Proc. Natl. Acad. Sci. USA
Vol. 78, No. 9, pp. 5729–5733, September 1981
Genetics

Frequency of thermostability variants: Estimation of total "rare" variant frequency in human populations

(genetic variation/human erythrocyte enzymes/health effects/mutation studies)

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Contributed by James V. Neel, April 29, 1981

ABSTRACT **Eight erythrocyte enzymes were examined for thermostability in an unselected sample of 100 newborn infants. Three thermolabile variants, one each of lactate dehydrogenase, glucosephosphate isomerase, and glucose-6-phosphate dehydrogenase, were identified, none of which was detectable as a variant by standard electrophoretic techniques. All were inherited. This frequency of 3.8 heritable thermostability variants per 1000 determinations is to be compared with a frequency of electrophoretically detectable variants of 1.1 per 1000 determinations, a frequency of 2.4 enzyme-deficiency variants per 1000 determinations, and a frequency of 1.1 hypo/hyperactive enzyme-activity variants per 1000 determinations in this human newborn population. The total measured frequency of individuals with rare enzyme deficiency or electrophoretic or thermostability (or both) variants at these loci is 8.4 per 1000 determinations. A similar distribution and frequency is seen when the comparison is limited to the seven loci studied by all techniques. It is clear that not all of the electrophoretic and thermostability variants present in the population are detected by the techniques used in this study. Accordingly, it is estimated that the true frequency of carriers of a rare variant for each of these enzyme-coding loci averages greater than 10/1000. Some implications of these frequencies for human disease are discussed.**

In recent years, we have been engaged in a systematic effort to determine the amount of inherited variation detectable in a relatively unselected series of human enzymes, not only because of the intrinsic interest of the question, but also because of the relevance of the data in developing a genetic monitoring system. Data have already been published on the frequency of electrophoretic variants (1) and of variants characterized by a near or total loss of enzyme activity (2) in a series of newborn infants. In this paper, we present data on the frequency of thermostability variants and then attempt a synthesis of the findings to date. We also consider the relative usefulness of these three types of variants in a program designed to study human mutation rates.

We are aware of no other survey data on the frequency of enzyme variants exhibiting altered thermostability. On the other hand, thermostability assays have been used frequently in successful attempts to divide electrophoretic variants into subclasses—e.g., in *Drosophila* (3–6), mice (7), and humans (8). For instance, in *Drosophila*, on the average, three thermostability types could be identified within each class of previously identified electrophoretic variant (3–6, 9), although large variation among loci is noted (10, 11). In human material, three clearly distinct thermostability types were identified within a group of 11 glucose phosphate isomerase type 1-4HIR variants, and two subtypes were identified within a group of four 1-5HIR variants of the same enzyme (8). Thus, there was good reason to believe that thermostability studies of erythrocyte enzymes from a series of unselected individuals should show occult variation.

MATERIALS AND METHODS

Placental cord blood samples were obtained from newborn infants and venous samples were obtained from both parents for all those deliveries at the University of Michigan Women's Hospital for which appropriate "informed consent" could be obtained from both parents as described (2, 12). Erythrocyte samples and hemolysates were prepared and stored, and enzymes were assayed as described by Fielek and Mohrenweiser (13).

The following enzymes were assayed: adenylate kinase (AK, EC 2.7.4.3), glucose phosphate isomerase (GPI, EC 5.3.1.9), glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49), 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), triosephosphate isomerase, (TPI, EC 5.3.1.1).

Thermostability experiments were conducted as follows. (*i*) Hemolysates were prepared by addition of 6 to 7 vol of 5 mM triethanolamine·HCl, pH 7.5/1 mM EDTA/2 mM 2-mercaptoethanol/0.05% Triton X-100 to 1 vol of packed cells. The hemoglobin concentration was 2.8–3.2 g/100 ml of hemolysate. (*ii*) Four 50-μl aliquots of the hemolysates were prepared. Three aliquots were incubated at appropriate temperatures in feedback-thermister-regulated oil baths for 20 min and the fourth was maintained at 4°C and served as the initial control. (*iii*) The tubes were quickly cooled in an ice bath and then centrifuged at 10,000 × *g* for 15 min to remove precipitated protein. (*iv*) The activity remaining in the supernatant in each of the three incubated aliquots was determined and compared with the activity in an aliquot maintained at 4°C during this period of time. The activity was expressed as percent activity remaining. Thus, a profile of stability at three temperatures was established. Temperatures were selected to minimize the total number of incubations necessary for the series and also provide at least two data points for which pilot studies had indicated an expectation of 40–70% remaining activity for the normal enzyme. As LDH-B is responsible for >80% of the total LDH activity in the erythrocyte, it is assumed that in effect only the gene product of the *LDH-B* locus is being monitored for thermostability. G6PDA$^-$ variants were excluded from the study.

