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Hereditary Disorders of Erythrocyte Metabolism

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HEREDITARY DISORDERS OF ERYTHROCYTE METABOLISM

Proceedings of the Symposium held February 13-15, 1967, at the City of Hope Medical Center, Duarte, California

Edited by **ERNEST BEUTLER, M.D.**

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Chairman, Division of Medicine, City of Hope Medical Center,
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City of Hope Symposium Series: Vol. 1

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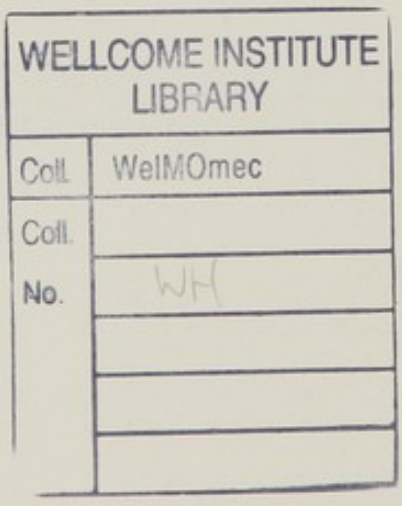
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Preface

MORE THAN THIRTY YEARS AGO, Warburg used the red blood cell as a metabolic model. It proved to be an invaluable means for understanding many processes of metabolism. Its use in man and other species has been responsible for the accumulation of a vast store of knowledge at an increasingly accelerated rate. Yet it has been recognized, for only slightly more than a decade, that metabolic abnormalities within red cells are the cause of disease. Many such abnormalities have been described by biologists in various parts of the world. Awareness of the widespread nature of these abnormalities has stimulated further vigorous research.

It seemed a worthwhile endeavor to provide an opportunity for those investigators who are actively concerned with problems in this field to exchange ideas. Support provided by the Institute for Advanced Learning in the Medical Sciences at the City of Hope, Duarte, California, made such a conference possible, and a meeting was held there February 13-15, 1967. Attendance was limited in order to maintain a relatively informal spirit of inquiry, with the hope that the participants would not only stimulate one another intellectually, but would also compare techniques and perhaps engage in collaborative studies in the future. All of those who were present were well versed in the fundamentals of the biochemistry of the human erythrocyte. Thus, most of the three days was spent in the presentation and discussion of advanced concepts and new theses about biochemical genetics of the erythrocyte.

This volume contains all of the lectures as well as the discussion that followed each. Like the Symposium itself, these proceedings are intended for those biochemists, geneticists, and clinicians who are already familiar with the fundamental aspects of erythrocyte metabolism. Each speaker has dealt primarily with one or a small group of genetically-determined abnormalities of red cell metabolism. He has presented a review of available information about the pathologic condition, including new data from his own laboratory. The ensuing discussions of each paper yielded a great deal of previously unpublished material and intriguing speculation.

The valuable editorial assistance of Mrs. Antreen Pfau is gratefully acknowledged.

ERNEST BEUTLER, M.D.

Abbreviations

AAC—adenine-adenine-cytosine, mRNA codon for aspargine
AAU—adenine-adenine-uracil, mRNA codon for aspargine
ACD—acid citrate dextrose
ADP—adenosine diphosphate
AK—adenylate kinase
AMP—adenosine monophosphate
APH—acetylphenylhydrazine
ATP—adenosine triphosphate

CNHD—congenital nonspherocytic hemolytic disease

DCIP—2, 6 dichlorophenol—indophenol; 2, 6 dichlorobenzenenone-indophenol
DEAE—diethylaminoethyl (cellulose)
DHAP—dihydroxyacetone phosphate
DNA—deoxyribonucleic acid
DPN—diphosphopyridine nucleotide (NAD)
DPNH—reduced diphosphopyridine nucleotide (NADH)
DTNB—5, 5′—dithiobis (2-nitrobenzoic acid)
1, 3—DPG—1, 3, diphosphoglyceric acid
2, 3—DPG—2, 3-diphosphoglyceric acid
2, 3—DPGM—2, 3-diphosphoglycero-mutase

EDTA—ethylenediaminetetraacetic acid
EEG—electroencephalogram

FAD—flavin adenine dinucleotide
FMN—flavin mononucleotide
F-1-P—fructose-1-phosphate
F-6-P—fructose-6-phosphate

GAC—guanine-adenine-cytosine, mRNA codon for aspartic acid
gal-1-P—galactose-1-phosphate
G-ald-3-P—glyceraldehyde-3-phosphate
GAU—guanine-adenine-uracil, mRNA codon for aspartic acid
GR—glutathione reductase
GSH—reduced glutathione
GSSG—oxidized glutathione
GSSG-R—glutathione reductase
G-1-P—glucose-1-phosphate
G 1, 6-di-P—glucose-1, 6-diphosphate
G-3-P—glyceraldehyde-3-phosphate
G-6-P—glucose-6-phosphate
G-6-PD—glucose-6-phosphate dehydrogenase

H_i—methemoglobin
HK—hexokinase
HMP—hexose monophosphate
HS—hereditary spherocytosis

IMP—inosine monophosphate
INH—isonicotinic acid hydrazide

LDH—lactic dehydrogenase

MB—methylene blue
MCHC—mean corpuscular hemoglobin concentration
MetHb—methemoglobin
MTT—[3 (4, 5 dimethylthiazolyl 1-2) 2, 5 diphenyltetrazolium bromide]

NAD—nicotinamide adenine dinucleotide (DPN)
NADH—reduced nicotinamide adenine dinucleotide (DPNH)
NADP—nicotinamide adenine dinucleotide phosphate (TPN)
NADPH—reduced nicotinamide adenine dinucleotide phosphate (TPNH)
NEM—N-ethyl maleimide
NSHA—nonspherocytic hemolytic anemia

PAS—para-aminosalicylic acid
PCMS—*P*-chloromercuriphenylsulfonic acid
PEP—phosphoenolpyruvate
PFK—phosphofructokinase
PGADH—phosphogluconic acid dehydrogenase
PGK—phosphoglycerokinase

PGM—phosphoglucomutase
P_i—inorganic phosphate
PK—pyruvate kinase
PNH—paroxysmal nocturnal hemoglobinuria
PNH—reduced pyridine nucleotide
PU—phosphatase units
3-PGA-3-phosphoglyceric acid
6-PGA-6-phosphogluconic acid
6-PGD-phosphogluconic dehydrogenase

RNA—ribonucleic acid

SH—thiol or sulfhydryl group

TPI—triosephosphate isomerase
TPN—triphosphopyridine nucleotide (NADP)
TPNH—reduced triphosphopyridine nucleotide (NADPH)
TRA—triethanolamine
Transferase—gal-1-P uridyl transferase

UDPG—uridine diphosphoglucose
UDP-Gal-uridine diphosphogalactose

Electrophoretic Variation in Erythrocyte Enzymes

HARRY HARRIS, M.D., F.R.S.; D. A. HOPKINSON, M.D.; JILL E. LUFFMAN, Ph.D.; and SANDRA RAPLEY, B. Sc.

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THE WORK of our laboratory during the last four years has been largely aimed at trying to assess the extent and the character of genetically-determined enzyme variation in human populations.[4,5] We have mainly studied enzymes present in erythrocytes because these are particularly convenient for population and family investigations. We have mainly used the method of starch gel electrophoresis because this is known to be capable of revealing relatively subtle molecular differences with quite small amounts of material, and is also very suitable for screening large numbers of samples. The enzymes were chosen on a quite arbitrary basis so that we might obtain, within the limits of the techniques applied, an unbiased picture of enzyme variation in general.

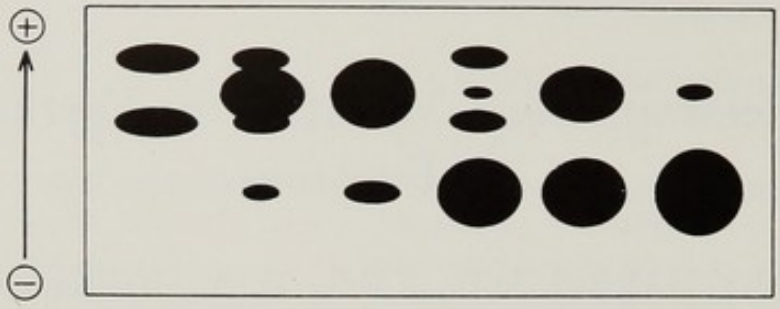
The results obtained with three very different enzymes, each of which, however, has been found to exhibit a striking degree of electrophoretic variation, will be outlined here. The enzymes are red cell acid phosphatase, phosphoglucomutase, and adenylate kinase.

RED CELL ACID PHOSPHATASE

This enzyme differs in a number of ways from the acid phosphatases present in other tissues, and it is probably peculiar to the red cell. Its functional significance is not known.

It may be detected after starch gel electrophoresis by incubating the surface of the gel with phenolphthalein diphosphate at pH 6.0.[8] The gel is subsequently made alkaline with ammonia vapor so that the sites of acid phosphatase activity appear as bright red zones due to the liberated phenolphthalein. α or β naphthyl phosphates, which are widely utilized as substrates for the detection of other phosphatases after electrophoresis, were not used because they are not hydrolyzed by this enzyme.

Figure 1 illustrates diagrammatically the electrophoretic patterns of red cell acid phosphatase isoenzymes that may be observed when hemolysates from different individuals are subjected to starch gel electrophoresis at pH 6.0. The patterns differ from one another in the number, the electrophoretic mobilities, and the relative activities of the isoenzymes present. They are characteristic of the individual and genetically-determined.[8,9] In the English population, five of the phenotypes, A, BA, B, CA, and CB, are relatively common and occur in



Phenotype	A	BA	B	CA	CB	C
Genotype	$p^a p^a$	$p^b p^a$	$p^b p^b$	$p^c p^a$	$p^c p^b$	$p^c p^c$
Frequency	0·13	0·43	0·36	0·03	0·05	0·0016

FIG. 1. Diagram of isoenzymes in different red cell acid phosphatase phenotypes. Electrophoresis carried out at pH 6.0 as described by Hopkinson, Spencer and Harris.[9] The approximate frequencies of the different types in the English population are indicated.

approximately 13, 43, 36, 3 and 5 per cent of people, respectively. The sixth, type C, is less frequent and is found in only about 1 in 600 individuals.

Extensive family studies have been carried out (representative data are shown in Table 1) and lead to the conclusion that the six phenotypes are determined by three common alleles P^a, P^b, and P^c. Phenotypes A, B, and C represent the homozygous genotypes (P^aP^a, P^bP^b and P^cP^c); and phenotypes BA, CA, and CB, the heterozygous genotypes (P^aP^b, P^aP^c and P^bP^c).

Homozygotes with type A acid phosphatase have two main isoenzyme components of roughly equal intensity. They are of different electrophoretic mobilities from those that occur in types B and C. Types B and C differ from one another in the relative activities of the two isoenzymes present. In type B the

TABLE. 1. *The distribution of red cell acid phosphatase phenotypes observed in 440 English families*

Parents	Number of matings	Children						Total
		A	BA	B	CA	CB	C	
A × A	13	22	—	—	—	—	—	22
A × BA	50	65	48	—	—	—	—	113
A × B	27	—	65	—	—	—	—	65
A × CA	6	4	—	—	5	—	—	9
A × CB	10	—	5	—	14	—	—	19
BA × BA	94	40	91	54	—	—	—	185
BA × B	109	—	106	96	—	—	—	202
BA × CA	16	14	9	—	6	4	—	33
BA × CB	16	—	10	7	6	10	—	33
B × B	55	—	—	141	—	—	—	141
B × CA	12	—	12	—	—	20	—	32
B × CB	24	—	—	33	—	25	—	58
CA × CB	5	—	2	—	2	3	1	8
CB × CB	3	—	—	0	—	4	1	5
Totals	440	145	348	331	33	66	2	925

faster component (towards the anode) is much more active than the slower component, whereas in type C the opposite is the case. The isoenzymes present in the heterozygous types BA, CA, and CB can be regarded as mixtures in roughly equal amounts of the isoenzymes present in the two corresponding homozygous types.

The types also differ from one another in total level of activity.[16] On the average, red cells of type B show about 50 per cent more acid phosphatase activity than those of type A, while type BA red cells are intermediate in this respect. Furthermore, the activity in type CB is on the average greater than that in types CA or B. The enzyme activity attributable to the three alleles P^a, P^b and P^c, appears to be roughly in the ratio 2:3:4. An interesting consequence follows from this (Figure 2). If one determines the red cell acid phosphatase activity in a randomly-selected series of individuals, one obtains a continuous and unimodal distribution, which is not unlike the distributions obtained when the activities of many other red cell enzymes are determined in normal people. However in the present case it can be shown that this distribution represents a summation of separate but overlapping distributions with different means, corresponding to each of the several qualitatively different enzyme phenotypes. Furthermore the genetically-determined component of the variance of the overall distribution can be largely if not entirely attributed simply to the effects of three alleles.

The acid phosphatase types also differ from one another in thermostability.[12] Types CA and CB have been found to be relatively more thermostable than

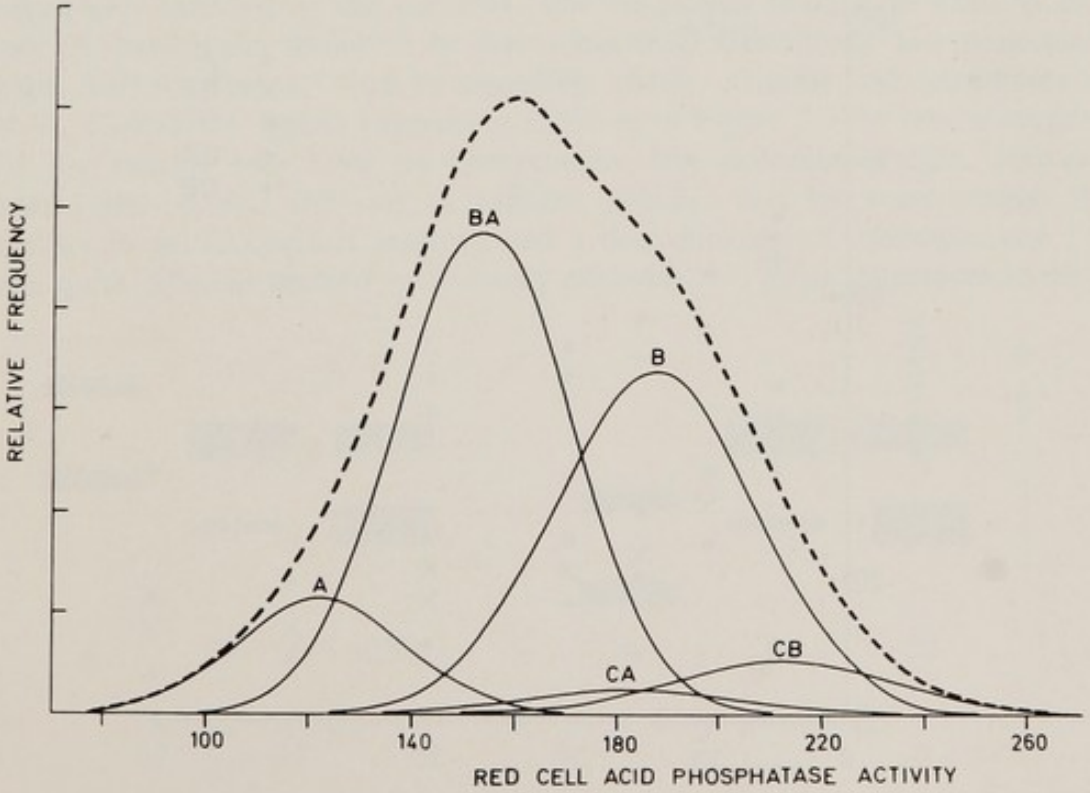


FIG. 2. Distribution of red cell acid phosphatase activities in the general population (broken line) and in the separate phenotypes. The curves are constructed from the data given by Hopkinson, Spencer and Harris.[9]

types B, BA, and A; and in the latter group, type B is slightly more stable than type A and type BA is intermediate. These experiments were carried out by determining the residual activity in hemolysates held at different temperatures for varying periods of time. Typical results obtained at 50°C are illustrated in Figure 3. During the course of these experiments, the samples were also examined electrophoretically. It seemed that in each type, the faster moving components were less stable than the slower moving components. Furthermore it appeared that, at least in type B, the faster moving component was first converted to a component with the mobility of the slower component normally present, before being completely denatured. Whether this represents a true conversion of the fast isoenzyme to the normal slow isoenzyme or whether it represents some quite different kind of change is not yet known.

