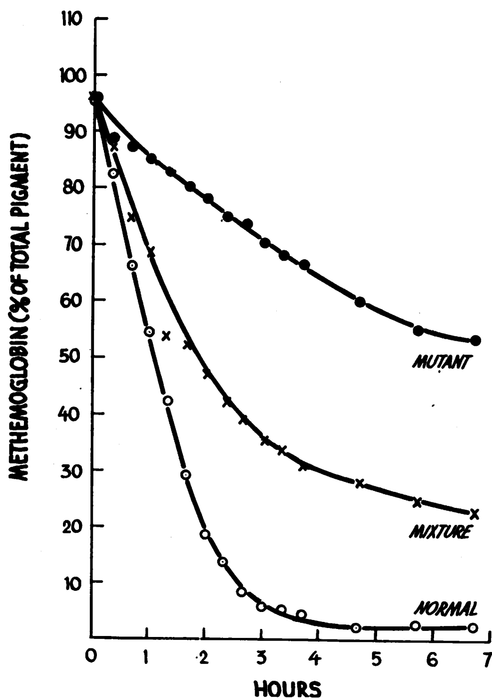

FIG. 5.—The rate of methemoglobin reduction in the red cells of a g-6-pd–deficient male, a normal subject, and an artificial mixture. The reaction mixture, incubated at 37°C, contained the following: glucose, 5 mg/cc; Nile blue sulfate, 11 mcgs/ml; red blood cells 29% suspension; all in isotonic potassium phosphate buffer pH 7.4.

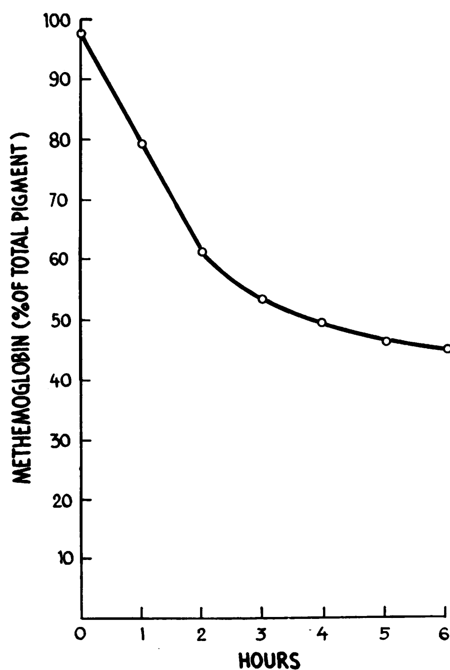

FIG. 6.—The rate of methemoglobin reduction in the red cell of a Negro female with "intermediate" red cell g-6-pd activity. Conditions as in Figure 5.

"intermediate" females is very close to that computed from the gene frequency as estimated on the male population, many exceptions have been observed. Analysis of the pedigrees originally reported by Childs *et al.*[18] demonstrates that of the 20 mothers of affected males examined, 6 had normal glutathione stability tests, 7 had intermediate tests and 7 had marked GSH instability, indicating gross enzyme deficiency. If only mothers known to be heterozygotes by pedigree analysis are considered, four mothers were normal, one was intermediate, and one was deficient. Similarly, glutathione stability tests reported by Sansone[19] revealed that three mothers of g-6-pd–deficient sons were normal, four intermediate, and three deficient. Gross *et al.*[20] have reported a pedigree in which there was well marked deficiency of g-6-pd in a mother who had both normal and enzyme-deficient sons. In a Sephardic Jewish family which we have recently examined, the mother of normal sons had total absence of g-6-pd.[21] We have also observed normal enzyme activity in the red cells of a female heterozygous for g-6-pd deficiency associated with congenital non-spherocytic hemolytic anemia. A similar case has recently been reported by Kirkman and Riley.[22] Such a large number of female heterozygotes with normal or grossly deficient enzyme may be explained on the basis of permanent inactivation of one or the other X-chromosome early in embryonic development. The fact that the sex chromatin is first found in the late morula stage of development[23, 24]

is consistent with the idea that the activity of the X-chromosome becomes fixed early. At this stage of development, it is entirely possible that only a very limited number of cells serve as progenitors of the yolk sac. It is possible that only a limited number of these serve as progenitors of the entire erythroid series in later life. If two cells at this stage of development form the basis of the erythroid series, one would expect by chance that one-quarter of heterozygotes would have entirely normal red cell enzyme activity, one-quarter would have absent red cell enzyme activity, and one-half would represent a red cell mosaic, as has been shown above. If three cells represented progenitors of the erythroid series at the time of X-chromosome inactivation, the expected ratio would, of course, be one-eighth marked deficiency, three-eighths containing one-third normal activity, three-eighths containing two-thirds normal activity, and one-eighth containing full activity. If the number of red cell precursors in the embryo at the time that one X-chromosome is inactivated is any greater number, one could predict the relative incidence of varying degrees of deficiency for any number of precursor cells at the time of inactivation according to the binomial expansion. There is, of course, no reason to believe that the number of red cell progenitors is exactly the same in each embryo at the time of inactivation. The reason, then, that an appreciable number of individuals heterozygous for g-6-pd deficiency have normal or grossly deficient red cell enzyme levels would be that a very limited number of cells in the embryo represent red cell precursors at the time of inactivation of one of the X-chromosomes. The effect might be quite different in other sex-linked abnormalities, such as hemophilia. Here, the number of cells in the embryo which represent the precursors of the antihemophilic globulin-producing cells may be considerably greater at the time of inactivation.

Our data are most consistent with the point of view that the human female is normally a genetic mosaic, containing cells with a genetically active maternal X-chromosome and cells with a genetically active paternal X-chromosome. The fixation of the role each chromosome is to play seems to occur at an early stage of embryonic development.

Summary.—1. To explain the lack of dosage effect in human sex-linked traits, it is proposed that the human female may represent a mosaic of cells, consisting of those with an active paternal X-chromosome and those with an active maternal X-chromosome.

2. To test this hypothesis, red cells of females heterozygous for glucose-6-phosphate dehydrogenase (g-6-pd) deficiency have been studied. If such females represent a genetic mosaic, one would expect two red cell populations, g-6-pd–deficient cells and normal cells. If, on the other hand, both X-chromosomes are active in all of their red cell precursors, a single population of red cells with intermediate g-6-pd activity would be predicted.

3. The glutathione (GSH) stability of red cells of heterozygotes was studied in two different systems. In each system, the disappearance of GSH in the presence of acetylphenylhydrazine followed the course predicted for, and demonstrated to occur with, a mixed red cell population consisting of g-6-pd–deficient and normal cells.

4. The rate of reduction of methemoglobin in the presence of Nile blue followed the course predicted for, and demonstrated to occur with, a mixed red cell popula-

tion consisting of g-6-pd–deficient and normal cells.

5. These findings support the hypothesis that only one of the X-chromosomes is active in each red cell precursor of the human female.

6. Early inactivation of one of the X-chromosomes will explain the markedly variable penetrance of this defect in females.

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