For present purposes, we have arbitrarily defined a thermostability variant as a variant characterized by a percent re-

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Abbreviations: AK, adenylate kinase; GPI, glucose phosphate isomerase; G6PD, glucose-6-phosphate dehydrogenase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; PGK, phosphoglycerate kinase; PK, pyruvate kinase; TPI, triosephosphate isomerase.

maining activity after incubation that was more than 2.5 SD below the mean at the intermediate incubation temperature and also at one of the other two temperatures. To eliminate false positives due to sample abnormalities, such as sample deterioration, change in distribution of cell types and such, all other enzymes examined from the same person were required to have a normal stability profile. As can be seen in Table 1, 2.6 SD is ≈10 percentage points of activity loss. Thus, at the middle incubation temperature, a thermostability variant, as defined, retained less than ≈80% of the activity of the normal enzyme. It is also possible for a variant to have increased stability, and the appropriate criteria would be similar except that the percent remaining activity would be correspondingly above the mean.

RESULTS

Normal Thermostability. The thermodenaturation profile for each of the eight erythrocyte enzymes is presented in Table 1. Differences in thermodenaturation profile between samples from adults and newborn infants are noted for four of the eight enzymes—AK, G6PD, MDH, and PK. These differences are associated with differences in the composition of the erythrocyte sample (percent reticulocytes and mean cell age) obtained from newborns and adults (14). In addition, the thermostability of two erythrocyte enymes (AK and PK) from adult males was greater than that of the enzyme from erythrocytes of pregnant females. This difference is associated with pregnancy status as the thermostability of these two enzymes in erythrocytes from nonpregnant adult females does not differ from the adult male profile (14). No such difference between sexes was noted in cord blood samples nor was a sex/pregnancy status difference observed for PGK, the third kinase enzyme studied.

The AK_1 2 variant is the only electrophoretic variant known to occur as a genetic polymorphism of any of the eight loci studied in this population. The thermostability of the enzyme from inividuals of the 1-2 phenotype is less than that of the more common 1 variant (Fig. 1). The two newborns with an AK_1 1-2 phenotype encountered in this study were not scored as thermostability variants.

Table 1. Thermostability profiles of erythrocyte enzymes

Enzyme	Temperature, °C	% activity remaining		
		Mother	Father	Child
AK	43.5	87 ± 6	92 ± 5	78 ± 5
	47	62 ± 5	73 ± 6	53 ± 6
	49	33 ± 6	41 ± 6	26 ± 8
G6PD	39	60 ± 6	61 ± 5	78 ± 5
	40	49 ± 5	65 ± 4	65 ± 4
	42	39 ± 4	51 ± 4	51 ± 4
GPI	51	76 ± 3	73 ± 3	77 ± 4
	52	49 ± 4	47 ± 5	52 ± 5
	53	34 ± 4	32 ± 4	37 ± 5
PGK	51	74 ± 5	75 ± 4	78 ± 5
	52	41 ± 4	43 ± 5	45 ± 5
	53	24 ± 4	24 ± 4	26 ± 6
LDH	60	77 ± 4	76 ± 2	74 ± 3
	62	56 ± 3	55 ± 2	52 ± 4
	64	39 ± 4	39 ± 4	36 ± 4
MDH	56	74 ± 3	75 ± 4	69 ± 4
	57.5	51 ± 3	51 ± 4	43 ± 4
	59	40 ± 2	40 ± 2	35 ± 3
PK	56	60 ± 5	68 ± 5	57 ± 5
	57.5	43 ± 5	49 ± 4	34 ± 4
	59	20 ± 4	29 ± 5	14 ± 4
TPI	56	76 ± 3	74 ± 3	75 ± 4
	57.5	56 ± 3	55 ± 4	55 ± 3
	59	35 ± 2	35 ± 4	36 ± 4

Level of enzyme activity remaining after incubation at specified temperature for 20 min is expressed as percent of control (mean ± SD). n = 100 for children and 27 for each parent.

