Kurland and Maaløe postulate that the effect is due to a constant small turnover of proteins. In the presence of chloramphenicol, the degradation would be unimpaired, but since the rebuilding of proteins is stopped, the amino acid pool would be replenished. For this idea experimental evidence is given; indeed they showed that in bacteria which had been starved for the required amino acid, the RNA synthesis initiated by chloramphenicol recovers later than in that case where both an amino acid and chloramphenicol were used. However, in the latter case, a similar but shorter lag was also observed. They showed also that small concentrations of chloramphenicol are not effective to initiate the recovery in absence of the amino acid. In our experiments with vegetative phage DNA, we found no such lag, although the infective cells had been starved for 10–15 min. (We have also observed similar stringency and lack of a lag in phage infected bacteria starved for only 5 min.)

Bendich, A., personal communication.

ELECTROPHORETIC HETEROGENEITY OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND ITS RELATIONSHIP TO ENZYME DEFICIENCY IN MAN*

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Communicated by Bentley Glass, August 14, 1962

The hemolytic anemia induced by the antimalarial drug, primaquine, is associated with inherited, X-linked deficiency of erythrocyte glucose-6-phosphate dehydrogenase (G-6-PD). It is uncertain whether such deficiency is produced by an alteration in enzyme structure or by an aberration in a regulatory mechanism. Qualitative enzymatic abnormalities have been observed in certain uncommon individuals with an apparently X-linked form of congenital nonspherocytic hemolytic anemia. Such abnormalities have not been detected among Negro and Mediterranean subjects with the more commonly occurring forms of G-6-PD deficiency. Recently, Marks, Gross, and Banks have described an Italian family in which several male members have intermediate levels of G-6-PD activity, altered enzymatic properties, and variant electrophoretic mobility of partially purified erythrocyte enzyme.

In a preliminary account, we described electrophoretic variation in G-6-PD of crude hemolysates obtained from normal Negro subjects. This observation has been independently reported by Kirkman, who has adduced evidence for X-linkage of such variation. Our preliminary data were consistent with autosomal inheritance; subsequently, inconsistencies to the autosomal hypothesis became apparent. These inconsistencies were resolved through the use of leukocytes as a source of G-6-PD. As will be indicated in this report, electrophoretic variation of G-6-PD is, in fact, X-linked, and the responsible structural locus appears to be either tightly linked or identical to the locus governing G-6-PD deficiency in Negroes.

Materials and Methods.—Subjects: Blood samples were obtained from healthy individuals, chiefly hospital personnel, prisoners, or pre- and postpartum women and their families.

G-6-PD assay: Crude hemolysates were assayed by a modification of Glock and McLean's method described by Zinkham. Purified erythrocyte and leukocyte enzymes were assayed in the usual manner. One unit of enzyme activity in either assay system was taken to represent...
generation of one micromole of TPNH per min at approximately 25°C. Total protein content was estimated by use of absorbency at 280 and 260 mp. 

Preparation of crude hemolysates: Blood was collected in heparin, citrated bovine fibrinogen, or an acid-citrate-dextrose solution. Erythrocytes were washed 3 times in normal saline, hemolysed with 2 volumes of water, extracted with 0.5 volumes of toluene, and centrifuged for 30 min at 40,000 x g at 4°C. Supernatant fluids were examined in duplicate by electrophoresis.

Preparation of crude leukocyte homogenates: Leukocytes were obtained by the fibrinogen sedimentation method.12 Washed leukocytes were disrupted at 0°C by sonic oscillation with Raytheon model DF-101 for 2/3 min. Prior to electrophoresis, supernatant fluids were adjusted to a final G-6-PD activity of approximately 0.5 unit per ml. Undiluted material had enzyme activity of approximately 0.8 unit per ml and a protein concentration of approximately 10 mg/ml.

