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Frequency of enzyme deficiency variants in erythrocytes of newborn infants

(genetic variation/enzymopathies/null variants/mutation studies)

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ABSTRACT **The frequency of enzyme deficiency variants, defined as alleles whose products are either absent or almost devoid of normal activity in erythrocytes, was determined for nine erythrocyte enzymes in some 675 newborn infants and in approximately 200 adults. Examples of this type of genetic abnormality, which in the homozygous condition are often associated with significant health consequences, were detected for seven of the nine enzymes studied. Fifteen inherited enzyme deficiency variants in 6142 determinations from the newborn population and 5 variants in 1809 determinations from adults were identified. Seven of the deficiency variants involved triosephosphate isomerase, a frequency of 0.01 in the newborn population. The average frequency of 2.4/1000 is 2–3 times the frequency observed for rare electrophoretic variants of erythrocyte enzymes in this same population.**

Recent years have witnessed a great wave of enthusiasm for the electrophoretic approach as a means of defining the frequency of "occult" variation in a random sample of a plant or animal population. Strong tool though this has been, it has been recognized that this technique reveals only the tip of the iceberg, in that it fails to detect variants not associated with a charge change in the molecule under scrutiny, and it usually fails to detect variation characterized by actual or functional loss of gene product. In this paper we will describe the results of an effort to determine the frequency of genetic variants resulting in total or near-total loss of activity for a series of nine human erythrocyte enzymes. We have previously reported on the frequency of rare electrophoretic variants in this sample, and in a companion paper (1) we will report the results of studies designed to estimate, for the same population and enzymes, the frequency of genetic variants exhibiting alterations in thermostability. Both investigations were undertaken in the context of an effort to move the study of human mutation rates to the biochemical level (2).

In 1932, Muller (3), in an attempt to classify types of genetic variation, distinguished between amorphs, hypomorphs, and hypermorphs. An amorph was a variant in which gene product (as then understood) was totally absent. In retrospect, Garrod in 1909 (4) defined the first amorph in man. Currently, over 100 metabolic diseases have been described in which a specific enzyme defect, either an amorph or a hypomorph in the terminology of Muller, has been identified (5–7). Almost all of these defects are detectable in the heterozygote or "carrier" individual through a significant reduction in enzyme activity, usually approaching 50% of the expected value (6). Although we have been unable to trace the origin of the term, these deficiences and in particular the amorphs, are often called "nulls."

Our knowledge of the population frequency of null alleles in humans has been largely indirect, in the sense that most estimates take as their point of departure a recessively inherited disease characterized by loss of enzyme activity, for which incidence–prevalence data are available, and derive a gene frequency from the application of Hardy–Weinberg principles. Notable exceptions to this have been surveys to determine heterozygote frequencies directly for acatalasemia (8) and Tay–Sachs disease (9). By contrast, the nine enzymes that have been the objective of this study were not chosen specifically because they are associated with a disease (although for seven of the nine enzymes deficiency diseases are known) but because of their relatively restricted variation in level of enzyme activity among individuals (10), which renders these enzymes favorable subjects for the study of deficiency variants. Accordingly, the frequency estimates to be presented should thus be relatively unbiased. Because our approach to detecting these variants does not differentiate between "true nulls" or amorphs and variants retaining low levels of activity, or hypomorphs, we have called them "deficiency variants," a term that encompasses both of these types of variants.

MATERIALS AND METHODS

Biochemical Procedures. The blood samples employed in this study were obtained in an effort to develop appropriate methods for monitoring changes in mutation rates (2). Umbilical cord blood samples were obtained at delivery from newborn infants at the University of Michigan Women's Hospital. Blood samples from parents were generally collected a few hours before obtaining the cord sample. The population consisted of all term females and their husbands presenting at the maternity service who agreed to donate a blood sample. Only when samples were obtained from both parents was a newborn included in the sample. The ethnic composition of this population is approximately 10% Negroid, 2% Mongoloid, and 84% Caucasoid, with the ethnicity of the remaining 4% being unknown or undefined. Samples were collected, cells were processed, and packed cells were stored in liquid N_2 until utilized. Hemolysates were prepared as described (10), except that the aliquots for the assay of aspartate aminotransferase and glucose-6-phosphate dehydrogenase were stored in the presence of 0.04 mM pyriodoxal 5-phosphate and 0.1 mM $NADP^+$, respectively.