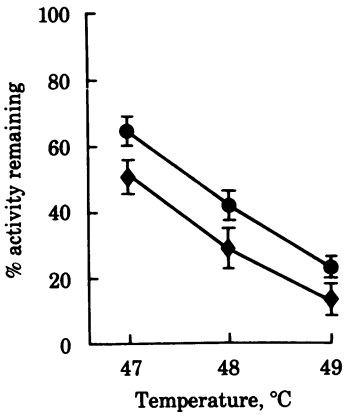

FIG. 1. Thermostability profiles of AK1 (●) and AK1-2 (◆) phenotypes. n = 6 for phenotype; samples were from newborn infants.

Thermostability Variants. The screening of 100 cord blood samples for the stability of eight enzymes resulted in the identification of three variants, each more thermolabile than the normal enzyme.

(*i*) In family 1793, the percent remaining activity of LDH in a male child was approximately 4 to 5 SD below the mean at each of the three temperatures studied (Fig. 2). The father exhibited similar findings, while the enzyme in the mother's erythrocytes exhibited a normal profile. The level of enzyme activity for the child was 9500 (units/g of Hb)/hr and for the father was 6200 (units/g of Hb)/hr. This activity is lower than the 11,002 ± 1067 and 8640 ± 1149 units expected for newborn and adult controls, respectively (2). Thus, the proband's value was 86% of normal compared with a level of activity which is 71% of normal in the father. We believe this reduction in activity in these two persons is real and, further, that the difference between father and son probably reflects the effect of differences in mean cell age between newborns and adults. A reduced activity that is apparently affected by mean cell age suggests a decreased *in vivo* half-life for this variant, which exhibits an increased thermolability. The electrophoretic pattern of erythrocytic LDH in this trio was normal. It is asssumed that the variant allele is at the *LDH-B* locus because the gene products of this locus are responsible for ≈80% of the LDH activity in erythrocytes, although positive identification of the locus involved will require further studies.

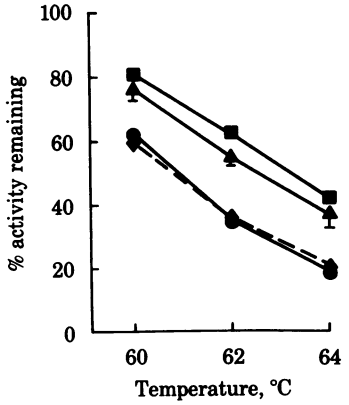

FIG. 2. Thermostability profiles for LDH. ▲, Percent remaining activity ± SD for control group; ●, proband; ◆, father; ■, mother.

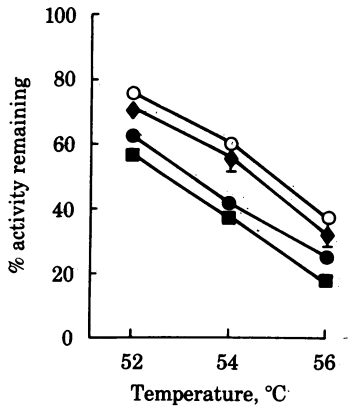

FIG. 3. Thermostability profiles for G6PD. ○ and ◆, Percent remaining activity ± SD for control groups of adults and newborns, respectively; ■, proband; ●, mother. Incubations and enzyme assays were as described in *Materials and Methods* except that the hemolysate was prepared in buffer/0.1 mM $NADP^+$.

this locus are responsible for ≈80% of the LDH activity in erythrocytes, although positive identification of the locus involved will require further studies.

(*ii*) A thermolabile variant of G6PD was observed in a newborn male in family 2018 (Fig. 3). The enzyme from the mother was also less stable than normal, while the thermostability of G6PD from the father was within the normal range. The thermolability data of Fig. 3 were obtained by using a hemolysate prepared with buffer containing 0.1 mM $NADP^+$. The results were similar in the absence of added $NADP^+$ although the stability curve was shifted to the left. The thermostability of these two samples was unaffected when the $NADP^+$ concentration was increased during the heating step to 1 mM or the preincubation time was extended. The electrophoretic mobility of the G6PD was identical to the normal B-type pattern. The levels of G6PD activity for mother and child were 310 and 620 (units/g of Hb)/hr, respectively. This is 110% of the activity expected for adults and newborns, respectively.

(*iii*) The GPI enzyme from a male child in family 1791, after heating, exhibited excessive enzyme activity loss to a value that was 3.4 SD below normal (Fig. 4). This labile variant was observed in the father also. Normal levels of enzymatic activity were observed in erythrocyte hemolysate from both individuals (98% and 106% of appropriate controls, respectively). The normal GPI electrophoretic pattern was observed for all members of the family.