Purification of erythrocyte enzyme: Erythrocyte G-6-PD was purified in a manner similar to that outlined by Kirkman.2 Before electrophoresis, purified erythrocyte enzyme was adjusted to 0.25-0.5 units per ml by dilution with the buffer of dialysis.

Electrophoresis: Starch gel vertical descending electrophoresis13 in a discontinuous buffer system14 at pH 8.5 was employed. An electrical potential of 3-5 volts/cm was applied for approximately 10-16 hours at 4°C.

Enzyme localization after electrophoresis: Cut gel surfaces of two 7-slot gels were developed for G-6-PD by overlay with 110 ml of a solution containing 100 ml of 0.5 M TRIS-HCl pH 8.6, 10⁻² M MgCl₂, 2 to 4 X 10⁻⁴ M TPN, 1 to 5 X 10⁻² M G-6-P, and 10 to 30 mg of Nitro-BT (Dajac Laboratories) in 10 ml of water, to which was added 8 mg of phenazine methosulfate (Dajac). Enzyme localization was carried on in darkness for 2-14 hr. Reaction was stopped by washing with cold tap water.

Results and Discussion.—Phenotypes of erythrocytic G-6-PD from normal Negro males: In the absence of enzyme deficiency, two commonly occurring G-6-PD phenotypes are distinguishable following vertical starch gel electrophoresis of crude hemolysates from Negro male subjects. At pH 8.5, the G-6-PD type designated A migrates just within the cathodic tail of hemoglobin A while the type designated B migrates approximately as far as hemoglobin S (Fig. 1). An uncommon G-6-PD phenotype, C, has a mobility intermediate to that of hemoglobins C and S.

The identity of the stained material as G-6-PD is corroborated by the absence of staining after electrophoresis of crude hemolysates from more than 40 subjects with known G-6-PD deficiency. 6-phosphogluconic acid dehydrogenase, the next enzyme in the metabolic sequence, is electrophoretically distinct from G-6-PD and, at pH 8.5, migrates just cathodic to hemoglobin A₂. G-6-PD phenotype was unchanging in repeated samples.

As an additional source of material for electrophoretic and enzymatic characterization, partially purified and concentrated erythrocytic G-6-PD was prepared from 16 type A Negro males, 13 type B Negro males, and 4 type B Caucasian males.
In every case, the patterns obtained with purified material provided the same type as observed with crude hemolysates. Figure 2 indicates that the A and B types of partially purified erythrocytic G-6-PD are more complex than observed with crude preparations. Each type consists of rapidly and slowly migrating components. The rapid, more anodic, components are designated according to type as A1 or B1. The occasional appearance of "1" components in crude hemolysates suggests that these components in partially purified material are not an artifactual preparation.

**Phenotypes of erythrocytic G-6-PD in Negro females:** Crude hemolysates obtained from Negro females usually typed as either A or B; however, a small pro-

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**Fig. 2.—**Starch gel electrophoresis of partially purified erythrocytic G-6-PD. G-6-PD A type is shown in the left and B type in the middle channel. Both A and B type enzyme were obtained from nondeficient Negro males. Erythrocytic enzyme from a G-6-PD-deficient Negro male is shown on the right. The "1" components of each type are shown at the top of the figure while the "2" components lie closer to the bottom.

**Fig. 3.—**Starch gel electrophoresis of G-6-PD from leukocytic hemogenates. Origin is at the bottom of the figure. From left to right are shown G-6-PD phenotypes C, B, A, AB, and AB. Variation in intensity of staining is due to slight inequality of enzyme concentration. All subjects had normal enzyme activity.

portion of samples apparently contained both A and B types. Latterly in this investigation, partially purified erythrocytic G-6-PD, prepared from five women, revealed the presence of both A and B components in two individuals. The detection of the AB class potentially invalidated our earlier assumption of dominance, and the limitation of this class to women suggested that electrophoretic differences in G-6-PD were X-linked rather than autosomal. However, the proportion of AB women typable using crude hemolysates was far less than anticipated for a heterozygous class, and we were thus led to seek a more satisfactory source of G-6-PD for typing.