Assays for adenylate kinase (AK, EC 2.7.4.3), aspartate aminotransferase (GOT, EC 2.6.1.1), glucosephosphate isomerase (GPI, EC 5.3.1.9), lactate dehydrogenase (LDH, EC 1.1.1.27), malate dehydrogenase (MDH, EC 1.1.1.37), phosphoglycerate kinase (PGK, EC 2.7.2.3), pyruvate kinase (PK, EC 2.7.1.40),

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Abbreviations: AK, adenylate kinase; GOT, aspartate aminotransferase (glutamate–oxaloacetate transaminase); GPI, glucosephosphate isomerase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; PGK, phosphoglycerate kinase; PK, pyruvate kinase; TPI, triosephosphate isomerase; G6PD, glucose-6-phosphate dehydrogenase; CV, coefficient of variation.

and triosephosphate isomerase (TPI, EC 5.3.1.1) were performed as described by Fielek and Mohrenweiser (10). Glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) was assayed by the method of Beutler (11). All assays utilized the centrifugal fast analyzer described by Burtis *et al.* (12). The units of activity are μmol of product/g of hemoglobin per hr.

Enzymes, substrates, and cofactors were obtained from Calbiochem. Other chemicals were of analytical or reagent grade.

Definition of an "Enzyme Deficiency" Variant. Our primary interest in this paper is to provide a first estimate of the frequency of carriers of null or amorph alleles in a human newborn population. This is not at present possible in a truly rigorous fashion. The most favorable opportunity for an approximation to the true frequency is provided by enzymes with a coefficient of variation (CV) of 11% or less. In principle, an individual heterozygous for a null variant should exhibit 50% of normal enzyme activity. On the assumption that the same CV should apply to the heterozygote for a null, then very few heterozygotes should, under these circumstances, exhibit values exceeding 66% of expected (+3 SDs) and few normals should have values below 67% of normal (−3 SDs).

In addition to null heterozygotes, one can anticipate by this convention detecting some fraction of a class of alleles characterized by reduced activity or marked instability of their gene product. The proportion of these that would be detected by the above-stated convention ranges from almost 100% of those with 1% activity through about 50% of those with 30% activity to a small proportion with 50% activity. Repeat determinations and family studies as well as biochemical studies should clarify the molecular basis for these enzyme deficiencies.

Six of the nine enzymes under study do have a CV of 11% or less in infants, so that they meet the criteria for this most favorable approximation to the frequency of carriers of nulls. However, three of the enzyme have CVs in excess of 11% (GOT, G6PD, and PK) and the two distributions will overlap. For these three enzymes we have employed the conservative approach of classifying as deficiency variants only those individuals with a level of activity more than three SDs below the mean, a criterion that will provide an underestimate of the frequency of both nulls and low-activity variants. Additionally, the residual activity for sex-linked traits in the hemizygous males would have to be more than 5.0 SDs below normal to be classified as an enzyme deficiency.

Data Acquistion Strategy. Previous studies by Fielek and Mohrenweiser (10), with the instrumentation used in these studies and with similar samples, indicated that the total "within sample" variation would be small and primarily due to small changes in assay conditions (e.g., temperature) on different days. Therefore, groups of samples were established consisting of approximately 13 families (in preliminary studies when mother, father, and newborn were routinely studied) or 52 newborns (in later studies) and all assays for a single enzyme within a group were completed within a single day's operation. Tentative identification of probands was made on the basis of the mean and variation within each respective group. All assays yielding an enzyme activity more than 2 SDs from the mean were immediately repeated. The next step in the identification of a presumptive deficiency variant involved preparation of a new hemolysate from cells stored in liquid N_2. At the same time, samples from both parents and also from a control group of at least 10 cord blood and 10 adult samples would be analyzed simultaneously. If upon repeat analysis the aberrant value was more than 3 SDs below normal and the values for the other enzymes were normal, this was defined as an enzyme deficiency variant. It was assumed to be an inherited variant if a similar enzyme activity profile was observed for one parent and the other parent had a normal enzyme activity profile. The identification of activity variants in this study was generally through the cord blood sample, although in preliminary efforts all three members of a family were studied simultaneously.