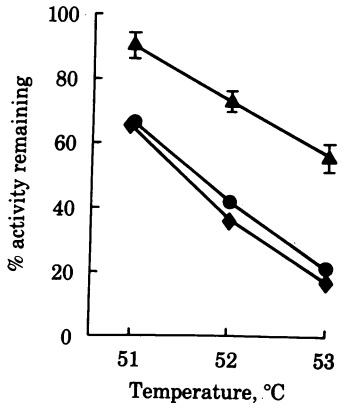

FIG. 4. Thermostability profiles for GPI. ▲, Percent remaining activity ± SD for control group; ◆, proband; ●, father.

Except for two infants exhibiting the AK1-2 phenotype, no electrophretic variants were identified in this group of newborns. However, two "enzyme deficiency" variants, one of TPI and one of G6PD, were present in this group of 100 newborns (cf. ref. 2). Neither deficiency variant was associated with an altered thermostability profile. Thus, no rare variant was detected by more than one technique.

DISCUSSION

With the criteria used in this study, thermostability variants have a frequency of 3.8 per 1000 determinations. These criteria are rather stringent, however, requiring that a heterozygote manifest, at the least, almost a 20% decrease (or increase) in the percent activity remaining after incubation at two of three different temperatures. This implies that the product of the variant allele retains, after heating, not more than 60% of the activity retained by the normal enzyme, corresponding to rather marked thermolability on the part of the gene product associated with the variant allele. It seems reasonable to postulate that there are lesser degrees of genetically determined alterations in thermostability not being detected by present procedures.

Although the analysis necessary to establish an alteration in the primary structure of the variant enzymes has not been completed, the profiles are not those obsserved to be associated with changes in mean erythrocyte age or increased reticulocyte count (14). For all three variants, the localization of the alteration to a single enzyme in an individual and the appearance of the alteration in two generations is consistent with the presence of an inherited structural gene variant. To the best of our knowledge, this is the first survey-type estimate of the frequency of individuals having thermostability variants of representative enzymes. Previous studies have always determined the number of different thermostability variants within a previously identified electrophoretic class (3–6, 9–11).

Of the eight enzymes studied for thermostability variants, seven have also been studied in this laboratory with respect to electrophoretic (from a total of 27 loci) and enzyme-activity variants (from a total of 9 loci). These seven are G6PD, AK, GPI, LDH-B, MDH, TPI, and PGK (as 80% of the activity of LDH is associated with the B subunit, we assume that we are *not* screening for LDH-A variants). The results are summarized in Table 2.

Relative to the question of the commonality of variants detected by electrophoresis, thermostability, and enzyme-activity measurements, none of the thermostability variants was identified as an electrophoretic variant or as an enzyme-deficiency variant as defined by Mohrenweiser (2). It should be pointed out that enzyme-deficiency variants (nulls) would be detected with these techniques only if structurally similar nonfunctional protein were present and combined with normal subunits, altering normal stability or electrophoretic profiles. Although (in theory) some fraction of the variants should be detectable by more than one technique, this did not occur in our limited sample. Each of the thermostability variants identified in this study would therefore be classified as a "hidden variant" (9), and each presumably results from the frequently discussed "silent" amino acid substitutions not detected by electrophoresis, whose mutational frequency is estimated to be 2 to 3 times greater than those that are so detected.

The observed total for these types of variants at the seven loci studied with all three techniques is 7.7 per 1000 determinations: there is, of course, a considerable sampling error, although this value is in good agreement with the 7.3 per 1000 determinations when all the loci studied are included. In addition, we have previously identified a group of "other activity variants" in this same sample, presumably also due to heterozygosity for a sin-

Table 2. Comparative frequency of rare variants at erythrocyte enzyme loci as determined by four criteria

	Frequency at seven loci			Frequency at all loci studied			
	Determinations, no.	Variants, no.	Frequency, no./1000	Loci, no.	Determinations, no.	Variants, no.	Frequency, no./1000
Electrophoresis*	11,982	8	0.7	27	48,043	54	1.1
Thermostability	700	3	4.3	8	800	3	3.8
Deficiency†	4,808	13	2.7	9	6,142	15	2.4
Activity†	4,808	5	1.0	9	6,142	7	1.1
Total			8.7				8.4

* Data from Neel *et al.* (1).
† Data from Mohrenweiser (2).

gle allele, that has a frequency of 1.0 or 1.1/1000, depending on the data included (Table 2). Thus, exclusive of polymorphisms, the current estimate of the frequency of variant individuals for any particular enzyme is 8.7/1000 at the seven loci or 8.4/1000 at all loci studied. The studies are not yet to the point where the degree of heterogeneity encompassed by that figure of 8.4-8.7/1000 can be estimated.