**Phenotypes of leukocytic G-6-PD in nondeficient Negroes of both sexes:** Crude...
leukocytic homogenates were found to possess several advantages as an enzyme source for electrophoretic analysis. Leukocytic G-6-PD is easier to prepare than partially purified erythrocytic enzyme, provides a clearer electrophoretic pattern than is realizable with either crude or purified erythrocyte preparations, and readily permits typing of the AB class.

Electrophoresis of leukocytic G-6-PD results in the patterns shown in Figure 3. Types A, B, and C each consist of multiple components similar to those observed with partially purified erythrocytic enzyme. As many as six components may be evident in a single sample. The type AB is easily discerned in leukocyte preparations (Fig. 3) and has thus far been confined to women. Type AB specimens contain as many as 12 components all of which correspond to either type A or B components.

After electrophoresis in 0.05 M acetate gels at pH 5.5, the relative migration of types A and B is reversed. At this pH, the A phenotype is slightly anodic while B is distinctly cathodic. Such reversal in relative migration of G-6-PD type with pH alteration suggests that the principal difference between A and B types is one of electrical charge.

Comparisons among 55 Negro males, 22 of type A and 33 of type B, indicate that the electrophoretically determined G-6-PD phenotype is identical in crude preparations of leukocytes and erythrocytes. Since the same gene apparently governs G-6-PD structure in these two types of cells, the G-6-PD phenotypes of males can be ascertained through using preparations of either erythrocytes or leukocytes. Comparison of G-6-PD phenotypes between leukocytes and crude hemolysates among 100 Negro females indicated considerable discrepancy. Most of this discrepancy could be attributed to our failure to detect the AB class in erythrocytes. In no case was a woman found to be type A in erythrocytes and type B in leukocytes. Moreover, in only one instance was a woman type B in erythrocytes and type A in leukocytes. Thus, the use of crude hemolysates in typing electrophoretic variants of female subjects allows recognition of at least one gene product.

Electrophoretically determined G-6-PD phenotypes of individuals with enzyme deficiency: Crude hemolysates obtained from G-6-PD-deficient subjects do not usually possess sufficient enzyme activity for detection on starch gels. Occasionally, a faintly staining zone has been detected in crude preparations and in every instance has been type A. On the other hand, partially purified erythrocytic enzyme resembled type B in material from each of 22 G-6-PD-deficient Negro males and 2 Negro females (Fig. 2). However, the fit of electrophoretic pattern to type B was imperfect and, moreover, slight variation existed between subjects. We have no satisfactory explanation for the apparent discrepancy in electrophoretic phenotype between crude and partially purified erythrocytic enzyme from G-6-PD-deficient subjects, but we note that protein purification potentially alters enzyme structure.

Because the activity of leukocytic G-6-PD is essentially normal in Negroes with erythrocytic enzyme deficiency,\textsuperscript{15} leukocytes were next employed as a source of material for phenotyping. Leukocytic G-6-PD phenotype has been indistinguishable from type A in all but one of 63 Negro males with erythrocytic G-6-PD deficiency and in all of 5 Negro females with similar deficiency. In contrast, the leukocytic phenotype of 2 Greek males and 1 Greek female with erythrocytic G-6-PD deficiency was indistinguishable from type B.
Leukocytic homogenate was also prepared from one individual with congenital nonspherocytic hemolytic anemia. Specific activity of leukocytic G-6-PD from D. Ey. was, 2 hours after venipuncture, within the normal range for nondeficient Caucasian subjects but quickly declined by approximately 50 per cent within the next 30 min despite storage on ice. Electrophoretic analysis, shown in Figure 4, indicated lack of fit to either A or B type providing potential support for the view that this disorder results from structural alteration of G-6-PD.2