The separations shown in Figure 1 were obtained with an electrophoretic system in which a citrate buffer at pH 6.0 was used as the bridge solution and a Tris/succinate buffer at pH 6.0 in the gel. This system has been routinely used in our family and population studies. Recently however H. E. Sutton (personal communication) has shown that if phosphate buffers at the same pH are used, the type A isoenzymes show a very different mobility relative to the types B and C isoenzymes. In the original system, the two type A components move relatively faster than the two type B or type C components, whereas in the phosphate buffer system, they move relatively more slowly. We have recently investigated this curious effect and found rather to our surprise, that it appears to depend simply on the presence or absence of citrate in the buffers used. Tris

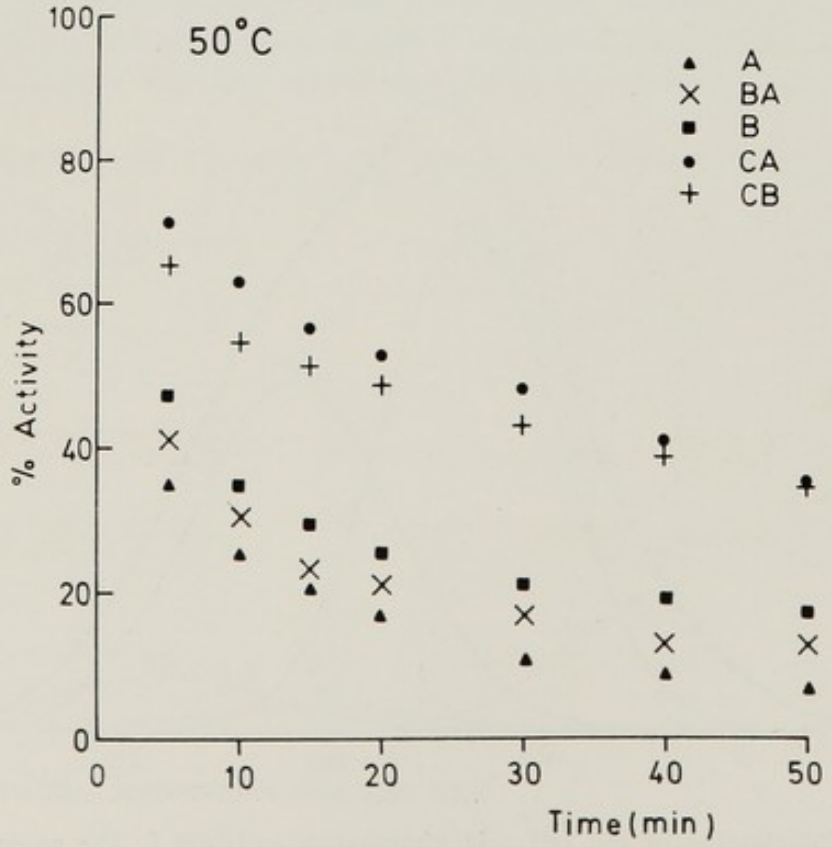


FIG. 3. Mean percentage residual red cell acid phosphatase activity of hemolysates of different phenotypes held at 50°C for varying periods of time.

and succinate alone, or in combination with one another or with phosphate, give the so called 'slow' A pattern. Citrate alone or in combination with Tris, succinate or phosphate gives the so-called 'fast' A pattern. All the experiments were carried out at pH 6.0. This quite unexpected result has led us to test a series of other organic acids to see whether any of them also behave like citrate. As a simple test system we have examined the mobilities of the type A and type B isoenzymes in a series of experiments at pH 6.0 in which the bridge buffer was 0.4M in phosphate and 0.1M in the particular organic acid, and the gel buffer was 0.01M in phosphate and 0.005M in the organic acid. So far, it has been found that a typical fast 'A' pattern (Figure 4) has been obtained when tricarboxylic acids such as citric, isocitric, cis-aconitic, trans-aconitic, tricarballylic and trimellitic were used. On the other hand, a 'slow' A pattern (Figure 4), indistinguishable from that obtained with phosphate alone, was found when certain dicarboxylic acids such as succinic, oxalic, α-oxoglutaric, maleic, itaconic, aspartic or glutamic, or a number of monocarboxylic acids were used. EDTA and phthalic acid appear to give an intermediate effect. It looks as though the phenomenon depends on some structural peculiarity in the A type isoenzymes because the relative mobilities of the B to the C isoenzymes do not appear to be modified by the buffer changes. We intend to extend the range of the buffer constituents used, in the hope of defining the nature of the effect more precisely. This unexpected and quite marked alteration in relative mobility produced by different buffer systems at a given pH is worth bearing in mind in electrophoretic studies of other enzymes.

Red cell acid phosphatase has also been examined by gel filtration. It is considerably retarded on the columns, and the elution position of each of the types, is consistently found to be later than that of relatively low molecular weight marker proteins,[12] such as myoglobin (M.W. 17,000) and cytochrome c (M.W. 12,400). A typical experiment is shown in Figure 5. The results suggest that the enzyme may have an unexpectedly low molecular weight, perhaps about 7,000-10,000. However, it remains possible that for some reason its behavior in gel filtration is atypical, and a determination of molecular size by some quite different method is obviously desirable. No gross differences in elu-

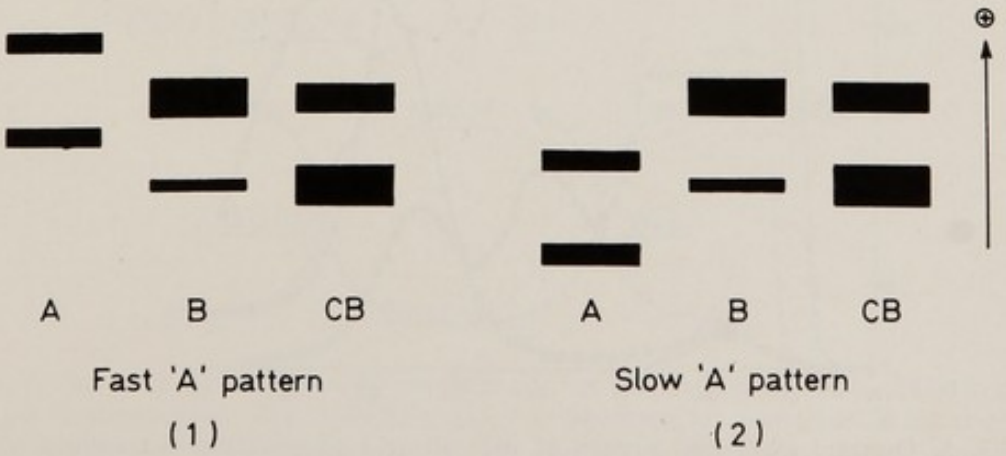


FIG. 4. Diagram showing relative mobilities of red cell acid phosphatase isoenzymes (types A, B and CB) in different buffer systems (see text). (1) Fast 'A' pattern. (2) Slow 'A' pattern.

tion position between the types were found, and the isoenzymes in a given type appeared to be present in the elution peak. But relatively small differences in elution position would have been difficult to detect in these experiments, and the matter is being further investigated.

Scott[15] has compared the kinetic properties of partially purified preparations of types A and B phosphatases. No marked differences were detected.

It appears that the alleles at the red cell acid phosphatase locus each determine at least two isoenzymes with qualitatively distinctive properties. In the English and other European populations, the alleles P^a, P^b, and P^c occur with frequencies of about 0.36, 0.60, and 0.04, respectively. In Negro populations P^a appears to be less common (*ca* 0.20), P^b more common (*ca* 0.80) and P^c virtually absent. However two other phenotypes, RA and RB[3] (E. H. Sutton, personal communication) have been seen in about 1 per cent of Negroes. They show a pair of relatively fast-moving zones, together with either the typical A or the typical B zones. They may be attributed to a further allele P^r in heterozygous combination with P^a or P^b. These R isoenzymes have not yet been studied extensively. However it is of interest that they resemble the B and C isoenzymes rather

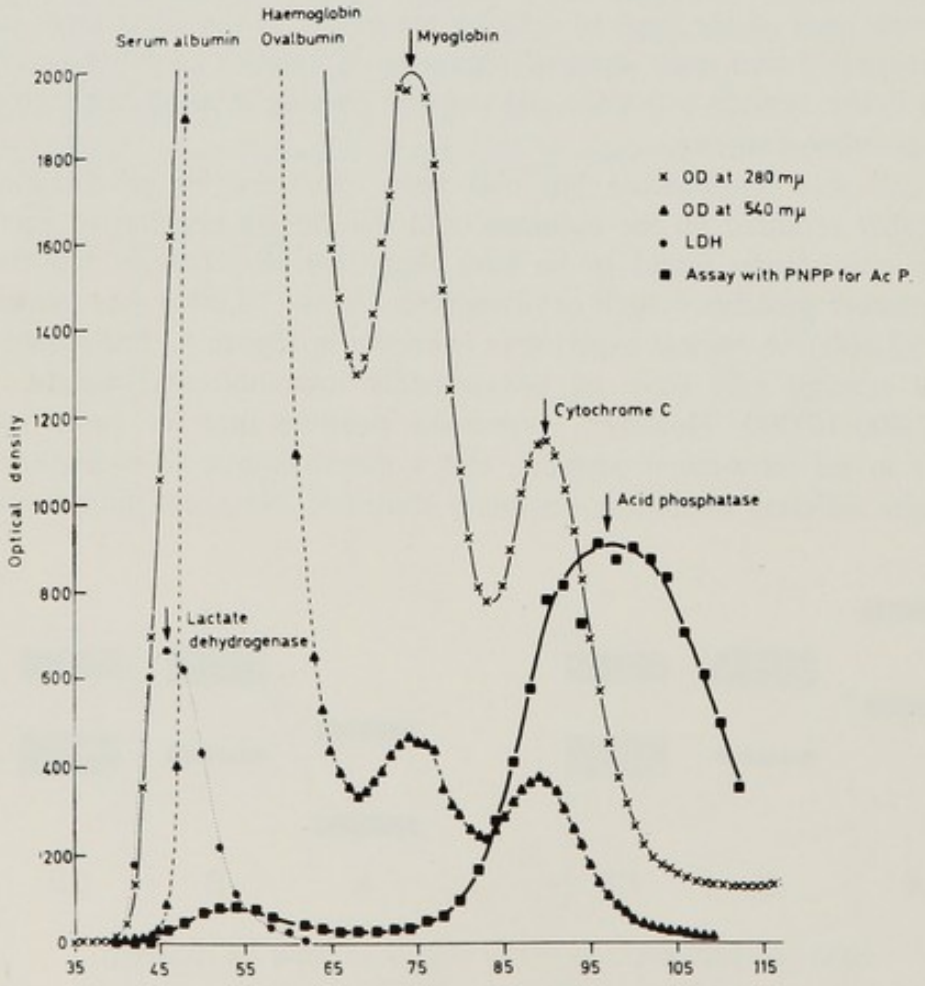


FIG. 5. Diagram of elution pattern of red cell acid phosphatase and various markers on Biogel P 60. Experimental details as in Luffman and Harris.[12] The position of the various protein markers was determined both by O.D. determination and by starch gel electrophoresis of the individual fractions.

than the A isoenzymes in their electrophoretic behavior in relation to the presence or absence of citrate in the buffer system.

PHOSPHOGLUCOMUTASE

Phosphoglucomutase (PGM) is a phosphotransferase that catalyses the transfer of a phosphate group between the 1- and 6- positions of glucose. It has an important role in carbohydrate metabolism and is widely distributed in mammalian tissues. Although its activity in erythrocytes is rather less than in many other cell types, red cells are nevertheless a valuable source of the enzyme for human population and family studies.

The reaction system used to detect PGM after starch gel electrophoresis[17] is shown in Figure 6. The underlined components are included in the reaction mixture, and the sequence of reactions results in blue-coloured insoluble formazan being deposited at the sites of enzyme activity. Glucose 1,6 diphosphate (G 1,6-di-P), although required only in small amounts, is a critical ingredient of the reaction mixture. It can now be obtained commercially, but is expensive, and we have in practice relied mainly on using as substrate glucose-1-phosphate (G-1-P) preparations, which happen to contain G 1, 6-di-P as an impurity. However, the amount present varies greatly in different G-1-P samples, and only those containing at least 1 per cent G 1,6-di-P are usable.

When human red cell lysates are subjected to starch gel electrophoresis at pH 7.4 and this reaction system is then applied, a series of distinct isoenzyme bands are seen (Figure 7 *a-g*). Three common patterns occur, and are referred to as PGM 1, PGM 2-1, and PGM 2. Isoenzymes *a* and *c* are present in PGM 1 and PGM 2-1 but not in PGM 2; isoenzymes b and d are absent in PGM 1 but are present in PGM 2-1 and PGM 2; isoenzymes e, f, and g are seen in all three types. In our own population, about 58 per cent of people are PGM 1, about 36 per cent PGM 2-1, and about 6 per cent PGM 2. These three common types have now been seen in a number of very different

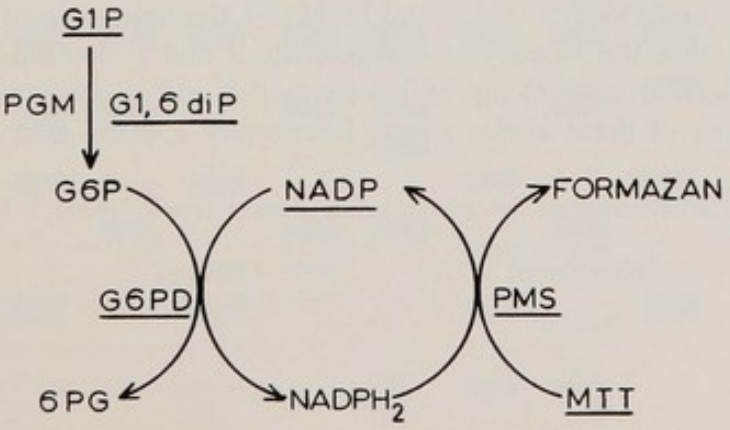


FIG. 6. Sequence of reactions in the detection of phosphoglucomutase (PGM) after starch gel electrophoresis. The underlined 6-di-P components are contained in the reaction mixture. G-1-P, glucose-1-phosphate; G1, 6-di-P, glucose-1,6-diphosphate; G-6-P, glucose-6-phosphate; G-6-PD, glucose-6-phosphate dehydrogenase; NADP, nicotinamide adenine dinucleotide phosphate; $NADPH_2$, reduced form of NADP; PMS, phenazine methosulphate; MTT, dimethyl thiazolyl diphenyl tetrazolium salt.)

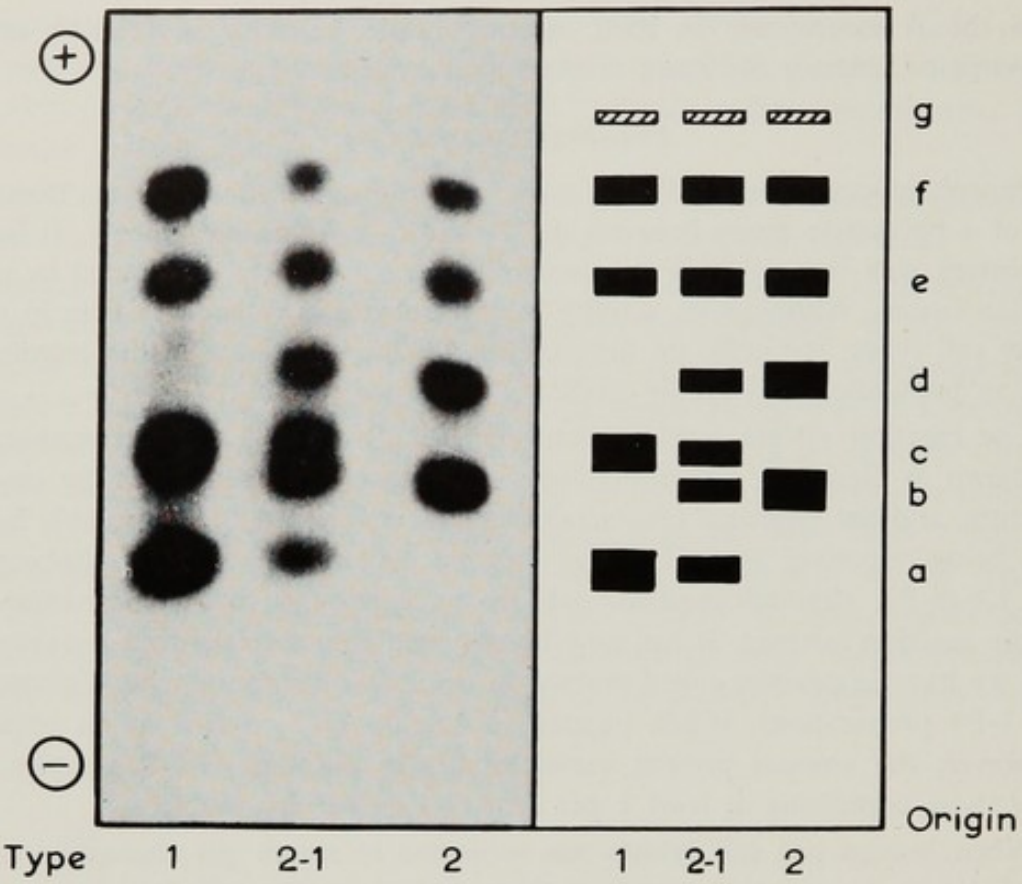


FIG. 7. Photograph and diagram of phosphoglucomutase isoenzyme patterns obtained by starch gel electrophoresis at pH 7.4.

human populations, and it is clear that this enzyme polymorphism is widespread throughout the species.

The segregation of these types in a large number of families has now been studied (Table 2). The findings are readily explained in terms of the simple hypothesis that the polymorphism is determined by two autosomal alleles (PGM_1^1 and PGM_1^2), PGM 1 and PGM 2 representing the homozygous types $PGM_1^1PGM_1^1$ and $PGM_1^2PGM_1^2$, and PGM 2-1 the heterozygote $PGM_1^1PGM_1^2$. This suggests that the isoenzyme components *a* and *c* determined by PGM_1^1, represent molecular equivalents of isoenzymes *b* and *d* determined by PGM_1^2, and that neither of these alleles affects isoenzymes *e*, *f*, or *g*.

TABLE 2. *The distribution of phosphoglucomutase types* (PGM_1) *in 537 families*

Parents	Number of matings	Children			Total
		1	2-1	2	
1 × 1	199	392	—	—	392
1 × 2-1	203	207	215	—	422
1 × 2	34	—	71	—	71
2-1 × 2-1	77	35	81	41	157
2-1 × 2	21	—	24	31	55
2 × 2	3	—	—	13	13
Totals	537	634	391	85	1110

In the course of examining blood samples from several thousand individuals a variety of other PGM types, mostly very rare, have been discovered. A number of these on the basis of the characteristic isoenzyme pattern they display, and on the manner in which they have been found to segregate in families, can be attributed to the heterozygous combination of either PGM_1^1 or PGM_1^2 with one or another of a series of rare alleles at the same locus.[7] Examples of such phenotypes are shown in Figure 8. The patterns can be interpreted most simply by supposing that in each type there are two unusual isoenzyme components determined by the particular rare allele concerned, and also either the *a* and *c* components determined by PGM_1^1 or the *b* and *d* components determined by PGM_1^2. So far, evidence for the occurrence of six different rare alleles at this locus has been obtained. Their individual gene frequencies in European populations are probably in most cases less than 0.0005.