This is certainly an underestimate of the total frequency of variants of these types. On the one hand, only a single type of electrophoresis for each enzyme was used; it is well established that more variants are detected when several electrophoretic procedures are used than when only a single method is pursued (15, 16). At the same time, as noted above, the criteria for detecting thermostability variants are rigorous; it is reasonable to assume that there are lesser degrees of inherited thermolability than were detected in this study. Finally, a similar argument can be made that the enzyme-activity assays only detect the extremes. We suggest for the present that the total frequency of all these types of variants, including the variants we were unable to detect, is not less than 10 per 1000 determinations.

On the assumption that the variants contributing to this estimate of 10/1000 all resulted from mutation involving the same locus, then the total allele frequency of variants of this type would be 0.005. Given that frequency at any locus, individuals homozygous or doubly heterozygous for variants of the type under consideration should have a frequency of 2.5 per 100,000 conceptuses. The implications of this frequency for health and disease are obscure, although the ramifications of some classes of variants are known. Most of the inborn errors of metabolism that come to clinical attention result from homozygosity for enzyme-deficiency variants as defined in ref. 2, but it is not clear to what extent the clinical sieve is coloring our picture of the disease impact of such variants in humans. In this context, it should be pointed out that, if there are 5000 enzymes in the human body, then, assuming for this calculation independent segregation of the alleles involved, the probability at conception of being homozygous or doubly heterozygous, (or both) for one or more of the three types of enzyme variants we have recognized is $1 - 0.999975^{5000}$ or 0.118.

In a discussion of the possible health implications of these variants, the conservative course is to consider only the enzyme-deficiency variants, for whose impact when homozygous there is abundant documentation (17). In an earlier calculation, one of us, extrapolating from the results of indirect estimates of the rate of mutation resulting in electrophoretic variants in Amerindians and the relative frequency of electrophoretic-type and null-type mutations in *Drosophila*, guesstimated a mutation rate for nulls in humans of 2×10^{-5} per locus per generation (18). If there was no selection for or against heterozygotes with a null allele but homozygosity in general conferred a severe survival/reproductive handicap, this implies at equilibrium that some 0.095% of all conceptus should be homozygous for one of more of these 5000 null enzymes. That calculation suggests a heterozygote frequency for nulls of any particular protein of ≈0.9%. If the above assumption regarding the phenotypic effect in humans of homozygosity for a null is correct, then our observed heterozygote frequency for enzyme-deficiency variants (which, for these purposes, we equate to nulls) of ≈0.25% suggests that either the assumed mutation rate was too high or selection operates against the null heterozygote. Both explanations may apply. Recently, we have reviewed evidence raising the possibility of higher mutation rates in tropical zone/tribal/nonindustrialized populations than in temperate zone/civilized/industrialized populations (19). If this is correct, then it is inadmissable to use Amerindian mutation rates as the basis for an extrapolation to rates in civilized populations. On the other hand, the lower frequency of null homozygotes than predicted could also be related to selection against nulls when heterozygous, not only in combination with a normal allele but especially by virtue of double heterozygosity for a null and another type of variant, such as described in this paper. Whatever the final explanation, it is clear that the end point of the previous calculation was too high; by the approach of the paragraph above, the frequency of individuals homozygous for one or more nulls at conception can now be placed at 0.031. Even this modified figure is somewhat greater than current estimates of the total frequency of null homozygotes at birth (presumably presenting as inborn errors of metabolism) and leads to reiteration of the suggestion of substantial loss during gestation of such fetuses (18).

Drosophila melanogaster is the only other organism for which survey data exist on the frequency of enzyme deficiencies (20). These nulls had a frequency of 39 in 19,399 determinations (0.0020) but, for autosomal loci only, the frequency was 0.0025. The human data were obtained by using erythrocytes and therefore would identify enzyme deficiencies due to enzyme instability as well as loss of functional protein while, in the *Drosophila* studies, only the latter class of variants would be identified. If future work shows tht most of the deficiency variants in humans result from loss of functional protein, then the similarity of the *Drosphila* value to the human value, despite the enormous differences in the population structure of the two organisms, poses several interesting questions in comparative population genetics. In this context, the results of studies on the frequency of enzyme-thermostability variants in wild populations of *Drosophila* should be of extreme interest.