RESULTS

Normal Range of Enzyme Activity. The data on level of enzyme activity in erythrocytes from three populations, adult males, pregnant females, and the cord blood of newborn infants are presented in Table 1. These means were calculated after removal of values for enzyme deficiency variants, as previously described. The variation associated with date of assay was removed by utilizing a nested analysis-of-variance technique before the coefficient of variation was computed and the individual's values within days were adjusted to this overall mean. No indication of a bimodal distribution was discernible (Fig. 1). The levels of enzyme activity and the cord blood to the adult blood activity ratios were similar to those in previous reports (10, 13). The higher enzymatic activities in umbilical cord blood samples, for all enzymes except adenylate kinase, generally reflect differences in mean red cell age and percentage of reticulocyte between adult and cord blood samples, although they may also reflect unique characteristics of fetal erythrocytes (e.g., GPI), (14–16).

The AK_1^2 allele is the only autosomally inherited electrophoretic variant occurring in polymorphic frequency in this study, occurring with a frequency of 0.045 in this population. The AK-2 variant is associated with reduced activity in adults (50–85% of normal) (17, 18), but a similar difference was not observed in newborns by Rapley and Harris (17). In the current study, cord blood samples from newborns of the 1-2 phenotype had an activity of 5294 ± 158 units or 93% of the activity observed in newborns with the 1-1 phenotype. No adjustment for this small difference in enzyme activity associated with electrophoretic phenotype was included in the calculation of variation. The reduced level of AK activity in newborns compared to erythrocytes from adults (Table 1) is similar to that in previous reports (17). Individuals of both sexes exhibiting the sex-linked $G6PDA^-$ trait (seven newborns and two unrelated adults) were excluded from the study only for that enzyme.

Enzyme Deficiency Variants. Examples of inherited enzyme deficiency consistent with the presence of an enzyme deficiency allele were observed for seven of the enzymes assayed. The data are presented in Table 2.

The most frequently identified deficiencies were of TPI. Seven children—1052, 1099, 1164, 1864, 2077, 2110, and 2215—were observed to have a level of enzyme activity more than 3 SDs below normal. In each of these families a parent with

Table 1. Activity of erythrocyte enzymes in cord and adult blood samples

Enzyme	Activity, μmol/g Hb per hr		
	Newborn	Mother	Father
AK	6,200 ± 690	7,300 ± 950	7,500 ± 1,100
G6PD	570 ± 75	290 ± 50	290 ± 50
GOT	650 ± 85	420 ± 65	320 ± 50
GPI	4,370 ± 440	2,990 ± 330	2,740 ± 290
LDH	11,500 ± 1,160	8,900 ± 1,370	8,600 ± 1,150
MDH	9,500 ± 840	8,400 ± 1,100	7,900 ± 990
PGK	11,500 ± 1,150	7,700 ± 940	7,700 ± 720
PK	1,400 ± 190	940 ± 140	880 ± 120
TPI	173,000 ± 19,100	125,000 ± 18,600	125,000 ± 17,900

The ± indicates SD; n = approximately 100 for mothers and fathers and 675 children.