Evidence for a second genetic locus determining phosphoglucomutase isoenzyme[6,7] came from the discovery of an unusual phenotype segregating in a Negro family and characterized by variation of the more rapidly moving components *e, f,* and *g*. The phenotype (Figure 9) is referred to as 'Atkinson' after the name of the family in which it was first seen. The *e* and *f* zones are present but are rather weaker than usual, a relatively intense zone with the mobility of *g* is also present, and there are two further distinct components moving even more rapidly than *g*. This pattern of components has been seen together with the *a* and *c* components typical of PGM 1, the *b* and *d* components typical of PGM 2, and also with *a, b, c,* and *d* components typical of PGM 2-1. The pedigree of the family in which it was originally found is shown in Figure 10. The phenotype occurs in individuals in three successive generations and appears to be determined by an uncommon allele for which these individuals are

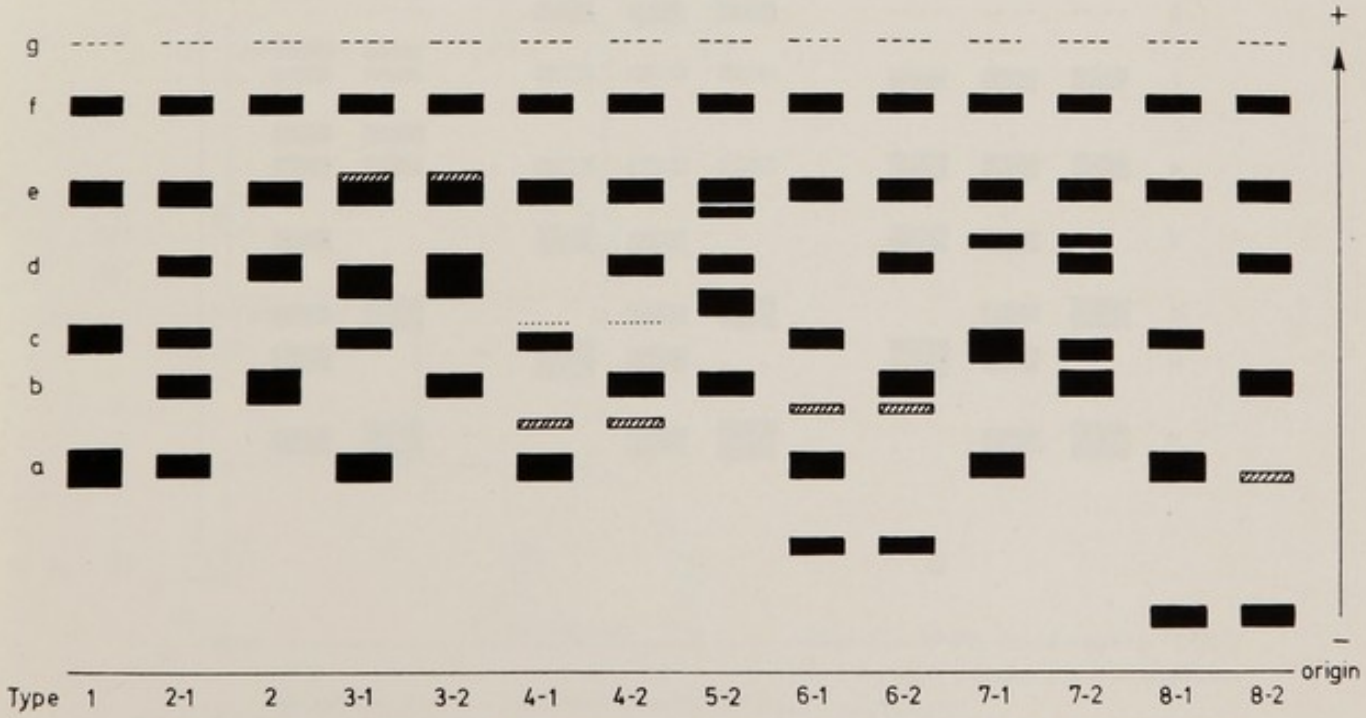


FIG. 8. Diagram showing PGM components observed in hemolysates of a number of rare phenotypes.

heterozygous. If so, it follows from the pedigree that this allele segregates independently of PGM_1^1 and PGM_1^2, and occurs at a separate and not closely linked locus. The allele appears to have been transmitted by the doubly heterozygous father II_1, to one but not the other of his two non-identical twin daughters, both of whom must have received the PGM_1^2 gene from their mother. The common allele at this second locus has been designated PGM_2^1, and the unusual allele, for which individuals showing the 'Atkinson' phenotype are heterozygous, PGM_2^2. The 'Atkinson' phenotype has been found to have an incidence in different Negro populations of 1-5 per cent. It has not been seen at all in the several thousand European individuals examined. A number of other families segregating for this phenotype have been studied. They confirm the hypothesis that individuals exhibiting it are heterozygous, but have provided no further information about the linkage relations with PGM_1.

The electrophoretic pattern in the 'Atkinson' phenotype can be most simply explained by supposing that the unusual allele PGM_2^2, causes a structural alteration of *e*, *f*, and *g* which increases their net negative charge, so that the altered component *e* now moves as rapidly as *g*, and the mobilities of the altered *f* and *g* components are increased correspondingly. Since the 'Atkinson' phenotype represents the heterozygote $PGM_2^1PGM_2^2$, both the usual and the altered *e*, *f*, and *g* components may be expected to be present in roughly equal amounts, and this would give the isoenzyme pattern observed.

Another variant affecting the *e*, *f*, and *g* components (the "Palmer" pheno-

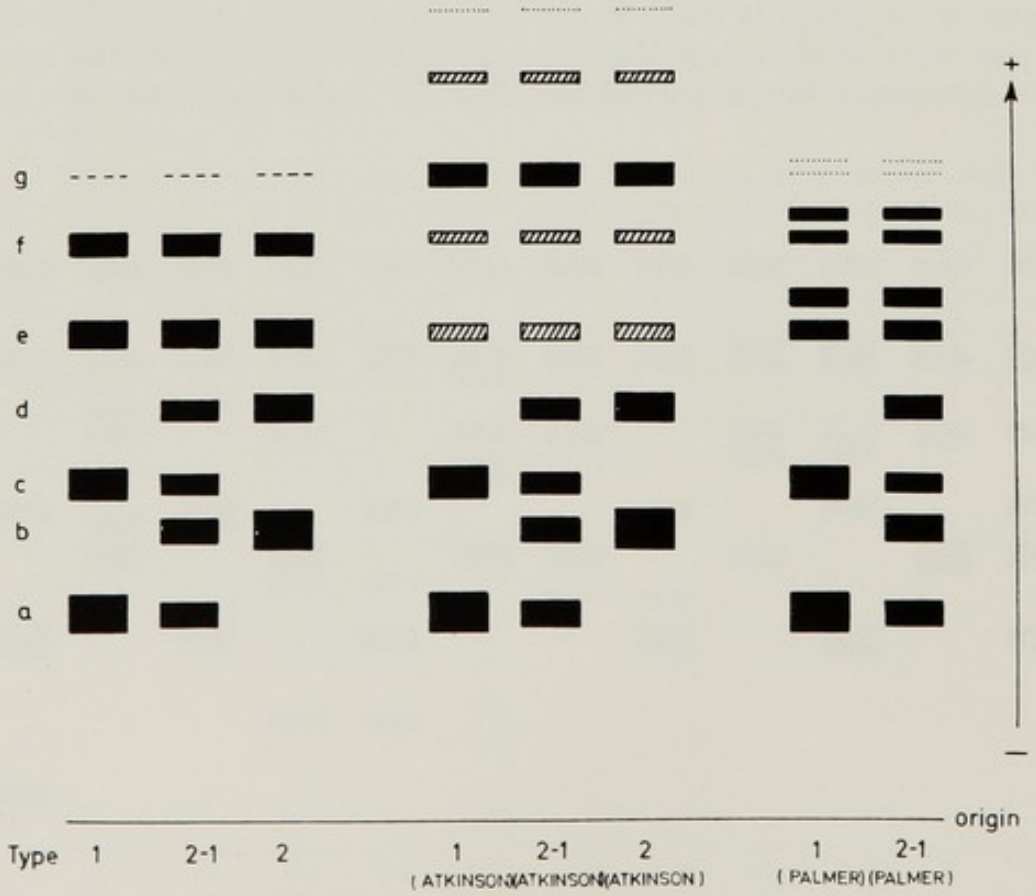


FIG. 9. Diagram showing PGM components observed in hemolysates of "Atkinson" and "Palmer" phenotypes.

type) is shown in Figure 9. This is very rare. It is thought to be due to a third allele at the PGM_2 locus, which causes a moderate increase in the mobility of *e*, *f*, and *g* so that each of the altered components lies just ahead of their usual counterparts.

The PGM isoenzymes and the common types, PGM 1, PGM 2-1 and PGM 2, seen in red cells, may also be readily demonstrated in a variety of other tissues. Those examined include liver, kidney, muscle, brain, skin, leucocytes, and placenta. They can also be demonstrated in cells grown in vitro in tissue culture. In many of these tissues the total phosphoglucomutase activity is considerably greater than in red cells, and this has enabled us to identify yet a third group of phosphoglucomutase isoenzymes not normally seen in erythrocytes. This group of components has been mainly studied in placenta though they have also been demonstrated in various other tissues. These isoenzymes are relatively weak and account for only a small proportion of the total phosphoglucomutase activity present. They move electrophoretically even faster than the *e*, *f*, and *g* components. Person-to-person differences in pattern occur and have been shown to be genetically-determined. The genetics has been studied by sib pair analysis of the types found in pairs of placentae derived from dizygotic twin births. Although this work is still in its early stages, it has been possible to demonstrate that at least two common alleles affect these components; that their frequencies are different in European and in Negro populations; and that they occur at a genetic locus that is separate and not closely linked to PGM_1. The linkage relations with PGM_2 have not yet been studied.

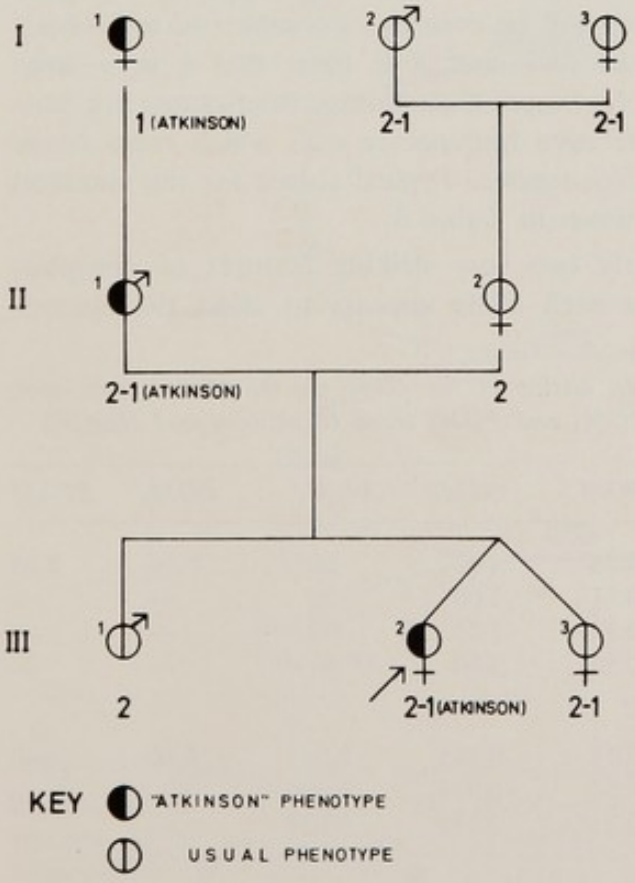


FIG. 10. Pedigree of family (MRC 225) showing segregation of "Atkinson" phenotype.

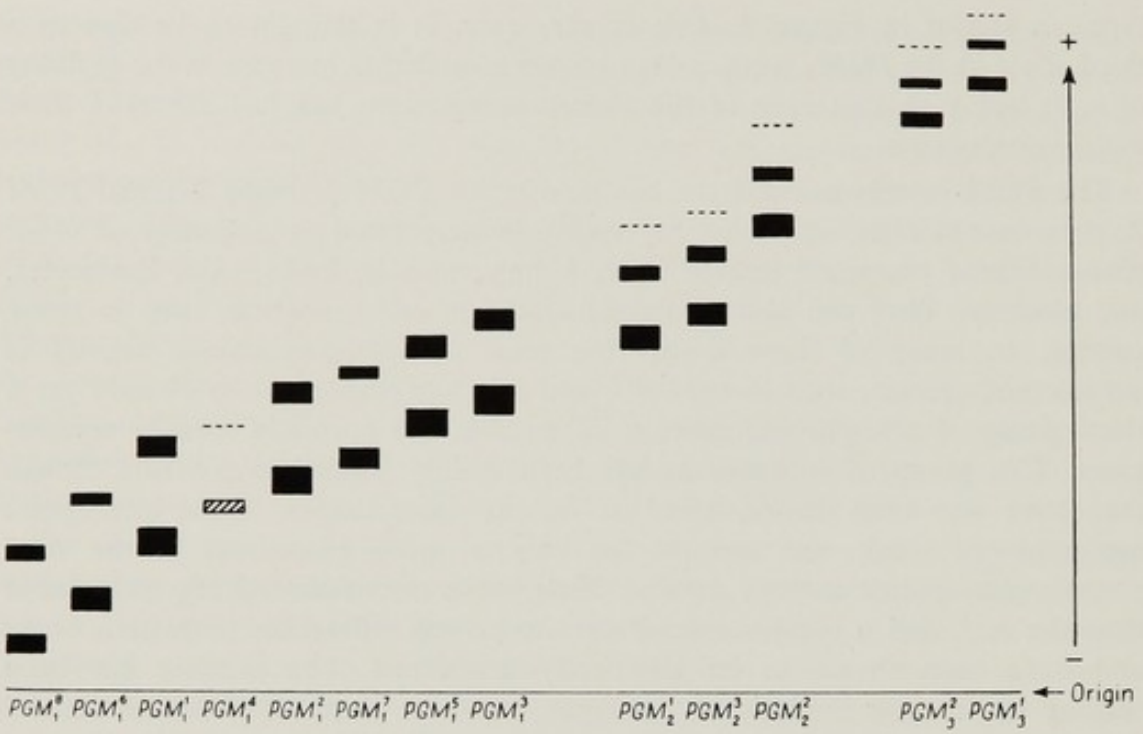


FIG. 11. Diagram showing phosphoglucomutase isoenzymes determined by different alleles at the three loci, PGM_1, PGM_2, and PGM_3.

Thus it appears that at least three different genetic loci determine phosphoglucomutase. The isoenzymes thought to be attributable to different alleles at each of these three loci are illustrated diagrammatically in Figure 11. An individual homozygous at all three loci shows at least eight distinct phosphoglucomutase isoenzymes. The patterns will be even more complex in individuals heterozygous at one or more of the loci, and it is clear that a very large number of different combinations of phosphoglucomutase isoenzymes are possible in different individuals. The relative frequencies with which these occur will of course depend on the gene frequencies. Typical values for the common alleles in various populations are shown in Table 3.

Figure 11 illustrates rather clearly two very striking features of phosphoglucomutase variations. One is that each allele appears to affect the electro-

TABLE 3. *Gene frequencies of common alleles at the three phosphoglucomutase loci; PGM_1, PGM_2, and PGM_3 (Data on PGM_1 and PGM_2 from Hopkinson and Harris[7])*

Population	PGM_1^1	PGM_1^2	PGM_2^1	PGM_2^2	PGM_3^1	PGM_3^2
'English'	0.76	0.24	1.00	.00	0.74	0.26
Greek	0.69	0.31	1.00	.00	—	—
Iraqi Jews	0.67	0.33	1.00	.00	—	—
Turkish Cypriot	0.70	0.30	1.00	.00	—	—
Negroes:						
a) Living in England	0.79	0.21	0.985	0.015	0.40	0.60
b) Yoruba, Nigeria	0.76	0.24	0.994	0.006	—	—
c) Bantu, S. Africa	0.79	0.21	0.975	0.025	—	—

phoretic mobilities of the two or three isoenzymes, determined by the particular locus, to the same extent. The other is the apparently remarkable similarity in the pattern of isoenzymes produced by the separate loci. This suggests that there may well be close structural homologies between the three sets of isoenzymes.

Preliminary studies have been carried out involving separations of the enzyme by zone electrophoresis in pevikon blocks (Superfosfat Fabriks A-B, Stockholm), by column chromatography on DEAE and CM cellulose, and by gel filtration in Sephadex G 100. The results so far suggest that the various PGM isoenzymes differ from one another in net molecular charge, but probably do not differ significantly in molecular size.

Adenylate Kinase

Adenylate kinase (AK) catalyses the reversible reaction

$$2\ ADP \rightleftharpoons ATP + AMP$$

The enzyme is widely distributed in human tissues but occurs with particularly high levels of activity in muscle and in red cells.