As noted above, the studies described in this and in refs. 1 and 2 were undertaken within the context of an effort to explore a variety of biochemical approaches to estimating the frequency of human mutation rates. We are now in a position to undertake a very preliminary analysis of the relative efficiencies of studies using electrophoretic, enzyme-activity, and thermostability determinations. Efficiency should be proportional to the quotient, mutation rate for the trait under study divided by cost per de-

termination. As, in the context of a screening operation, no certain example of spontaneous mutation in humans with respect to any of these three classes of variants has yet been documented, clearly no final decision is possible. We can, however, on the basis of experience to date, estimate the ratio of the relative effort involved in detecting electrophoretic, enzyme-deficiency, and thermostability variants as 1:1.5:4. The position of thermostability variants in this hierarchy holds even when the first point in the thermostability curve is supplied by the activity determination (and the effort not "charged" against the thermostability studies). Thermostability variants equaled activity variants in frequency, but the latter are probably more subject to negative selection and hence supported in the population by higher mutation rates. We conclude that the characterization of protein thermostability is a less efficient way to search for mutations than the determination of electrophoretic mobility or enzyme activity, at least within the context of a sampling protocol such as used in this experiment. Preliminary estimates indicate that the costs of the electrophoretic and enzyme-activity approaches are similar. Thus, if the rate of mutation resulting in deficiency variants is higher than that for electrophoretic variants, it could be the most cost-effective approach. In addition, the two techniques are complementary in that they detect different classes of variants. Accordingly, given the evidence for mutagen specificity in mutagenesis, an argument can be made for the simultaneous use of both techniques in any effort to detect an altered mutation rate. At the same time, it should also be obvious that enzyme-deficiency variants have a higher probability than electrophoretic variants of being associated with a health risk and thus would be more important in estimating the cost (as in cost/benefit ratios) of an increased mutation rate in human populations.

The technical assistance of S. Fielek is gratefully acknowledged. This work was supported in part by Department of Energy Contract EY-77-C-02-2828.

1. Neel, J. V., Mohrenweiser, H. W. & Meisler, M. M. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 6037–6041.
2. Mohrenweiser, H. W. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 5046–5050.
3. Singh, R., Hubby, J. L. & Lenwontin, R. C. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 1808–1810.
4. Thörig, G. E. W., Schoone, A. A. & Scharloo, W. (1975) *Biochem. Genet.* **13**, 721–731.
5. Cobbs, G. & Prakash, S. (1977) *Genetics* **87**, 717–742.
6. Trippa, G., Loverre, A. & Catamo, A. (1976) *Nature (London)* **260**, 42–44.
7. Bonhomme, F. & Selander, R. K. (1978) *Biochem. Genet.* **16**, 287–297.
8. Satoh, C. & Mohrenweiser, H. W. (1978) *Ann. Hum. Genet.* **43**, 283–292.
9. Bernstein, S. S., Throckmorton, L. H. & Hubby, J. L. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 3928–3931.
10. Singh, R. S., Hubby, J. L. & Throckmorton, L. H. (1975) *Genetics* **80**, 637–650.
11. Beckenbach, A. T. & Prakash, S. (1977) *Genetics* **87**, 743–761.
12. Neel, J. V., Mohrenweiser, H. W., Satoh, C. & Hamilton, H. B. (1979) in *Genetic Damage in Man Caused by Environmental Agents*, ed. Berg, K. (Academic, New York), pp. 29–47.
13. Fielek, S. & Mohrenweiser, H. W. (1979) *Clin. Chem.* **25**, 384–388.
14. Mohrenweiser, H. W., Fielek, S. F. & Wurzinger, K. H. (1981) *Am. J. Hematol.* **11**, in press.
15. Johnson, G. B. (1976) *Genetics* **83**, 149–167.
16. Johnson, G. B. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 395–399.
17. Stanbury, J. B., Wyngaarden, J. B. & Fredrickson, D. S. (1978) in *The Metabolic Basis of Inherited Diseases*, ed. Stanbury, J. B., Wyngaarden, J. B. & Fredrickson, D. S. (McGraw-Hill, New York), pp. 2–31.
18. Neel, J. V. (1978) *Can. J. Genet. Cytol.* **20**, 295–306.
19. Neel, J. V. & Rothman, E. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 3108–3112.
20. Voelker, R. A., Langley, C. H., Brown, A. J. L., Ohnishi, S., Dickson, B., Montgomery, E. & Smith, S. C. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 1091–1095.