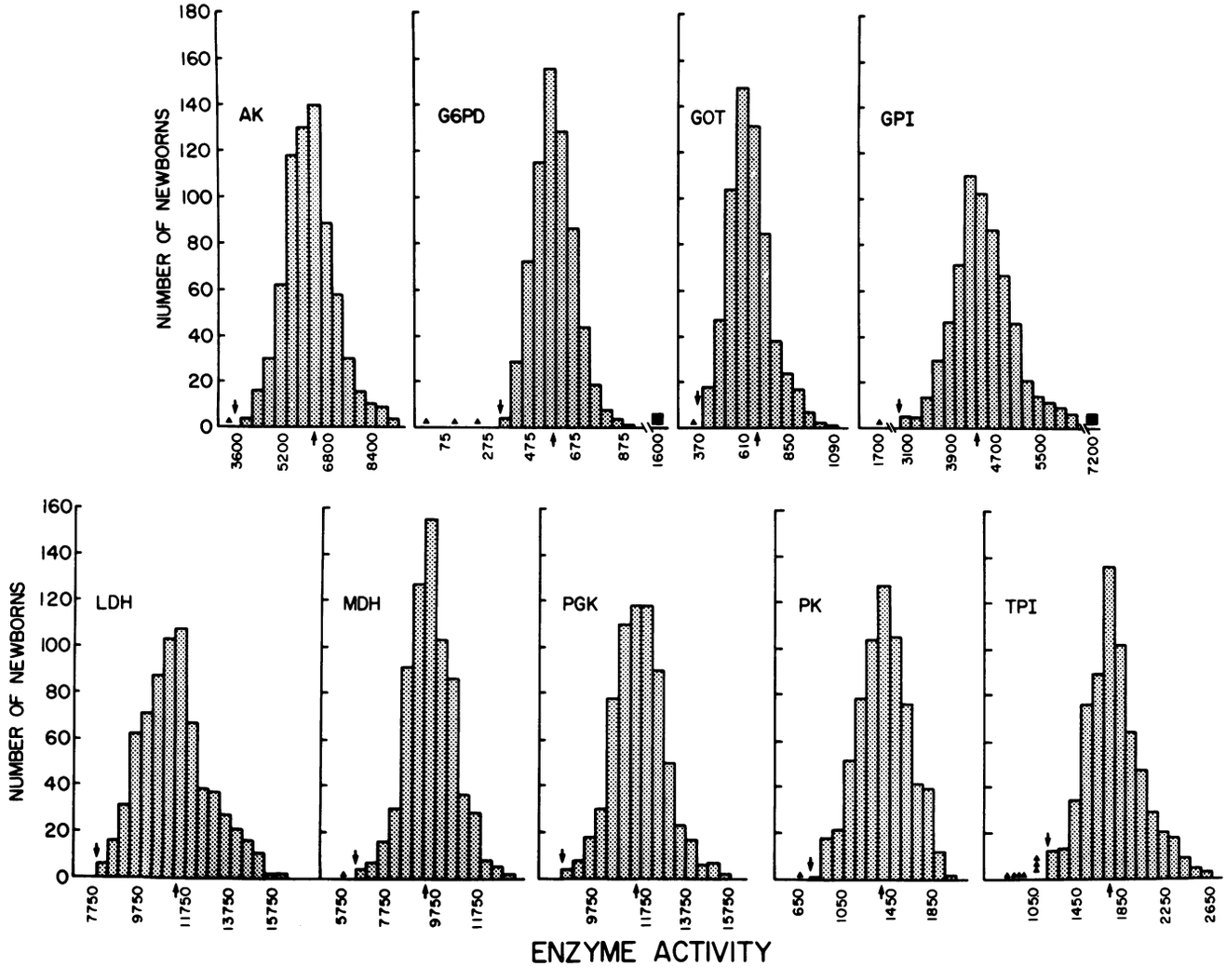

FIG. 1. Adjusted distribution of enzyme activity in erythrocytes from the cord blood of newborn infants. The activity is expressed as μmol/g Hb per hr and has been adjusted to remove date of assay as a source of variation. ↑ indicates the mean, ↓ is 3.0 SD below the mean. ▲ indicates an enzyme deficiency variant described in Table 2. ■ indicates a hyperactivity variant.

a TPI activity of less than 65% of normal was identified, whereas the other parent had normal levels of TPI activity. The male children in families 1062 and 2215 are also of the G6PDA$^-$ phenotype, and the activities of pyruvate kinase and aspartate aminotransaminase in erythrocytes from these infants are increased, although within the normal range. This increased activity would suggest a reduced mean cell age in these samples and could explain why these two newborns have almost 65% of expected TPI activity. It should also be noted that in family 2215 the TPI deficiency is inherited from the father, while the G6PD deficiency is inherited from the mother, indicating the two deficiencies are independent events. The children in families 1164 and 2077 are half siblings, with both children inheriting the variant allele from the mother.

A male child (family 1189) had a level of G6PD activity that was 22% of normal (120 units). The electrophoretic mobility of this enzyme is slightly reduced relative to the standard B type. The mother had an enzyme activity of 52% of normal adult levels (150 units) and a normal B-type electrophoretic pattern. Subsequent analysis of a sample obtained when the child was 24 months of age confirmed the original observations of an inherited enzyme deficiency and the discrepancy between mother and son in electrophoretic phenotype. Electrophoretic studies of G6PD from leukocytes of the mother and a daughter showed that the mother was actually a G6PD heterozygote individual but because of the instability of the variant it was not detectable in the erythrocytes obtained from the mother (19). A second male child (1718) was observed to have a level of G6PD activity 4% of that expected for newborn infants. This is less than the 20% residual activity observed for male newborns of the G6PDA$^-$ phenotype. Additionally, electrophoretic analysis suggested that the low residual activity in this individual had G6PDB-like mobility, although a definitive conclusion was not possible because of the low activity. Neither of these variants is a true "null," because residual activity associated with the product of the variant allele expressed in these hemizygous individuals is detected. A third G6PD deficiency variant was detected in a female child (family 1490). Both mother and child had enzyme activity levels of approximately 50% of normal, and the residual enzyme activity had normal electrophoretic mobility. In none of these families could normal activity be restored by increasing the substrate concentration in the assay or with preincubation in the presence of 1 mM NADP$^+$.