Two procedures for the detection of this enzyme after starch gel electrophoresis have been developed.[2] One uses ADP as substrate. The reaction

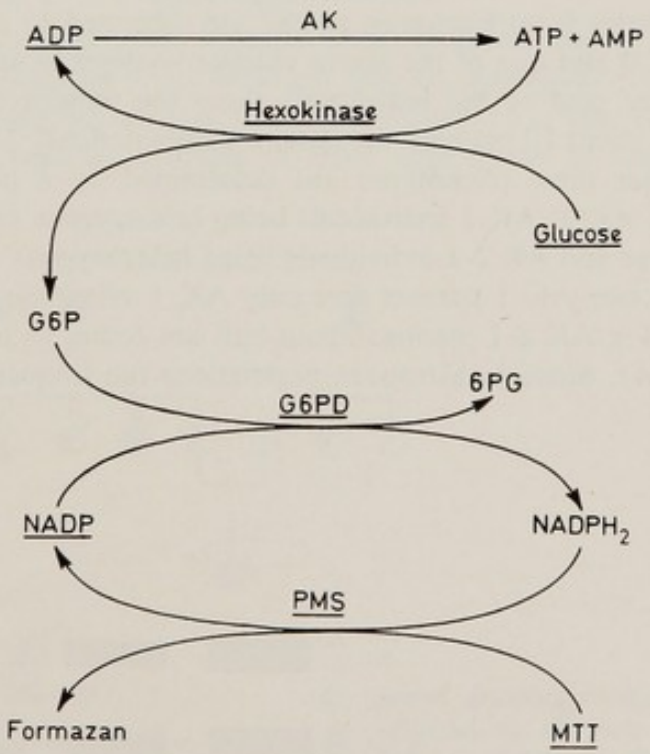


FIG. 12. Reaction system used in the detection of adenylate kinase after starch gel electrophoresis (first method). The underlined components are contained in the reaction mixture. (ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; other abbreviations as in Figure 6.)

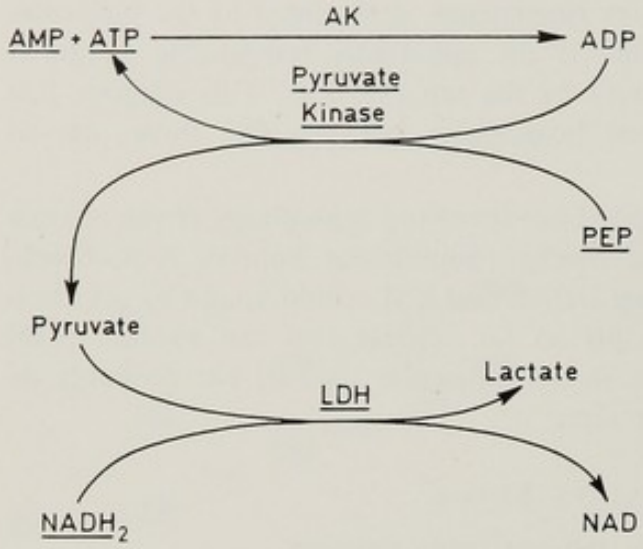


FIG. 13. Reaction system used in the detection of adenylate kinase after starch gel electrophoresis (second method). (PEP, phosphoenol pyruvate; LDH lactate dehydrogenase; other abbreviations as in Figures 6 and 12.)

sequence involved is shown in Figure 12, the underlined components being included in the reaction mixture. The system leads to the deposition of blue formazan at the sites of enzyme activity. The second procedure uses ATP and AMP as substrates. The reaction system is shown in Figure 13. The sites of AK activity are seen as dark bands (due to NAD) on a fluorescent background (due to NADH) when the gels are viewed in ultraviolet light. Both systems give essentially the same patterns. However, the first is rather more sensitive and is also more convenient, so that it is the one generally used in practice.

When hemolysates from European people are subjected to starch gel electrophoresis at pH 7.0 and one of the above reaction systems is applied, it is found that about 90 per cent of the individuals show the pattern designated AK 1 (Figure 14) and about 10 per cent the pattern designated AK 2-1. Family studies make it clear that these phenotypes are determined by a pair of autosomal alleles (AK^1 and AK^2), AK 1 individuals being homozygous (AK^1AK^1) for the commoner of these and AK 2-1 individuals being heterozygous (AK^1AK^2). Thus matings between two AK 1 parents give only AK 1 offspring, while among the children of AK 1 x AK 2-1 matings about half are found to be AK 1 and half AK 2-1 (Table 4). Since in European populations the frequency of AK 2-1 is



FIG. 14. Diagram showing isoenzyme patterns observed in hemolysates of types AK 1, AK 2-1, and AK 2. The dotted areas probably include two separate but rather weak and poorly defined zones.

TABLE 4. *Distribution of adenylate kinase (AK) types in 75 families in which at least one parent was type AK 2-1*

Parents	Number of matings	Children			Totals
		1	2-1	2	
1 × 2-1	69	97	91	—	188
2-1 × 2-1	6	3	6	4	13
	75	100	97	4	201

about 0.1, the incidence of the homozygote for the less common allele (AK^2AK^2) should be about 1 in 400. A phenotype designated AK 2 and shown in Figure 14 has, in fact, been found with about this frequency, and appears from family studies, representative examples of which are shown in Figure 15, to represent this homozygote. In Negro populations, the AK^2 allele is very infrequent, and nearly all individuals have the AK 1 phenotype[5] (J. E. Bowman & P. E. Carson, personal communication).

Both AK 1 and AK 2 show at least three zones of activity. The slowest of these is by far the most active. The fast zone, indicated in Figure 14, is much weaker than the others. It tends to be rather diffuse and appears to include two separate but rather poorly-defined components. The pattern of isoenzymes seen in AK 2-1 can be regarded as representing a mixture of the isoenzymes present in AK 1 and AK 2, in roughly equal amounts.

Two rare phenotypes have recently been identified. One of them (designated AK 3-1) was found by J. E. Bowman and his colleagues (personal communication). The other designated AK 4-1 (Figure 16), was found in our own laboratory, and another example of what appears to be the same phenotype

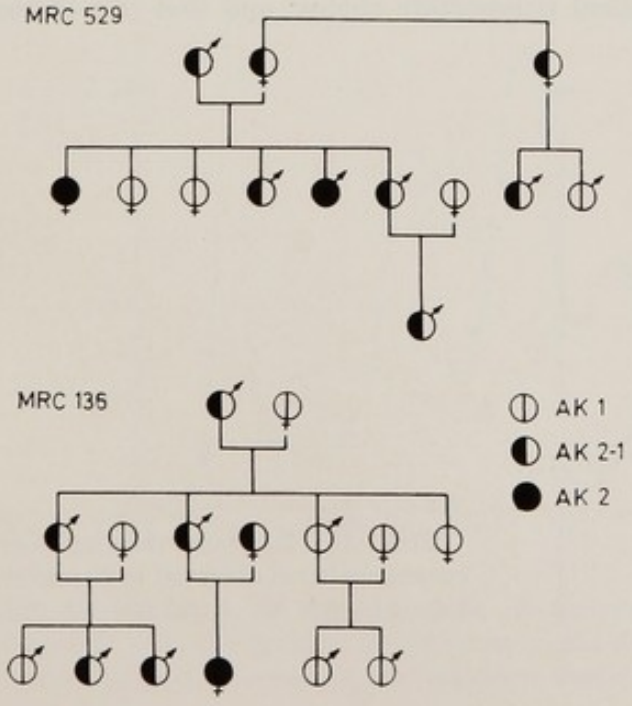


FIG. 15. Pedigree of families (MRC 136 and MRC 529) showing segregation of AK 1, AK 2-1, and AK 2.

has been found by Dr. E. Giblett (personal communication). Further examples of AK 4-1 were found in our family, and the segregation pattern suggests that the individuals showing this phenotype are heterozygous for a rare allele AK^4. This apparently determines a series of adenylate kinase components, each of which migrates slightly more slowly than the homologous components determined by AK^1.

Studies of the total adenylate kinase activity in red cells of AK 1 and AK 2-1 individuals have so far shown no marked difference between the two phenotypes. The two phenotypes also behave similarly on gel filtration, using Sephadex G 100. In both cases the activity emerges from the column as a single peak, in a position which, by reference to the elution positions of added markers of known molecular sizes, corresponds to a molecular weight of about 24,000, (Figure 17). This is similar to the value reported for rabbit muscle adenylate kinase and which was obtained by a quite different method.[13] The elution peak was shown on rerunning in starch gel to contain the several isoenzymes, so they are all likely to be very similar if not identical in molecular size.

Discussion

An important general point that emerges from this work is that a single allele at a particular locus may apparently affect two or more electrophoretically-distinct components with a given enzyme activity. This appears to be the case for the alleles at each of the five loci which have been found to affect the enzymes discussed here, and examples of the same sort of thing involving other enzymes are also known. Indeed, it seems likely that it is a very common phenomenon.

The effect may, in principle, arise in several different ways, and there is no reason to think that it will have the same molecular basis in all cases. It is perhaps useful, therefore, as a guide to further studies to consider some of the possible explanations.

One possibility is that each member of a set of multiple components (isoenzymes) contains two non-identical polypeptide chains; and that one of these

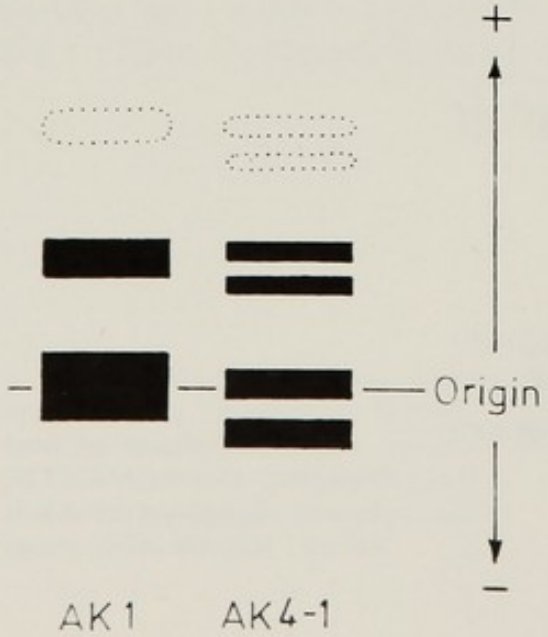


FIG. 16. Diagram showing isoenzyme patterns observed in haemolysates of types AK 1 and AK 4-1.

is common to all the isoenzymes of the set, whereas the other is peculiar to the particular isoenzyme. Each isoenzyme will have its own unique composition. Nevertheless they will all be affected by a mutation at the locus determining the amino acid sequence of the common polypeptide. More complex situations involving more than two polypeptides can be easily envisaged. This is in essence the situation known to occur in hemoglobins A, A_2, and F, and also in lactate dehydrogenase.

A second possibility is that the several isoenzymes determined by a given locus differ in their properties because of some secondary change that occurs subsequent to the primary synthesis of the polypeptide chains. They may, for example, differ by an acetyl or a phosphate group, or in the nature of an attached carbohydrate moiety. They would, however, all be affected by an alteration in the primary polypeptide sequence.

A third possibility is that the multiple enzyme components affected by a single locus differ from one another in molecular size. They may, for example, represent monomers, dimers, trimers, etc. of a single polypeptide chain. A structural alteration in this basic subunit would result in a simultaneous alteration in the several components.

A fourth possibility is that the several isoenzymes, determined by a given locus, have exactly the same primary structures, but differ in their tertiary or quaternary conformations. The existence of such stable conformational isomers

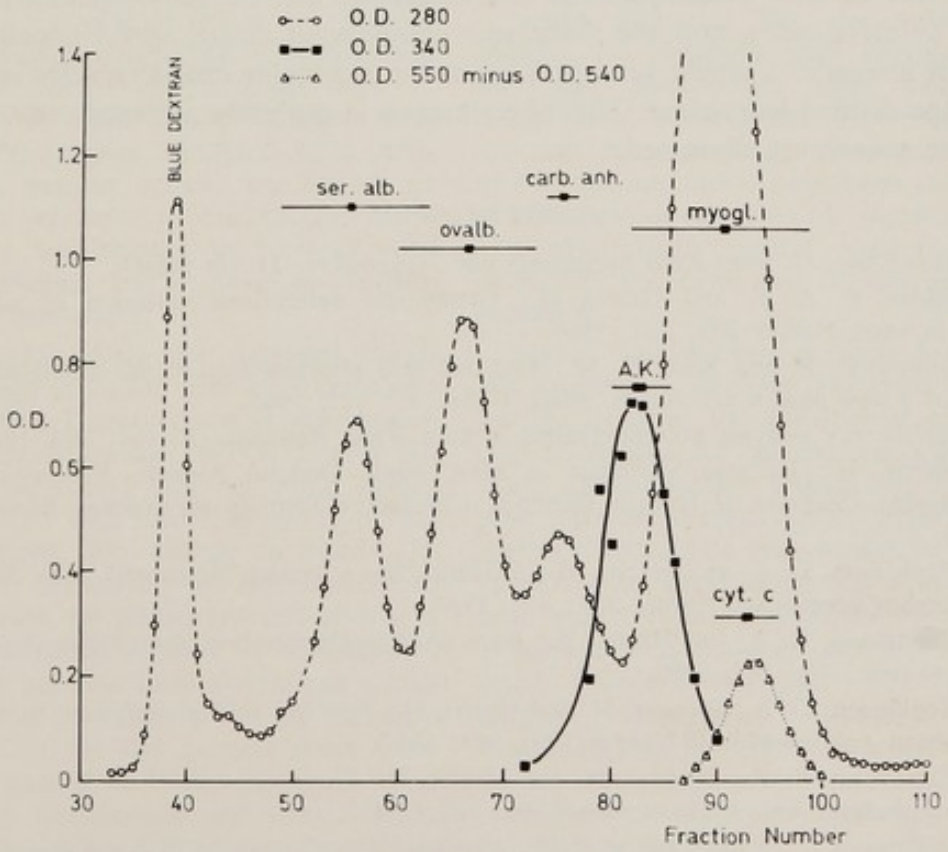


FIG. 17. Diagram of elution pattern of adenylate kinase and various markers on Sephadex G 100. The position of the markers was determined by OD determinations and by starch gel electrophoresis of the individual fractions.

has recently been postulated[11] account for the multiple isoenzymes seen in mitochondrial malate dehydrogenase, and it may well be a very general phenomenon.

A fifth possibility is that an enzyme may be more or less firmly bound to other quite different molecules (which could be large or small). A single enzyme species would then appear to exist in multiple electrophoretically-distinct forms, all of which are altered by a mutation affecting the structure of the enzyme itself.

A further possibility raised by recent studies on certain animal hemoglobins[1,10,14] is that a single allele may give rise to multiple structurally-distinct polypeptide chains with specific differences in aminoacid sequence, because of ambiguity in the translation of one or more messenger RNA codons.

Unfortunately, this list probably does not exhaust all the possibilities. Although in the particular enzymes discussed here the available data make some of these possibilities (e.g., molecular size differences) seem less likely than others, it is apparent that detailed structural studies will be required before the situation is clarified.

Summary

Genetic and biochemical studies on three human red cell enzymes, acid phosphatase, phosphoglucomutase, and adenylate kinase, are reviewed. Each of these enzymes is polymorphic in human populations, and clear-cut person-to-person differences in their electrophoretic characteristics can be demonstrated readily. In all of these cases, multiple isoenzyme components occur, and it appears that in each instance, a single gene may alter the molecular characteristics of more than one distinct isoenzyme. This phenomenon is probably a general one and its possible causes are discussed.

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DISCUSSION

JAFFÉ, CHAIRMAN, NEW YORK: Thank you, Dr. Harris.

CARSON, CHICAGO: I would like to add something about adenylate kinase and to ask Dr. Harris a question about multiple bands. In Chicago during comparison of the incidence of AK 2-1 in Caucasians and American Negroes (performed by Dr. Bowman), the incidence in Caucasians, which Dr. Harris found in London, was confirmed. Dr. Harris also found a low incidence of AK 2-1 in the Yoruba Nigerians, I believe. We went to Africa last summer and, in randomly-obtained blood samples from about 800 healthy unrelated West Africans of various tribes, we found no incidence whatever of the AK 2-1 form. This has not proven to be true with East Africans, but I have no further data on that point.

The other thing that interests me is the change in bands with heat denaturation. When I worked on 6-phosphogluconic dehydrogenase (6-PGD) with Dr. Ajmar, we found that we could inactivate 6-PGD with heat, but not to a very large extent. No change in bands occurred in the starch gel electrophoresis. Then, when we added red cell ghosts to our incubations, at first in hemolysates, but later purified hemoglobin-free ghosts to purified fractions from gel filtration for 6-PGD, in the normal most common variant of 6-PGD, the front band gradually disappeared and moved backwards, providing TPN was also added. This very clear-cut difference occurs only in the presence of the red cell membranes and TPN. We have never been able to produce this effect with any other combination. Even in the 6-PGD variant of other types that are more labile to heat, this kind of change does not occur unless we have this combination. Actually, we have not yet been able to go further into what factor or factors might be present in these membranes to cause this. Do you get shifts in bands of phosphatases, for example, by altering the preparation of your samples?

HARRIS, LONDON: The electrophoretic changes we observed after heating red cell acid phosphatase were obtained using whole hemolysates. However, we are now doing a study on preparations of the enzyme which have been through a considerable degree of purification, and preliminary results indicate that essentially the same sort of changes are occurring.

JAFFÉ: Dr. Harris, perhaps I might ask a question. Is there any evidence of linkage between the phenotypic expression of a gene for an enzyme and any other characteristic? Eventually, I suppose, one could map out a "fingerprint" for each individual that would be more specific than the fingerprints which are used now for purposes of identification.

HARRIS: Yes, certainly one can "fingerprint" people in this sense. The various enzyme polymorphisms appear to occur essentially independently of each other. So a very large number of different combinations of enzyme types may occur in different individuals. It is not difficult to show (see reference 4, preceding presentation) by simply taking the enzyme polymorphisms that have already

been discovered, that the probability that any two randomly-selected individuals will have the same combination of enzyme phenotypes is very low. It seems almost certain that many other enzyme polymorphisms remain to be discovered, and one may plausibly imagine, therefore, that in the last analysis every individual will be found to have a unique combination of enzyme types.