Frequency of phenotypes: Frequencies of G-6-PD electrophoretic variants: A+ (the A type occurring in the absence of erythrocytic enzyme deficiency), B+, and AB are shown in Table 1. These proportions were derived from a random sample of unrelated Maryland Negroes. Phenotypes of males were obtained from crude hemolysates or leukocytic homogenates, while those of females were determined entirely from leukocytes. The frequency of G-6-PD deficiency, ascertained by failure of enzyme to develop on gel staining and corroborated in many instances by direct enzyme assay, is also shown and designated A−. The single instances of a B− phenotype and a C+ phenotype are omitted.

The B+ type, typed with leukocytic and erythrocytic enzyme, was the only one observed among 80 Americans, 40 males and 40 females, of European origin.

Inheritance of phenotypes: The mode of inheritance of G-6-PD electrophoretic variation is presumably X-linked. Three lines of evidence support this assumption. Firstly, limitation of the AB phenotype to females strongly suggests that such women are heterozygous at an X-linked locus. Secondly, the phenotype frequencies observed in Negro females agree reasonably well with those predicted from male phenotype frequencies when assuming a panmictic population and X-linkage (Table 1). Thirdly, results of family studies now favor an X-linked hypothesis. Family members were initially phenotyped by use of crude hemolysates. Seventy Negro families were accumulated with this method of typing.17 The resulting proportions of mating types and children were compatible with the assumption that type A is inherited as an autosomal recessive. However, among 8 families with a type A father and a type B mother there was, in a total of 11 sons, only one instance of apparent father-to-son transmission of the gene governing type A. In contrast, among 6 families with a type B father and a type A mother there were 4 kindreds.

![Figure 4](image-url)
with apparent mother-to-son transmission of the gene for type A. These observations and the appearance of the AB type in leukocytes caused us to review our family collection. Two assumptions, supported by comparisons of erythrocyte and leukocyte types referred to above, were made in order to simplify the task of re-evaluation. These simplifying assumptions were, firstly, that women with type B in crude hemolysates are, without regard to enzyme deficiency, genetically either B/B or A/B; and secondly, that type A women are genetically either A/A or A/B. With these assumptions in mind, the mothers of all families presenting exception to X-linked inheritance were re-examined by the method of leukocyte typing. Since it was not possible to re-examine all 70 kindreds, our data are biased and thus not susceptible to analysis by the methods of population genetics. Nonetheless, with the addition of new families, the final collection of 58 Negro families has a total of 123 sons and contains no instance of apparent father-to-son transmission of the gene for type A. In three families, inconsistencies to an X-linked hypothesis remain between the phenotypes of a mother and her sons. The exceptionable kindreds are of interest since they suggest that additional factors may be involved in the appearance of G-6-PD electrophoretic variants. In two families, a type B+ mother bore an A+ son. These women, heterozygotes by an X-linked hypothesis, failed to express an A gene product in either leukocytes or erythrocytes. In the third exceptionable kindred, three mothers are obligate heterozygotes having gene combinations A+/B+, A-/B+, and A-/B+ respectively. All of these women have leukocytic G-6-PD type A and do not express a B gene product. Such exceptionable subjects possibly account for the slight deficit in observed numbers of AB type women shown in Table 1. Although the explanation for such exceptions is unknown, they are, in principle, analogous to the occasional appearance of women who are heterozygous for the gene for G-6-PD deficiency yet possess normal enzyme activity. Beutler has recently proposed that such women are X-chromosome mosaics and that only the chromosome bearing the wild-type G-6-PD gene is operative.18

The relationship between G-6-PD electrophoretic variation and enzyme deficiency: Nine families were observed in which a heterozygous mother (A-/B+) had one G-6-PD-deficient son and at least one other son. Eight of these mothers have leukocytic phenotype AB. Among the total of 28 sons in these families, 16 are type A−, 2 are enzyme-deficient but not otherwise typed, and 10 are type B+. There are no A+ or B− sons. In 2 additional sibships where the mother's type is unknown, there are a total of 4 enzyme-deficient males and 5 type B+ males. Type A+ males are absent. When the propositi are omitted from the aggregate of 10 families, there are a total of 31 males, 16 of whom are G-6-PD-deficient and 15 of whom are type B+.