Additional instances of inherited enzyme deficiency variants were detected for five enzymes, AK, GPI, MDH, PK, and GOT. Again in each family, one parent exhibited the deficiency while the other parent had normal activity for all nine enzymes. The GOT deficiency could not be reversed by extensive preincubation in the present of pyridoxal 5'-phosphate. The electrophoretic phenotypes of both the proband and affected parent

Table 2. Description of enzyme deficiency variants identified in erythrocytes from newborn infants

Enzyme	Family no.	Child			Mother			Father		
		Activity	%	Deviation	Activity	%	Deviation	Activity	%	Deviation
AK	1578	3,240	57	−3.3	7,700	106		4,290	57	−4.1
G6PD	1189	120	22	−5.3	150	52	−2.7	320	109	
G6PD	1490	235	42	−4.3	155	54	−2.6	310	106	
G6PD	1718	20	4	−6.3	130	45	−3.1	385	130	
GOT	1489	355	57	−3.0	200	48	−3.4	300	92	
GPI	2018	1,870	43	−4.7	1,100	39	−3.3	2,810	102	
MDH	1678	5,720	60	−4.4	5,040	60	−3.0	10,570	134	
PK	1415	660	55	−3.1	280	35	−4.6	900	103	
TPI	1062	108,100	65	−3.3	72,700	58	−2.8	117,200	94	
TPI	1099	88,700	52	−4.3	104,800	84		80,600	64	−2.5
TPI	1864	80,900	48	−4.7	73,300	59	−2.8	111,900	90	
TPI	2077*	105,400	62	−3.4	78,100	64	−2.5	155,500	124	
TPI	1164*	92,000	53	−3.8	78,000	64	−2.5	139,000	112	
TPI	2210	85,800	50	−4.1	60,900	48	−3.4	155,200	123	
TPI	2215	105,700	62	−3.1	104,800	113		71,200	57	−3.0

Activities are μmol/g Hb per hr; % is that of mean of appropriate controls; deviations are number of SDs from the mean.
* Children are half sibs.

with the AK deficiency were normal, thus the reduced activity of the AK-2 type enzyme was not relevant to this case.

No enzyme deficiency variants were identified for two enzymes, LDH and PGK. The frequency data are summarized in Table 3.

No instances were observed of an enzyme deficiency being present in a child but not identifiable in one of the parents; that is, no presumptive mutations were ascertained. Electrophoretic analysis was conducted for all enzymes except for PK. The only variant that was detectable as an electrophoretic variant in erythrocytes was the G6PD variant in family 1189.

The adult populations in Table 1 are the parents of the first 100 children studied (1809 enzyme determinations). Five deficiency variants were detected in this sample. Three of the variants (in families 1099, 1062, and 1189) were also present in the child, while two fathers were identified with enzyme deficiency variants (TPI and G6PD) that were not inherited by the child. This frequency in the parents, 2.8 variants per 1000 determinations, is similar to the frequency observed in the newborn population.

Other Enzyme Activity Variants. In addition to the deficiency variants, two examples of variants characterized by significantly increased activity were identified. A male child in family 1889 had a G6PD activity of 1600 units, a level 3 times and 10 SDs above the normal level in newborns. The G6PD activity in erythrocytes from the mother was 577 units or 5 SDs above normal for adults. The electrophoretic mobility of G6PD in erythrocytes from both mother and son was of the normal B type, and thus this variant is probably not of the G6PD Hektoen type previously described (20).

Table 3. Frequency of enzyme deficiency variants in newborn infants

Enzyme	n	Enzyme deficiency	
		No.	Frequency
AK	676	1	0.0014
G6PD	684	3	0.0044
GOT	637	1	0.0015
GPI	671	1	0.0014
LDH	702	0	0
MDH	702	1	0.0014
PGK	701	0	0
PK	697	1	0.0015
TPI	671	7	0.0104
Total	6142	15	0.0024

A hyperactive GPI variant with normal electrophoretic mobility was also observed. The enzyme activity in the child was 7260 units, which is 168% of normal and 7SDs above the mean for the newborn population. The father had a GPI activity of 3965 units, which is 45% and 4 SDs above normal. The activities of all other enzymes in both individuals, as well as the GPI activity in the mother, were normal.