PRINS, AMSTERDAM: Is it possible that we could use polymorphisms to study the function of, for instance, phosphatase in the red cell? Is there any reflection of the differences in red cell enzyme activity, for instance, in the phosphatase activity in the concentration of red cell metabolites, red cell metabolism, or increased hemolysis?

HARRIS: I agree entirely. I think that this field is quite wide open. It would certainly be worthwhile, taking these sets of red cells, differentiated by one or another of these systems, and examining the range of concentrations of different metabolites, the physiology of the red cells, their fragilities, and so on.

BEUTLER, DUARTE: I would like to ask Dr. Harris two questions. One concerns relationship between acid phosphatase phenotypes and activity on the one hand, and G-6-PD deficiency on the other. As you know, Oski and Diamond* reported a deficiency of red cell acid phosphatase in G-6-PD deficient patients. Subsequently, a study by Sinascalco suggested that this was really related to the particular acid phosphatase phenotypes that the G-6-PD-deficient patients had. I wonder, first of all, whether you have had any information on this point.

My second question concerns the heat stability of the acid phosphatase types. I notice from your graph that the type A was unstable, but that AC was stable. Do these enzymes behave independently in the heterozygotes? That is, does a heterozygote hemolysate behave just like a mixture of the hemolysates of the two homozygotes, or is there evidence of interaction of the polypeptides?

HARRIS: In regard to the last question, all of the evidence that we have is easily explained on the assumption that heterozygotes behave as a mixture, and that we don't get interactions. I think more work needs to be done, however, because of these curious changes in mobilities that you can see in the course of denaturation. Until we know what's happening there, we can't be absolutely sure.

In regard to the other question, Dr. Oski and his colleagues showed that on the average, acid phosphatase levels were lower in individuals with the Caucasian type of G-6-PD deficiency than in normal individuals, but not in the Negroes with G-6-PD deficiency. Subsequently, this work was confirmed by Dr. Kattamis† in Greece, but was not confirmed by Professor Sinascalo and his group in Sardinia. I don't know the reason for the discrepancy between the data of the Kattamis group and the Sardinian group because both seem to be working on the same type of G-6-PD deficiency. As to the cause of it, I think, that is probably irrelevant to the acid phosphate polymorphism. The point is that red cell acid phosphatase does seem to have free SH groups upon which, to some extent, it is dependent for its activity. These groups are probably maintained in the red cell by the presence of GSH. In the Caucasian type of G-6-PD deficiency, you get marked increase in oxidized glutathione. I think the effect is some way connected with this. As far as I understand it from Dr. Kattamis's results, the effect occurs in the same way with all types of red cell acid phosphatases.

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*Oski, F. A., Shahidi, N. T., and Diamond, L. K.: Erythrocyte acid phosphomonasterase and glucose-6-phosphate dehydrogenase deficiency in Caucasians. Science 139: 409-410, 1963.

†Kattamis, Ch. A. and Zannos-Mariohea, L.: Acid phosphatase activity in g-6-pd deficient Greeks. Proc. 10th Cong. Int. Soc. Blood Transf. Stockholm 1964, pp. 603-606, 1965.

Acatalasemia in Japan

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ACATALASEMIA, a rare congenital abnormality characterized by an apparent lack of catalase enzyme, was first discovered by Takahara in 1947. The lack of catalase activity in these cases was initially noted in whole blood,[22] but subsequent studies of the tissue of other organs such as the nasal and oral cavities, pharynx, bone marrow[20] and liver[10] have revealed a similar absence of activity for this enzyme. Some scholars suggest that "acatalasia"[2,28] is a better name. However, we have not yet had a single autopsy case to prove definitely that catalase is lacking in all the organs of the body and therefore, for the time being, we prefer the term "acatalasemia."

Toward the end of 1946, an 11-year-old, pale-looking girl came to our Ear, Nose and Throat Outpatient Clinic with high fever and complained of severe ulcers in her mouth. On examination, I found a peculiar type of gangrene starting around the neck of one of the molars of the right upper jaw and extending into the maxillary sinus and the nasal cavity. There was a foul-smelling and fetid granulation in her nose. Judging her condition to be very serious, I decided to remove the diseased part completely. After the radical operation, I poured hydrogen peroxide on the wound for cleansing.

To my great surprise, the blood coming in contact with the hydrogen peroxide immediately turned a brownish-black color and the usual bubbles did not appear. I thought that by mistake the nurse might have handed me a bottle of silver nitrate, commonly used in the ear, nose and throat clinics. So I at once poured saline solution into the wound to neutralize it. I was much relieved to know that nothing serious had happened. Then I applied hydrogen peroxide several times but obtained the same results. Thus I came to believe that there was some abnormality in the blood of this patient.

She was the second child of seven siblings. Four of them had mouth gangrene of varying degrees, and their blood all reacted in the same manner as described. The other three had no oral lesions and their blood reacted to hydrogen peroxide in the normal fashion. Thus I presumed that there must be some close relationship between the blackening of the blood and the oral disease. Thereupon, I decided to study the blood samples of this patient and her siblings. It was demonstrated by a series of qualitative and quantitative analyses of the blood that catalase was lacking in the erythrocytes of these subjects.

To date, 77 cases of acatalasemia from 39 Japanese families and 3 cases from a pure Korean family have been found in Japan. Recently, 11 Caucasian

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TABLE 1. *Number of acatalasemic cases that have been reported*

	No. of family	Male	Female	Total
JAPAN	39	38	39	77
KOREA	1	1	2	3
SWISS	3	3	3* 5	11
ISRAEL	1	1	0	1
	44	43	3* 46	92

(up to 1966) * not described

cases from three Swiss families were reported by Aebi[1] and one from Israel was reported by Szeinberg.[19] The total of 92 cases are summarized in Table 1. It is noteworthy that most of the acatalasemics are the offsprings of consanguineous marriages and that acatalasemia is sporadic in distribution and not restricted to any limited area of Japan.

The following three factors are interesting and worthy of further discussion: (1) Oral disease specific to acatalasemia, (2) Genetic aspects, and (3) Biochemical and immunochemical problems.

Oral Disease Specific to Acatalasemia

In reviewing the reported cases statistically,[25] it was found that about one-half of the acatalasemic individuals have a peculiar progressive gangrene of the mouth now known as Takahara's disease. These patients were mostly under the age of 10 years, some even in their infancy. Apparently the gangrene seldom occurs after puberty. This disease usually originates as a small painful ulcer in the crevices around the neck of a tooth or sometimes from the tonsillar lacunae. In severe cases, inflammation develops into a far-advanced gangrene of the maxillae (as in the first case) or may develop in the soft oral

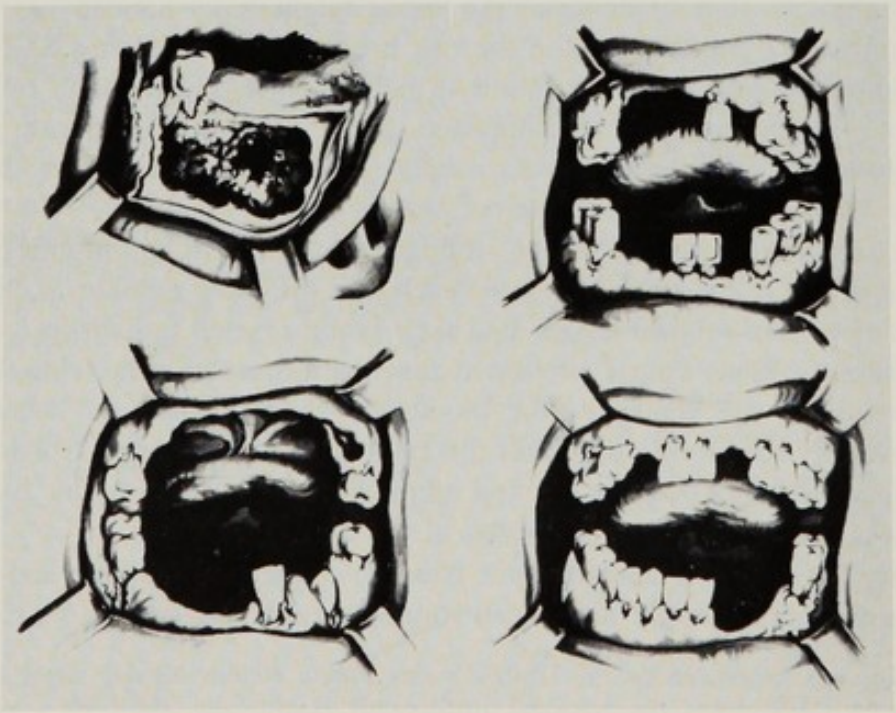


FIG. 1. Oral lesions found in patients with acatalasemia.

tissues. These findings are similar to textbook descriptions of so-called Noma. After healing, there is a remarkable recession of gum, which is characteristic of this disease, or there may be scarring, which causes difficulty in opening the mouth. Various examples of these oral lesions are shown in Figure 1. Pathogenesis of this type of oral ulceration is attributed mainly to the lack of catalase in the blood.[4] That is, bacteria in the crevices of the tooth or tonsillar lacunae produce hydrogen peroxide during their metabolic proliferation, and, as there is no catalase in the patient's tissues to decompose the hydrogen peroxide so produced, hemoglobin is decomposed by oxidation by the hydrogen peroxide, thus causing ulceration and decay of the oral tissues. An early, radical removal of diseased teeth or tonsils and the application of antibiotics are very effective in checking the progress of the disease. The healing rate of the wound, however, does not differ from the healing rate in otherwise normal individuals.

The other half of the acatalasemic individuals who do not develop oral lesions are designated as having asymptomatic acatalasemia.

Genetic Aspects

In close collaboration with Atomic Bomb Casualty Commission groups in Hiroshima and Nagasaki, we tested the catalase activity of the blood of acatalasemic family members and found that there are two types of individuals in such families. In one type, catalase activity is normal and in the other the activity is clearly intermediate between normal and acatalasemic values. This intermediate group was designated as "hypocatalasemia," Figure 2 shows the distribution of catalase values for the members of 13 acatalasemic families. As there is no overlapping of catalase values between the hypocatalasemic,

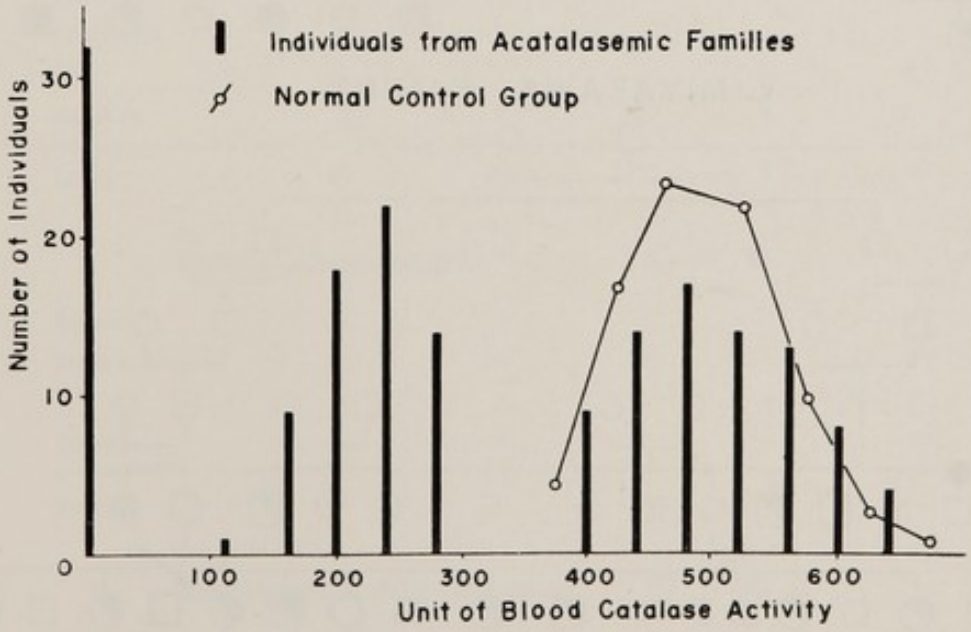


FIG. 2. Blood catalase activity of 175 siblings and 13 acatalasemic families compared with the distribution curve of enzyme activity of 273 normal subjects. The black column on the left represents acatalasemic subjects, the five columns in the center, hypocatalasemic subjects, and the columns on the right represent normal individuals in these families with catalase values within the control distribution curve.

normal, and acatalasemic groups, we can readily distinguish hypocatalasemics from normal and acatalasemics individuals by biochemical assays.[13]

By plotting the values for the hypocatalasemics in relation to their respective family tree, we verified four combinations of mating following the Mendelian recessive law in the offspring (Figure 3). These findings suggest that this acatalasemia is of incomplete recessive monogenetic inheritance and that hypocatalasemia is heterozygous and is a carrier state for the acatalasemic gene.[21] As is obvious from Table 2, the number of hypocatalasemic individuals is relatively large as compared with the number of normal individuals in these families. Therefore, when consanguineous marriage takes place in such an acatalasemic family, there is a greater chance of mating acatalasemic with hypocatalasemic or hypocatalasemic with hypocatalasemic, rendering it possible to produce an acatalasemic state in the offspring. There is also a possibility of producing acatalasemia by the marriage between two hypocatalasemics even

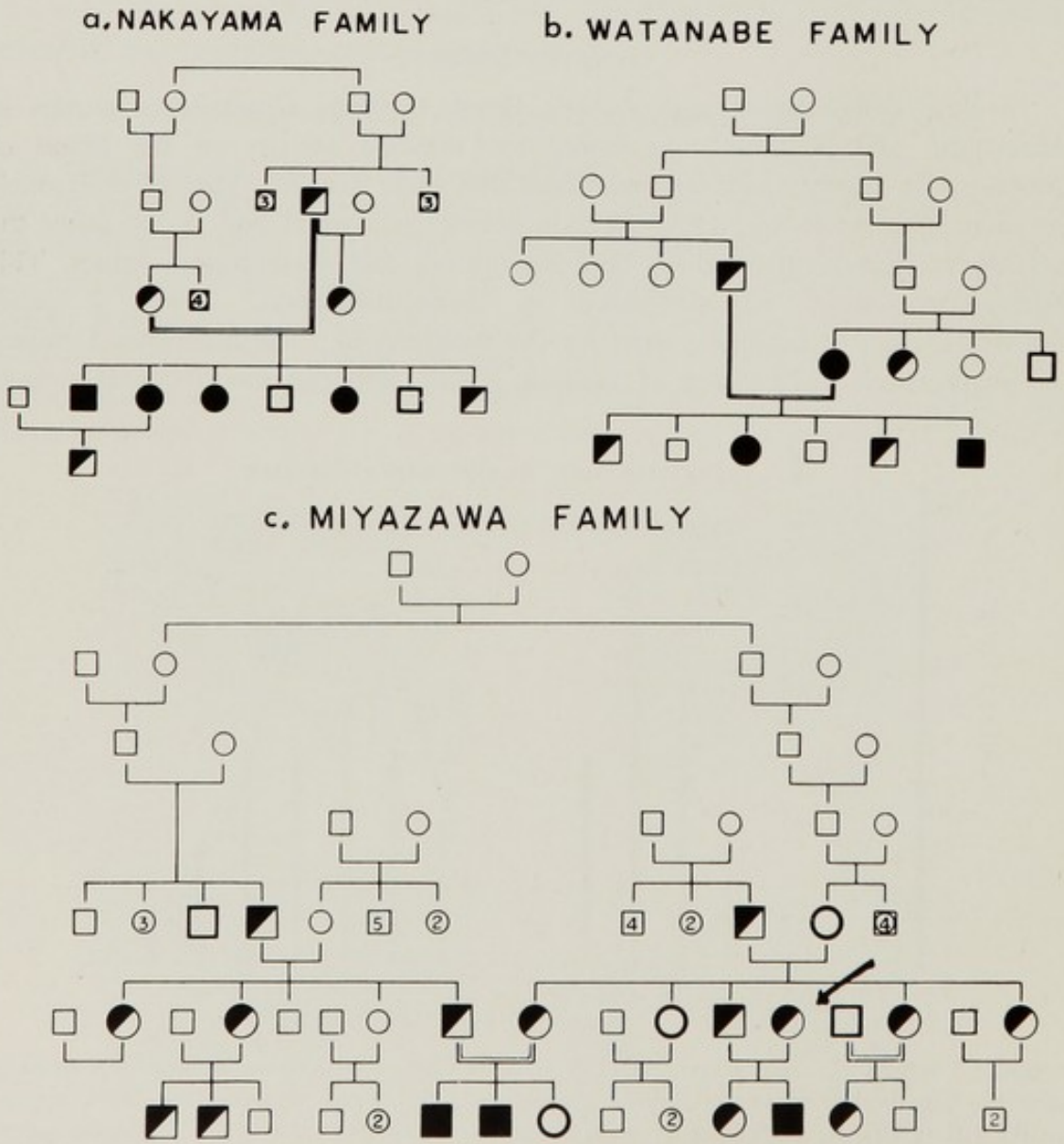


FIG. 3. Pedigrees of three families with acatalasemia. The arrow in Figure 3c indicates the mating of two apparently unrelated hypocatalasemic subjects.

though they may not be related consanguineously, as indicated by arrow in Figure 3c.