<table>
<thead>
<tr>
<th>Class</th>
<th>A+</th>
<th>A−</th>
<th>B+</th>
<th>AB*</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males—observed</td>
<td>50</td>
<td>55</td>
<td>206</td>
<td>0</td>
<td>311</td>
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<td>(0.1608)</td>
<td></td>
<td></td>
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<tr>
<td>Females—observed</td>
<td>10</td>
<td>3</td>
<td>52</td>
<td>35</td>
<td>100</td>
</tr>
<tr>
<td>(0.1768)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Females—expected</td>
<td>8.27</td>
<td>3.12</td>
<td>43.88</td>
<td>44.72</td>
<td>99.99</td>
</tr>
</tbody>
</table>

Expected female frequencies are calculated from the Hardy-Weinberg equilibrium on the assumptions that A+, A−, B+ are co-dominant X-linked characters.

* The AB class includes genotypes A-/B+ and A+/B+.

TABLE 1

FREQUENCY OF G-6-PD PHENOTYPES IN U. S. NEGROES
There are several possible explanations for our failure to observe type A+ and B− sons among the children of others with gene combinations A−/B+ and our further finding that all but one of 63 G-6-PD-deficient Negro males were type A. Firstly, the AB and deficiency loci might be widely separated and the combination B− lethal in Negroes. This seems unlikely, since our own data when pooled with those from published reports1, 19 indicate that 39 G-6-PD-deficient and 39 nondeficient males appeared among sibs and collateral male relatives of enzyme-deficient propositi. There is thus no distortion of the 1:1 expectation. Secondly, the AB locus and the deficiency locus may be closely linked such that the genes of type A and G-6-PD deficiency are in coupling. This last explanation is favored. It necessarily assumes that coupling has sufficiently recent origin in Negroes to preclude the appearance of the recombinant B−. The lack of recombination is compatible either with two distinct loci, each determining a separate product, or with one locus producing a single product which is variable at two points.

The association between A and − characters may provide additional explanation for our inability to detect A−/B+ females through the use of crude hemolysates. If the A and − phenotypes represent separate alterations of the same gene product, then it might be expected that erythrocytes of A−/B+ women, usually possessing intermediate G-6-PD activity, would manifest principally the B character but not the A. On the other hand, if A and − phenotypes represent separate alteration of two gene products, then no necessary correlation exists between detectable electrophoretic phenotype and the − character in heterozygous women. Adequate analysis of erythrocytic G-6-PD electrophoretic phenotype and accompanying enzyme assay in such women provides a potential means of delineating the genetic relationship between the two kinds of characters.

The one observed B− Negro male has a form of G-6-PD deficiency which resembles that occurring in some Mediterranean peoples. Enzyme-deficient persons of the latter origin have very low levels of erythrocytic enzyme and depressed levels of leukocytic enzyme.15 The exceptional Negro B− male had no detectable erythrocytic G-6-PD while his leukocytic enzyme activity per mg protein was the lowest observed among 88 Negro subjects, including 20 with erythrocytic enzyme deficiency. The mother and four maternal half brothers of the B− subject were all B+. The B− Negro may represent a mutation, be an instance of European genetic admixture in an American Negro, or result from recombination in a maternal ancestor. If the last explanation is correct, then the quantitative G-6-PD differences existing between enzyme-deficient Mediterranean and Negro subjects may be due to the presence of B character in the former and A character in the latter.