In five other parent–child pairs, a level of enzyme activity more than 2.5 SDs but not 3.0 SDs below the mean, and restricted to a single enzyme, was observed. The enzymes and level of activity are as follows: (child/parent) GPI: 74%, −2.6 SDs/76%, −2.3 SDs; AK: 69%, −2.8 SDs/78%, −2.2 SDs; G6PD: 45%, −3.5 SDs (male child)/80%, −2.0 SDs; GOT: 61%, −2.7 SDs/61%, −2.5 SDs; PK 62%, −2.7 SDs/55%, −3.35 SDs. These latter five newborns, although obviously exhibiting a reduced level of enzymatic activity, which is similar to the level of activity in a parent, were not classified as "enzyme deficiency" variants but will be referred to simply as "low activity" variants.

Some 7 of 65 Negro children were ascertained, by activity measurements and electrophoretic analysis, to be of the G6PDA$^-$ phenotype. They were excluded from further consideration only for G6PD studies. This agrees with previous reports on the frequency of this variant in populations in the United States (21).

DISCUSSION

Abnormal enzyme activities in erythrocytes may be due to several causes in addition to a structural alteration involving a single enzyme locus. Most of these causes, including changes in mean cell age or cell type (15, 16), exposure to cytostatic drugs (22), and other dyserythropoietic conditions (23, 24) usually result in alterations in the activity of several enzymes simultaneously and usually involve both increases and decreases in activity. Therefore, it is unlikely that an acquired enzymopathy would fulfill our requirement that the level of enzymatic activity for eight of the nine enzymes assayed be within the normal range (excluding the G6PDA$^-$ variant). Also, the probands ascertained to be enzyme deficient have a level of enzyme activity more than 3.0 SDs below the mean, and it is unlikely that an acquired erythroenzymopathy or a "normal" level of enzyme

activity would meet this stringent definition of an enzyme deficiency. Additionally, care has been taken to exclude the possibility that cofactor deficiency, as has been observed for glutathione reductase (25), is causing the reduction in enzymatic activity.

Each of the variants identified in a newborn infant was also present in one of the parents. We take this evidence of genetic segregation for an extreme variant as evidence that a single locus is involved in the defect. We assume this locus to be the structural locus encoding for the protein in question or possibly the flanking regions, but of course we cannot exclude the possibility that the finding is due to a codominantly segregating repressor-type gene. For each of the described enzyme abnormalities with reduced activity, a unique, specific alteration exists, without a generalized enzymopathy. In each instance, the evidence suggests the existence of an allele, the gene product of which has no function, reduced function, or is nonexistent in the erythrocyte. The percent remaining activity in either the affected parent or the newborn is approximately 55 ± 7. This range of remaining activity is consistent with the existence of both null and hypomorph alleles in this group of affected individuals.

This frequency of enzyme deficiency variants of 2.4/1000 is some 2–3 times the average frequency of rare electrophoretic variants for erythrocyte enzymes (19). This frequency of deficiency variants is similar to the frequency of enzyme null alleles observed by Voelker *et al.* (26) in natural populations of *Drosophila melanogaster*, although variation in the frequency among loci is noted in both studies. It should be noted that the deficiency variants identified in the newborn population exist at loci that are generally observed to exhibit restricted electrophoretic variation (19, 27), and most of these enzymes are critical to normal erythrocyte function.

Mukai and Cockerham (28) and Voelker *et al.* (29), in studies of the spontaneous mutation rate in *Drosophila*, found that the frequency of mutation to null variants was 3 times higher than that observed for electrophoretically detectable variants. Similar results were obtained in studies of radiation-induced mutations in *Drosophila* (30) and chemically induced mutations in mice (31, 32).

The ability to detect enzyme deficiency variants adds a dimension to mutation monitoring programs in that it expands the types of genetic damage that can be readily detected and includes a class of variants with potential health implications. This ability also provides further insight into the extent of genetic variation in the human population, as discussed by Mohrenweiser and Neel in an upcoming paper (1).

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