Hypocatalasemic individuals have catalase in their blood, though its amount is only one-half that in the blood of the normal person. Accordingly, they have no particular disease to speak of and live normal lives. So, I think the acatalasemic gene of these individuals has been handed down from one generation to the next over a long period of time. Therefore, if we can check closely the frequency of hypocatalasemia over wide geographic areas, we shall be able to determine gene flow or the flow of races. For the past seven years we have checked the frequency of hypocatalasemia in about 83,000 Asian individuals; Japanese and Koreans residing in Japan, residents of the Ryuku Islands, and Chinese living in Taiwan—both those living in these places for generations and those who migrated there recently during the war. The distribution and frequency of hypocatalasemia in Asia is shown in Table 3. The frequency of hypocataslasemia has been found to be the highest among Koreans, followed by the Chinese of Northern China, Central China, Southern China, and those Chinese in Taiwan residing there for many generations. (Figures 4 and 5) The lowest

TABLE 2. *Genetic combinations involved in transmission of acatalasemia*

Family	Siblings	Acat.	Hypo.	Normal	Not tested
Hypocatalasemia × Hypocatalasemia ◐ × ◐					
Nakayama	7	4	1	2	0
Miyazawa	3	2	0	1	0
Nagase	5	2	1	1	1
Kanzaki	2	1	1	0	0
Hayashi	9	2	0	1	6
Kobayashi	2	1	1	0	0
Ito	7	2	2	1	2
Inadera	3	2	1	0	0
Total	38	16	7	6	9
Normal × Hypocatalasemia ○ × ◐					
Nagase	6	0	3	3	0
Kobayashi a	5	0	4	1	0
b	2	0	1	0	1
Inadera	6	0	2	1	3
Total	19	0	10	5	4
Acatalasemia × Hypocatalasemia ● × ◐					
Watanabe	6	2	2	0	2
Acatalasemia × Normal ● × ○					
Nakayama	1	0	1	0	0

TABLE 3. *Distribution and frequency of hypocatalasemia in Far-Eastern populations*

Race	No. exam.	No. of hypocat.	Freqency (%)		Year
FREQUENCY OF HYPOCATALASEMIA					
Japanese	46878	116	0.25		1960-66
Ryukyan					
Miyako	10083	1	0.01	0.007	1965
Okinawa	3297	0	0		1965-66
Korean (in Jap.)	922	11	1.29		1960-62
Chinese (in Taiwan)	20565	68	0.33		1966
Aborigines	792	1	0.13		1966
(Tayal	84	1)			
FREQUENCY BY ORIGIN					
Chinese emigrant					
North China	461	4	0.87		
Middle China	1630	7	0.43		
South China	3863	13	0.34		
Taiwan	11674	36	0.31		

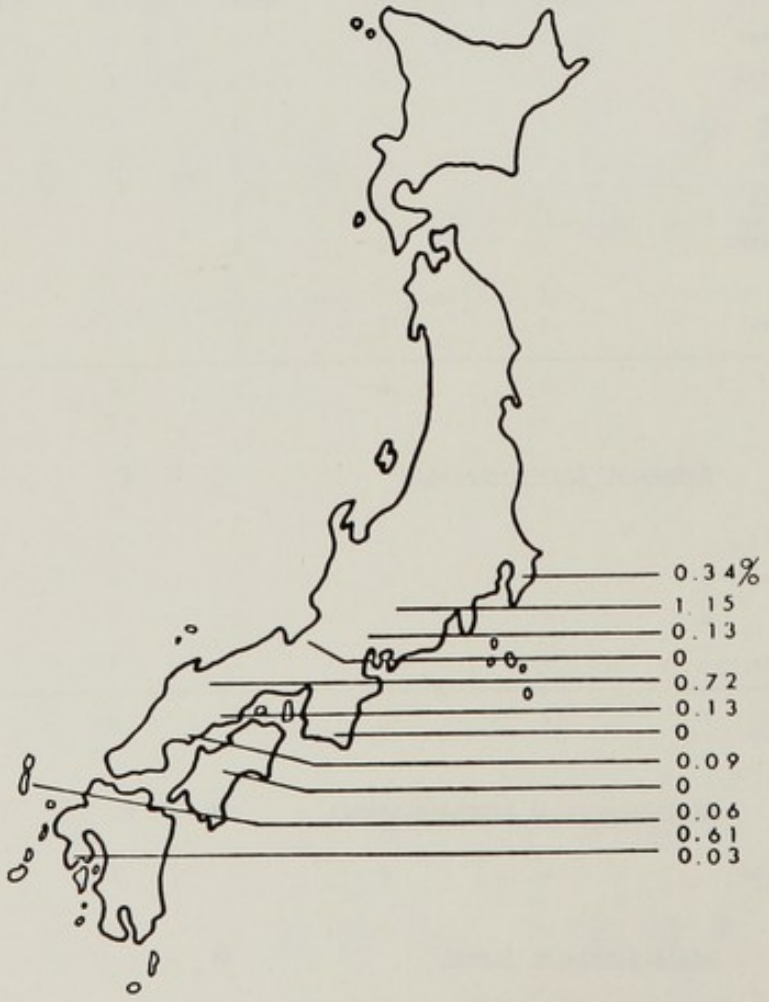


FIG. 4. The distribution of hypocatalasemia in various portions of Japan.

rate is found in the Okinawans, namely, only one hypocatalasemic person was found in about 13,380 persons that we examined. I believe, therefore, that Okinawans have had little contact with other peoples in Asia as far as their acatalasemic gene is concerned.

Biochemical and Immunochemical Aspects

Acatalasemic blood shows no difference in gross appearance when it is compared with normal blood, but it has peculiar and striking characteristics when exposed to hydrogen peroxide. The color changes instantly from red to brownish black without emitting any bubbles of oxygen. This happens because of the lack of catalase in the erythrocytes, and the blackening of the blood is caused by the formation of methemoglobin (Figure 6). When excess peroxide is added, oxidation continues, and within 20 or 30 minutes, the color changes to lemon yellow and then disappears, leaving a colorless sample. This clear liquid contains the final decomposition product propentdyopent, which gives a positive pentdyopent reaction. Rapid spectrophotometric scanning permits observation of this decomposition process in detail.[15,23]

With the exception of the above findings, this blood has no other obvious difference in its components when compared with normal blood. The quantity of peroxidase enzyme is of a normal amount and its reaction is essentially the same as that of normal blood. The ratio between methemoglobin and hemoglobin also is almost normal. However, there is a slight increase in Heinz body formation. In other respects, the metabolic processes of acatalasemic individuals seem to be normal with the exception that coproporphyrin and

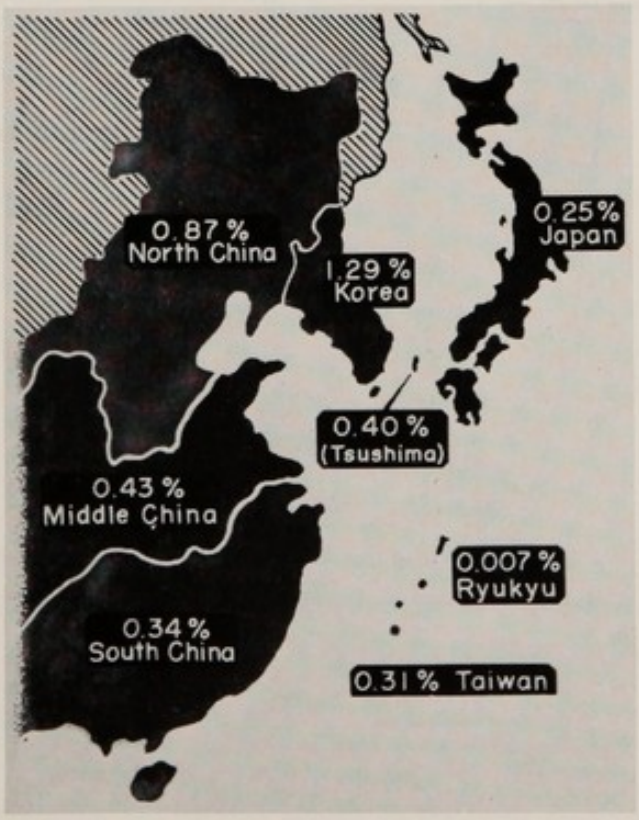


FIG. 5. The distribution of hypocatalasemia in various portions of Asia.

bilirubin excretion in the urine is five times that of normal subjects. There is no abnormality of hemoglobin itself.

In our search for evidence of G-6-PD deficiency in acatalasemic and hypocatalasemic individuals,[19] using the method described by Loehr and Waller,[12] we found no deficiency of this enzyme among three hypocatalasemic and four acatalasemic individuals. We did find low G-6-PD values in one case each of the hypocatalasemic and acatalasemic subjects from the same family as compared with the control values of six normal persons who were tested.

The test for determining the anomalous state of catalase has been conducted by assaying the catalase activity in erythrocytes, using the modified method from von Euler and Josephson[27] and Bonnichsen et al.[3] However, there are important questions relative to whether the catalase protein is actually deficient or highly altered or whether its activity might be inhibited by some unknown factors. In order to clarify these points, we conducted a series of studies by spectrophotometric, paper electrophoretic, and immunochemical methods. Paper electrophoretic patterns of crude catalase solutions, (stage 2 of Herbert-Pinsent) extracted by an ethanol-chloroform mixture, showed a main peak of slow mobility, which contains the protein fraction of hemoglobin, and an additional small peak of faster mobility containing the catalase fraction (Figure 7). The protein concentration of the faster peak from the acatalasemic

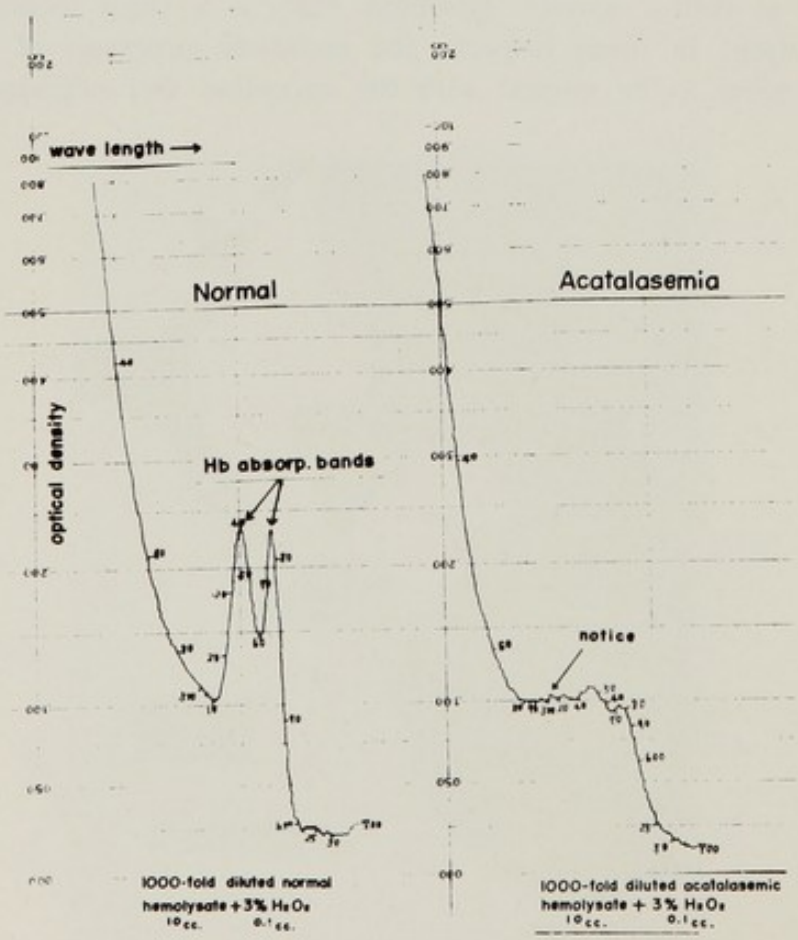


FIG. 6. The absorption spectrum of diluted normal and acatalasemic hemolysate one minute after the addition of hydrogen peroxide.

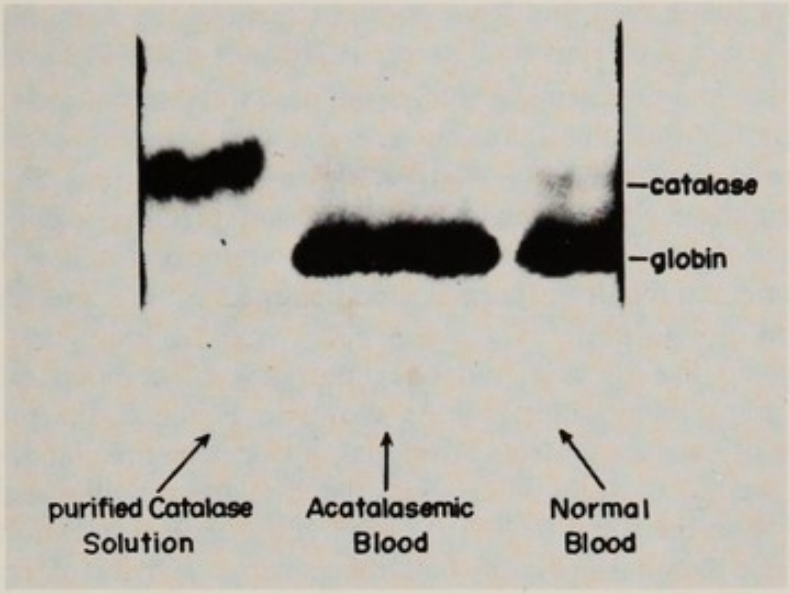


FIG. 7. Paper electrophoresis of crude catalase solution isolated from normal and acatalasemic red blood cells (stage 2 of Herbert-Pinsent).

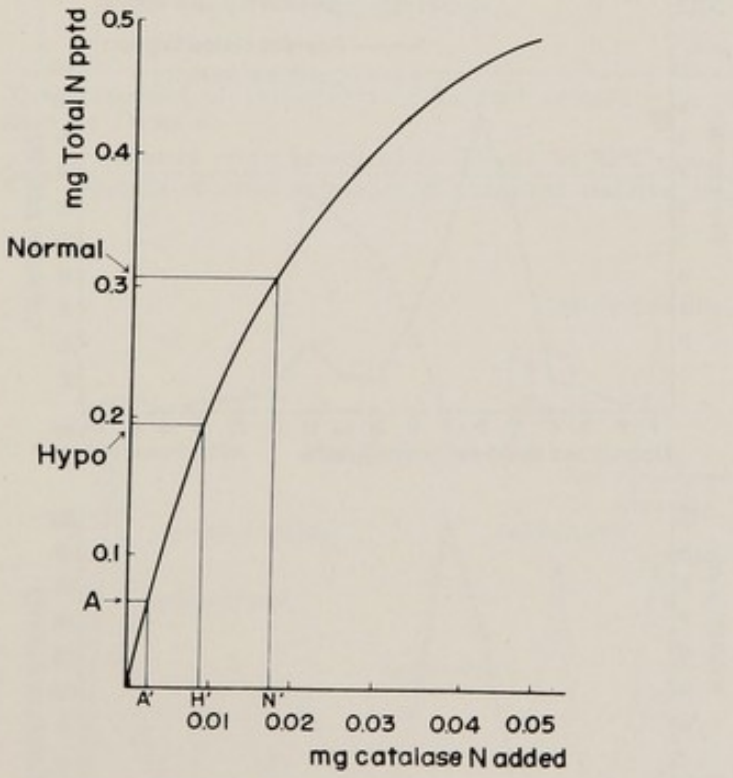


FIG. 8. The reaction of an acetone extract of normal, hypocatalasemic, and acatalasemic red cells with anti-human-red-blood-cell-catalase rabbit serum. Normal, hypo, and A, represent the nitrogen content of the precipitate between the complexed catalase antibody and the extract of normal, hypocatalasemic, and acatalasmic blood samples, and N′, H′, and A′ each represent the nitrogen content of the antigen, catalase.

blood is much lower than that from normal blood. In the more highly-purified preparations (stage 3 of Herbert-Pinsent), the faster peak is not seen.[24]

In conducting immunochemical studies, we used three techniques, the capillary ring test, agar micro-double diffusion test, and the quantitative precipitin test. In the precipitin ring test, extract (stage 2) from acatalasemic blood does not show a precipitin reaction with rabbit antiserum prepared against a highly-purified preparation (Kat f 60,000) of human erythrocyte catalase.[24] In contrast to this, acetone extracts from normal blood showed definite precipitin reaction at the dilution of 1 to 40.[14,16,17]

Now, I would like to point out one interesting observation related to the quantitative precipitin reactions.[14,17] As shown in Figure 8, the proportions of the quantities of catalase proteins in normal, hypocatalasemic, and acatalasemic hemolysates were 1.0: 0.5: 0.07, while the catalase activities showed nearly the same proportions of 1.0: 0.5:0. From this finding it can be said that hypocatalasemic blood shows one-half the catalase activity of normal blood because it apparently has only one-half the quantity of catalase protein, and acatalasemic blood lacks catalase activity because catalase protein is absent.