If the A− combination represents close linkage between two coupled genes, then the presence of A− combinations among the Yoruba in Nigeria29 as well as among American Negroes suggests that the A− combination originated before the effective close of the slave trade, that is, at least eight generations ago. Accordingly, recombination between X-linked AB and deficiency loci cannot be greater than approximately 2 per cent and still be in accord with the observed data.

Additional properties of G-6-PD electrophoretic variants: The mean G-6-PD activity in the crude hemolysates of 10 A+ type and 10 B+ type Negro males was respectively 144.3 ± 19.1 and 146.5 ± 20.8 units/100 ml erythrocytes. The enzyme activity of the individual with type C was within the normal range. Thus,
the structural change responsible for phenotypes A, B, and C apparently does not involve an enzymatically active site. Support for this view derives from similarities in other enzymatic properties. As indicated in Table 2, preparations of types

<table>
<thead>
<tr>
<th>TABLE 2</th>
<th>PROPERTIES OF PARTIALLY PURIFIED G-6-PD-A AND -B TYPES FROM ERYTHROCYTES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type A</td>
<td>Type B</td>
</tr>
<tr>
<td>Control activity remaining after 6 minutes: 40°C.*</td>
<td>63%</td>
</tr>
<tr>
<td>Heat of activation (calories/mole)*</td>
<td>10,200</td>
</tr>
<tr>
<td>pH optimum*</td>
<td>8.8</td>
</tr>
<tr>
<td>( K_m ) G-6-P ( \times 10^{-4} M^* )</td>
<td>5.17</td>
</tr>
<tr>
<td>Control activity in presence of dehydroisoandrosterone†</td>
<td>53%</td>
</tr>
<tr>
<td>10(^{-4}) M</td>
<td>91%</td>
</tr>
</tbody>
</table>

Methods of analysis followed those of Kirkman* (ref. 3) and Marks† (ref. 21).

A and B enzyme have properties which approximate those reported from the study of enzyme from nondeficient subjects.2–4

Summary.—Several electrophoretic variants of glucose-6-phosphate dehydrogenase are recognizable among Negro subjects in the absence of enzyme deficiency. Such variation is present in both erythrocytic and leukocytic enzyme and is not accompanied by alteration in other enzymatic properties. Leukocytic enzyme provides a convenient source of material for typing subjects with erythrocytic G-6-PD deficiency. Limitation of the heterozygous class to females suggests that the responsible structural locus is X-linked. Population and family studies support this conclusion but also indicate that additional factors may influence phenotype. The association of a particular G-6-PD variant, type A, with enzyme deficiency in all but one of 63 G-6-PD-deficient Negro males suggests that the locus controlling deficiency and the structural locus are closely linked. Evidence from population studies suggests that the distance between loci is not greater than two map units. Since no recombination between loci has been observed, it is possible that the two loci are part of the same functional unit. In contrast to Negroes, Greek G-6-PD-deficient subjects possess electrophoretic phenotype B. The B phenotype is the only type observed in Americans of European origin. One subject with congenital nonspherocytic hemolytic anemia presented a unique electrophoretic pattern, thus supporting the view that structural alteration of G-6-PD can produce this disorder.

* This investigation was supported by Grant No. B2053 from the National Institutes of Health. I. H. Porter was in receipt of a Wellcome Trust Foundation traveling grant.


2 Kirkman, H. N., H. D. Riley, Jr., and B. B. Crowell, these PROCEEDINGS, 46, 938 (1960).


6 Presented by one of us (I. H. P.) before the Second International Conference of Human Genetics, Rome, September, 1961, and to be published in the Proceedings of that Conference.


16 Studied by W. Zinkham, who found G-6-PD activity absent in hemolysates.
17 Blood groups were obtained from all of these families and presumptive illegitimate children excluded. Grouping was provided through the generous assistance of F. H. Allen, Jr., of the Blood Grouping Laboratory, Boston, Mass.
18 Beutler, E., M. Yeh, and V. F. Fairbanks, these Proceedings, 48, 9 (1962).