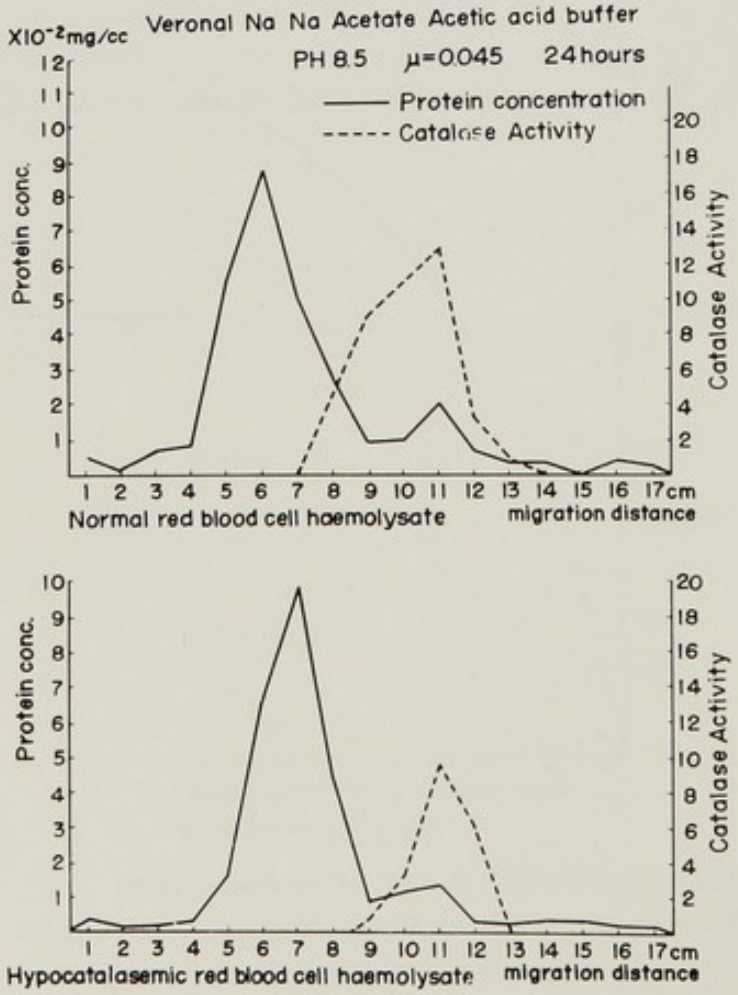


FIG. 9. Zone electrophoresis of red cell hemolysate prepared from normal blood (upper panel) and hypocatalasemic blood (lower panel). The area enclosed by the dotted line represents the catalase activity, which is approximately ½ of normal in the case of the hypocatalasemic blood. Similarly, the area under the solid line, representing protein concentration, is approximately ½ of normal in the hypocatlasemic sample.

TABLE 4. *Quotient of catalase of blood, oral, and nasal tissue in acatalasemic and normal persons estimated by Warburg's method*[8]

Acatalasaemia					Normal persons
Case no.	Sex	Age (yr.)	Material examined	Qcat	Qcat
1	♀	16	Blood from earlobe	7	4500
2	♂	18	Venous blood	5	About 4500
			Blood from earlobe	6	4500
3	♀	8	Blood from earlobe	10	4500
			Normal oral tissue	0	250
4	♀	14	Blood from earlobe	10	4500
5	♂	17	Venous blood	9	About 4500
			Erythrocytes of venous blood	8	12000
			Normal oral tissue	0	250
			Marginal tissue of oral ulcer	0	..
			Tissue of oral ulcer	0	..
			Tissue of ethmoidal sinus	0	450
			Nasal polyp	0	200

Qcat (quotient of catalase) is calculated according to the following formula:

$$Qcat = \frac{\text{Quantity of } O_2 \text{ generated in 30 min. at 38°C (c. mm.)}}{\text{Weight of dried substance of examined material (mg.)}}$$

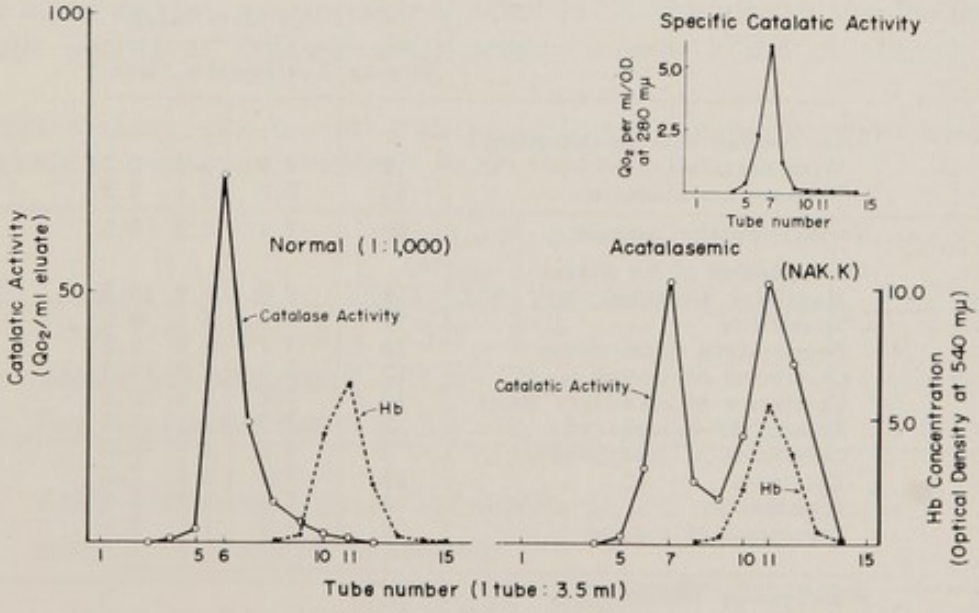


FIG. 10. The separation of minimal catalatic substance in an acatalasemic hemolysate by Sephadex G-100 gel filtration. The abscissa denotes the tube number of the fraction, and catalase activity of normal blood is expressed as 1/1000 of the actual value. The dotted line represents the hemoglobin concentration expressed in optical density at 540 mμ. Tube diameter, 097 cm.; height, 143 cm.; $V_0 \rightarrow 34.8$ ml. (blue dextran method); $V_1 \rightarrow 65.0$ ml.; and $V_t \rightarrow 106.3$ ml.

This point can be further substantiated by zone electrophoresis, using acatalasemic and hypocatalasemic hemolysates, as shown in Figure 9. These findings strongly suggest that substances suppressing or inhibiting the enzyme activity in these blood samples do not play an important role.

So far, I have briefly outlined facts known about acatalasemia and hypocatalasemia. I must add that the term acatalasemia denotes complete deficiency of catalase in the blood. But, as I described in Lancet in 1952,[20] actually there is 1/500-1/1000 of normal catalatic activity which may be observed by manometric methods and by the permanganate titration procedure of Inoue (Tables 4 and 5). What does this minimal catalatic activity reflect? Does this activity represent the action of the actual catalase molecules themselves or does it perhaps mean that substrate decomposition occurs from other actions? These are important questions from the standpoint of human genetics.

In 1964, Professor Aebi isolated the catalase-active fraction from acatalasemic hemolysate using Sephadex G-100 gel filtration and demonstrated the existence of residual catalase in the fraction isolated free of hemoglobin.[1] We also separated a catalase-like substance from the acatalasemic hemolysate, using the method employed by Dr. Aebi—the so-called residual catalase of Aebi. In addition to this, we also recognized catalytic activity in the hemoglobin fraction which may be separated from acatalasemic hemolysate by gel filtration. In our study, the catalatic activity was tested by the manometric method.[18]

The gel filtration pattern of a normal hemolysate is shown on the left in

TABLE 5. *Quantity of catalase in blood of acatalasemic and normal persons estimated by Inoue method*[7]

Cases	No. of persons	Catalase index		
		Average	Min.	Max.
From families with acatalasaemia :				
With acatalasaemia	9	0.0	0.0	0.1
Without acatalasaemia.. ..	45	5.5	3.4	7.2
Normal healthy persons.. ..	20	7.4	4.3	9.5
100 inpatients of the clinic :				
Maxillary sinusitis	19	7.6	5.2	10.2
Tonsillitis	17	7.8	5.5	9.5
Suppurative otitis media ..	12	7.7	6.9	9.2
Carcinoma of larynx	12	8.9	6.8	10.9
Carcinoma of maxillary sinus	10	7.4	3.3	10.5
Tumour of nasopharynx ..	3	7.7	7.5	7.9
Carcinoma of oesophagus ..	3	6.9	4.9	8.0
Epistaxis..	3	6.2	4.1	7.3
Diphtheria	2	7.5	7.3	7.7
Carcinoma of tongue	2	7.6	7.1	8.1

Miscellaneous diseases :

Brain abscess, 10.9; glioma of temporal lobe, 8.6; cyst of cerebellum, 9.; Ménière's disease, 6.3; tumour of cheek, 8.8; perforation of nasal septum, 6.2; spontaneous gangrene of nose, 7.6; sarcoma of maxillary sinus, 7.2; cystoma of dental root, 9.6; ranula, 8.9; lymphosarcoma of tonsil 7.5; carcinoma of pharynx, 8.7; paralysis of larynx, 7.8; Mikulicz's disease, 6.9; nocturnal laryngeal dyspnoea, 4.4; carcinoma of thyroid gland, 8.2; struma cystica, 8.7.

Figure 10, and on the right side the pattern for acatalasemic hemolysate. There are two peaks for each sample. In the normal hemolysate, the first peak was found to contain catalase-like substance when tested by Warburg's method, and the second peak was hemoglobin itself. In the acatalasemic hemolysate there are two peaks, indicating the catalytic activity. This first peak was found in the tube number which corresponded to the one with the highest catalase activity of the normal hemolysate. This catalatic activity was completely inhibited upon the addition of 10^{-4} M of sodium azide. In the second peak, the catalytic activity also was detected by the manometric method. In this instance, the catalatic activity was due to the formation of methemoglobin-hydrogen peroxide complex as described by Keilin and Hartree[11] in 1950.

These findings indicate that the minimal catalatic activity detected in the Japanese acatalasemic blood is composed of two fractions. One is from some as yet undefined catalase-like substance, which is represented by the first peak, and the other, from methemoglobin-hydrogen peroxide complex derived from hemoglobin in the presence of hydrogen peroxide. Heat-stability and azide tests have verified the view that catalatic activity of the second peak is undoubtedly derived from a substance that is distinct from that of the first peak. The material from the second peak resisted heat longer than did the first (Table 6 and Figure 11). Similarly, the second substance resisted higher concentrations of azide than did the first.

The presence of catalatic action revealed in this second peak also has been demonstrated in four other cases of acatalasemia (Figure 12). The activity of the second peak does not fluctuate as much as that of the first peak. The activity of the first peak varies considerably from one individual to the next. The first peak represents about ½-¼ of the total catalatic activity of acatalasemic blood and has a mean value of about ⅓. If one assumes that the residual catalatic activity of Japanese acatalasemic cases is 1/500 of normal blood

TABLE 6. *Heat inactivation (67° C for 10 min.) on first catalatic substance and second hemoglobin of acatalasemic hemolysate by gel filtration*

	cat.activity before inactivation	activity after heat inact.	theoretical activity (1) + (2)	O.D. at 280 mμ
First frac. (cat. sub)	19.5	(1) 2.6		0.079
Second frac.(hemoglobin)	53.6	(2) 46.2		11.342
First frac.+Second frac.	73.1	51.7	48.8	11.421

Azide inactivation (1.25×10^{-4} M for 10 min. at 37°C) on first catalatic substance and second hemoglobin

	cat. activity before inactivation	activity after azide inactivation	% of activity inhibited	O.D. at 280 mμ
First frac.(cat. sub)	24.4	0	100	0.10
Second frac.(hemoglobin)	75.9	61.1	39	18.68

(by M. Ogata T. Takehisa, S. Takahara unpulished data)

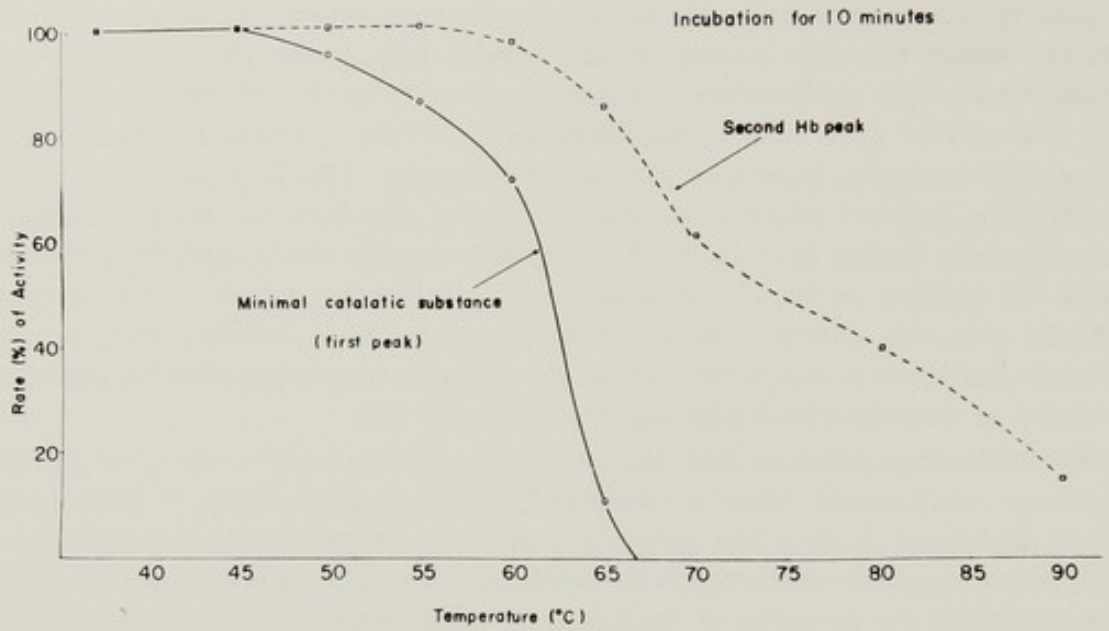


FIG. 11. The heat stability of minimal catalatic substance in the first peak and hemoglobin in the second peak obtained by Sephadex G-100 gel filtration from acatalasemic hemolysates.

activity as we originally reported,[20] it can be said that about ⅓ of residual catalatic activity, namely about 1/1500 resides in the first peak and the remaining 2/3 of the activity, namely, the 2/1500 is from the hemoglobin in the second peak.

Now, there arises a question as to whether or not the catalase-like substance in the first peak is identical to normal blood catalase. In trying to find answers to the question, the following facts should be considered:

1. We find no appreciable difference in heat stability when we compared the first fraction of normal blood with that of acatalasemic blood.

2. When the first-peak fractions are treated with aminotriazole, which is an inhibitor of catalase, using a concentration of 5×10^{-4} M, the mean activity

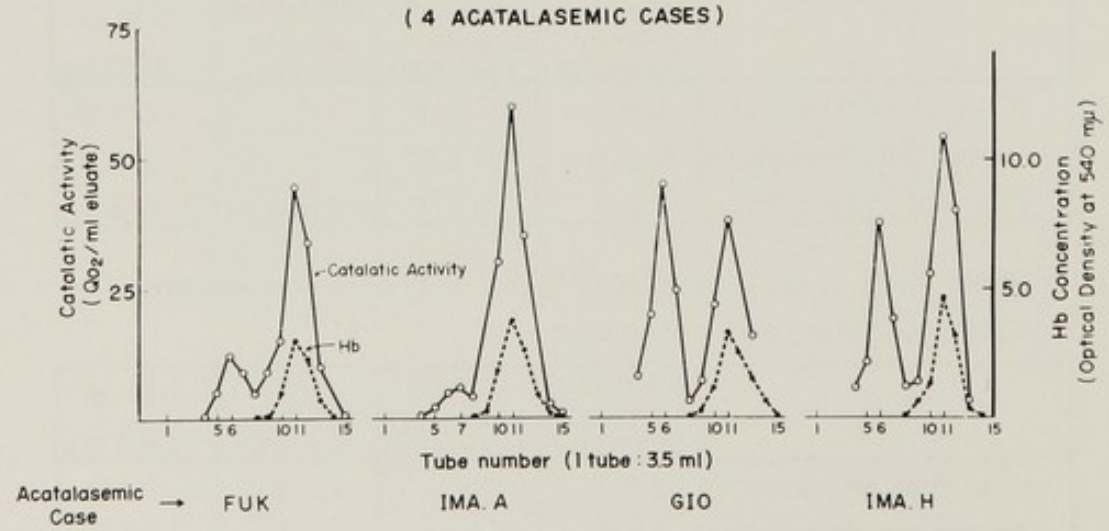


FIG. 12. The separation of minimal catalatic substance in acatalasemic hemolysates by Sephadex G-100 gel filtration.

TABLE. 7. *Aminotriazole inactivation (5 x 10^{-4} M for 30 min. at 37°C) on first catalatic substance of normal and acatalasemic hemolysate*

	cat. activity before inactivation	activity after inactivation	% of activity inhibited	O. D. at 280 mμ
Acat. first fraction	44.4	23.8	46	0.06
Norm. first fraction	102.5	50.5	51	0.13 *

* diluted with serum-alb. solution

(by M. Ogata, T. Takehisa, S. Takahara, unpublished data)

of normal blood is decreased to 46 per cent while that of acatalasemic blood is reduced to 51 per cent, indicating no significant difference between these (Table 7).

3. When the antibody from purified human blood catalase is made to react with the first fraction of acatalasemic blood, the supernatant of the absorbed sample shows no catalatic activity. This finding again suggests that the catalase-like substance in acatalasemia resembles that of normal blood catalase.

4. Next, DEAE column chromatography, as described by Thorup et al.,[26] was applied to our study. Using .001 M phosphate buffer, the eluate recovered first is the hemoglobin fraction. With increasing concentration of phosphate buffer by the discontinuous method, we can obtain a fraction free of hemoglobin that corresponds to the first peak of the Sephadex gel filtration. This fraction

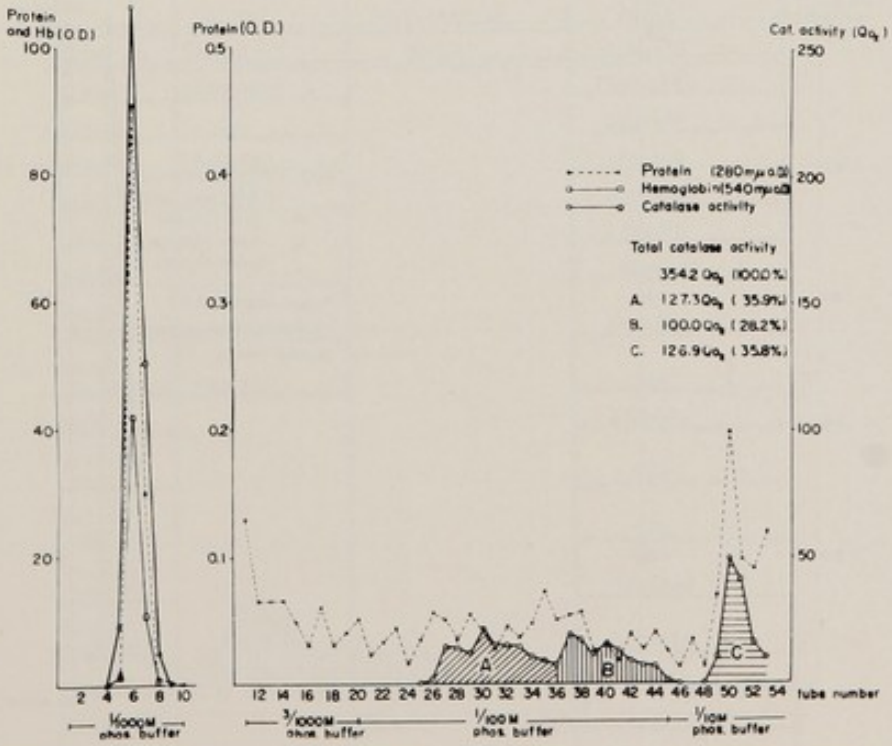


FIG. 13. Chromatographic pattern of erythrocyte catalase in an acatalasemic hemolysate.

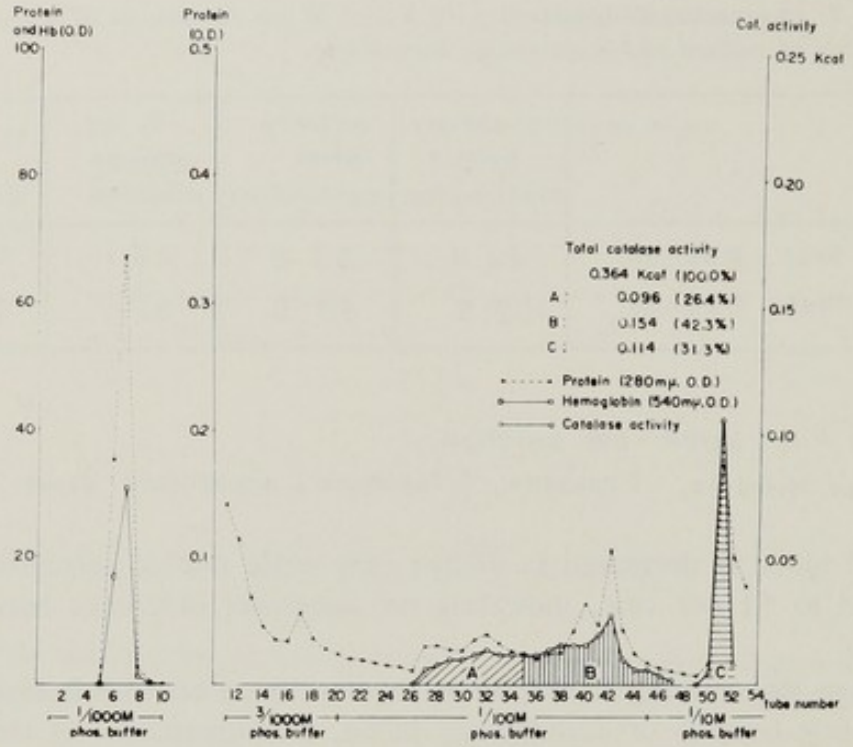


FIG. 14. Chromatographic pattern of erythrocyte catalase in a hypocatalasemic hemolysate.

shows catalatic activity and can be further separated into three peaks; A, B, and C (Figure 13). The same phenomenon can be demonstrated with normal blood as well as with hypocatalasemic blood (Figures 14 and 15). These findings seem also to support the assumption that the catalase-like substance in the acatalasemic blood resembles normal blood catalase.

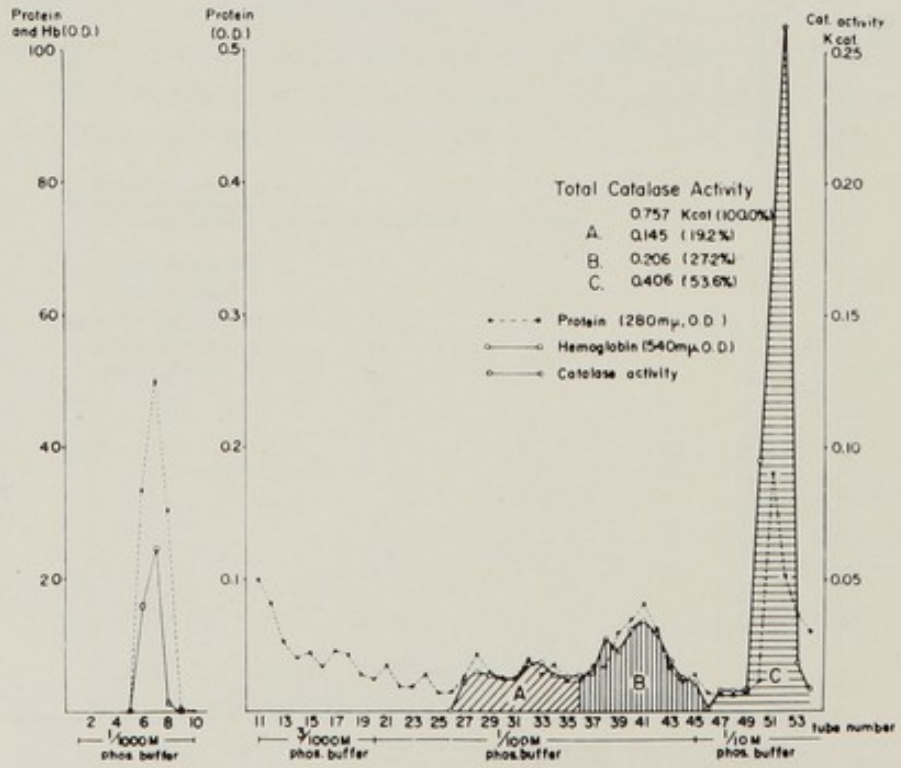


FIG. 15. Chromatographic pattern of erythrocyte catalase in a normal hemolysate.

Let us suppose that Japanese acatalasemic blood has a residual catalatic activity in the amount of 1/500 of normal blood; then it may be assumed that ⅓ of this (amounting to 1/1500 of the activity of normal blood) represents the catalatic action that is derived from catalase-like substance. This material very closely resembles normal blood catalase. The remaining ⅔ of the activity, or 2/1500 of the activity of normal blood, are derived from the action of hemoglobin.

From a genetic standpoint, it is very important to determine whether or not acatalasemia is due to a mutation involving the gene or factors responsible for the repression of synthesis of catalase. Therefore, the identification of the residual catalase-like substance in the acatalasemic blood and catalase of normal blood is important. For this reason, further investigations must be carried out in the study of the enzyme molecule and its physical chemistry.

Next, I should like to comment regarding the semantics and nomenclature of acatalasemia. As far as the blood of acatalasemic individuals is concerned, it is an established fact that its gene is transmitted to progeny by hypocatalasemic individuals who serve as carriers of the defective gene. This strictly follows the Mendelian recessive law. There is little reason to deny the existence of acatalasemia. But for this condition, some investigators proposed the name "acatalasia" or "anenzymia catalasea," a term which implies a total deficiency of catalase in all the tissues of the body. As you know, the radiation-induced mutant mice (M1 line, Cs^b) bred by Feinstein, Seaholm, et al.[6] also follow the recessive law of Mendel.[7] Cross-breeding of the acatalasemic mouse

TABLE 8. *Catalase activity (30° quotient of catalase) of human tissues using Warburg manometer*

	Normal	Acatalasemia	Osada
Blood	5280-7140	20-40	230-270
Liver	3670-4020		1320-1650
Kidney	520		270-290
Spleen	665-680		230-340
Heart	330-360		110-130
Lung			130-280
Stomach			90-110
Small intestine	490-510		140-170
Large intestine	520-540		160
Pancreas			230-390
Arterial wall			370
Veinal wall			370
Greater mentum			220-230
Lesser mentum			150-260
Bladder			160-180
Uterus			115-150
Abdominal muscle			240-410

with a normal mouse gives rise to heterozygous, hypocatalasemic offspring. These radiation-induced mutant mice are called acatalasemic mice. In these mice, while the catalase value of the blood is about 1/50 of the normal, the catalase activity in the internal organs is about 1/5 normal.[5]

In addition, I have a very interesting autopsy record of an acatalasemic woman (Osada) whose blood catalase showed 1/25 of the normal value. Contrary to my expectations, her liver and spleen at post mortem examination revealed 2/5 of the normal catalase value and her kidney had almost ½ of the normal value. This means that catalase values observed in acatalasemic blood are not necessarily reflected in the catalase values of internal organs. (Tables 8 and 9).

For this reason, I think the term acatalasemia is preferable, and the term acatalasia should be reserved for acatalasemic cases only when the catalase activity is proved to be completely absent in the entire body. We must, therefore, wait for more autopsy findings.

Summary

Acatalasemia is a constitutional abnormality characterized by lack of catalase activity in the blood and follows incomplete recessive inheritance. Ninety-two cases have been reported, including 12 Caucasians. It is noteworthy that acatalasemic individuals, with the exception of occasional characteristic lesions in their childhood, appear to live quite normally.

There are two types of individuals in acatalasemic families; in one, catalase activity is normal, and in the other, the activity is intermediate between normal and acatalasemic values. The intermediate group shows one-half the catalase activity of normal blood due apparently to having only one-half the quantity of catalase protein and it is designated as hypocatalasemic. This hypocatalasemia is heterozygous and the carrier state for the acatalasemic gene. The incidence of hypocatalasemia has been determined in 83,000 people living in Asia and its percentages were as follows: Koreans, 1.29; Chinese in northern China, 0.87; Chinese in middle China, 0.43; Chinese in south China, 0.34; in Taiwan, 0.31; Japanese, 0.25; Ryukyuans, 0.007 per cent.

Minimal residual catalastic activity, 1/500 to 1/1000 of normal detected in the blood of Japanese acatalasemics, arises from two sources. One is from some

TABLE 9. *Quotient of catalase ratio of several tissues using Warburg manometer*

	Normal	Osada
Blood	100	4
	(25:1)	
Liver	63	25
	(5:2)	
Kidney	9	5
	(2:1)	
Spleen	12	5
	(5:2)	

as yet undefined catalase-like substance, and the other from hemoglobin in the presence of hydrogen peroxide. The catalase-like substance closely resembles normal blood catalase in tests of heat stability, aminotriazol inhibition immunoreactions, and DEAE column chromatography. However, complete identification requires further investigations.

Acknowledgments

I would like to express my hearty thanks to collaborators like Dr. Ogata, Professor of Public Health, and the staffs of our own Department, Okayama University Medical School, as well as to Dr. Ohkura, Assistant Professor of Tokyo Medical and Dental University. Also, acknowledgment is due to Dr. Nishimura, Dr. Kobara, and Dr. Hamilton who collaborated with us in the past as the members of Hiroshima and Nagasaki Atomic Bomb Casualty Commission.

The discussion of Dr. Takahara's presentation is combined with the discussion of Dr. Aebi's paper, page 62.

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Acatalas(em)ia in Switzerland

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THE EXISTENCE of individuals in Japan lacking catalase in blood and possibly in tissues has been and still is a challenge for all scientists interested in this enzyme. The occurrence of this rare anomaly seemed for more than a decade to be limited to Japanese and Koreans. The first cases of this "classical" form of acatalas(em)ia observed in western countries were those detected as a result of screening performed in the Army Blood Group Laboratory of the Swiss Red Cross Blood Transfusion Service.[5] The blood specimens, examined by a simple qualitative test, as recommended by Dr. Takahara,[42a] covered essentially all male subjects of the Swiss population reaching the age of 19 years in 1961. At first, the result of this screening program seemed to be disappointing, because no case was found among the first 15,000 samples tested. At about the same time that a discussion was held as to whether the screening should be stopped, two cases were detected almost simultaneously. Propositus number one was Francois V., a farmer from Riddes (Canton Valais); the second case was Andreas B., a student from Flerden (Canton Graubünden). In order to increase the number of available blood donors, screening was repeated in 1965. One more case, Albin G., a worker from Altdorf (Canton Uri) was detected. Therefore, in these screening programs 3 persons out of 73,661 proved to be acatalas(em)ic; thus the frequency of homozygotes is about 0.04 per thousand. A comparison with figures obtained in screening for hypocatalas(em)ia cases in Japan permits the assumption that there is no gross difference between gene frequencies in these countries. However, such a comparison is in no way conclusive, because there is an uneven distribution of the acatalas(em)ia gene(s) even within the population of the same nation.[23,35,41] Whether the concept of "pockets," wherein certain rare recessive genes occur with unusually high frequency, can be applied to acatalas(em)ia in Switzerland, remains to be elucidated.

The examination of all members of the families V., B., and G. and their distant relatives has led to the detection of 8 more cases. Thus, a total of 11 (6 females and 5 males) individuals homozygous for acatalas(em)ia and a considerable number of subjects with partially reduced blood-catalase activity, most likely representing the heterozygous carrier state ("hypocatalasemia"), have been detected. None of these "acatalatic" subjects has, or apparently ever

Research on the biology of catalase and on acatalasia performed in this laboratory has been supported by grants from the "Schweizerischer Nationalfonds zur Förderung der wissenschaftlichen Forschung" (Projects A 142, 2879 and 3785).

had, severe gangrenous oral infections (Takahara's disease), which has been reported to occur in about 50 per cent of the Japanese cases.[40,42a] Up to the present time, all of the acatalatic individuals detected in this country are in good health, including their dental status. Because most of them are cooperative, an appreciable number of experiments has been performed in the past five years; e.g., (a) loading tests with formate[2]; (b) measurements of catalase activity in tissue specimens, such as liver, tonsils, placenta, and skin[2]; (c) tissue culture studies using acatalatic fibroblasts from biopsy specimens[27]; and (d) analysis of serum, white cell, and red cell enzymes.[2,8] Only the following aspects are discussed in some detail in this paper: 1. The level of catalase activity in blood of heterozygotes and homozygotes. 2. Analysis of enzymes other than catalase in blood. 3. Hemoglobin pattern and hemoglobin stability in acatalatic red cells. 4. Cellular distribution of catalase activity in normal and acatalatic erythrocytes. 5. Studies on the chemical nature of catalase in blood of heterozygotes and homozygotes.

The Level of Catalase Activity in Blood

The detection of homozygotes is based on the fact that their blood is virtually free of catalase. In the screening test this is manifested by absence of foaming and a rapid change in color from red to brown followed by complete decolorization of the sample within 15 minutes after addition of a few drops of a red cell suspension to approximately 1% H_2O_2 in phosphate buffer (approximately pH 7). However, the enzyme deficiency is not complete. The level of residual catalase activity, as measured by a micromodification of the perborate method [19] varies between 0.1 and 1.3% of that of normal blood.[2] For the measurement of such low activities in relatively concentrated hemolysates, this titrimetric technique is more convenient than UV-spectrophotometry,[13,14] which we prefer for collecting normal data. The main drawback in the former procedure, fading of the end point when titrating with permanganate, can be avoided if test and control assays are deproteinized before titration.[1] In a comparative study covering some 50 members of the Swiss acatalatic sibships, it has been observed, however, that the results obtained by titration and by spectrophotometry are not exactly identical. Whereas the decrease in extinction at 240 $m\mu$ within 30 seconds is a function of v_0 alone, the quantity of H_2O_2 (i.e., perborate) not decomposed after the incubation period of 5 minutes reflects not only enzyme activity, but also enzyme stability. By separation and purification of the material, it could be demonstrated that residual catalase activity in blood of homozygous cases ("acatalas(em)ia") is due to the presence of true catalase which is heat-sensitive and can be completely inhibited by azide. This is in full agreement with the observations made in most of the Japanese cases. According to Takahara[42] the corresponding figures for the relative level of residual activity vary between 0 and 3.2%.

The activity of blood catalase in the other members of the Swiss acatalasia-families varies within a rather wide range, from 15% of normal activity up to the normal level. As shown in Figure 1, there is no trimodal distribution without overlap as has been reported for the Japanese cases.[33] Because the normal activity range and the figures presumably representing heterozygotes

overlap considerably, it is difficult to distinguish heterozygotes and normal individuals in these families, unless it is evident from the pedigree. It is assumed that individuals with a blood catalase activity level of less than 2000 perborate units (2000 PU/g Hb) have to be considered as heterozygotes. With the exception of one single case (G.V., born 1884; 450 PU/g Hb) all those considered as heterozygotes have a blood catalase activity of 1500-2000 PU/g Hb, i.e., 60-80% of normal level. The relative level of enzyme activity in the homozygote as well as in the heterozygous carrier state is one of the main criteria for the distinction of different types of acatalasia (see concluding paragraph of this discussion).

It is assumed that the physiological function of catalase—possibly also in the red cell—is acting as a peroxidase in the presence of suitable hydrogen donors, such as ethanol, methanol, and formate. In a suspension of normal human red cells C^{14}-formate is readily converted to $C^{14}O_2$, provided that a suitable H_2O_2-generating system, such as glucose-glucoseoxidase or a thiol compound is added to the incubation medium. In contrast to normal cells, acatalatic cells are almost completely unable to perform coupled oxidation of formate although excess H_2O_2 is present.[8] The level of residual peroxidase-type activity in acatalatic red cells is slightly higher (2-4%) than that of catalase activity, as measured by H_2O_2-decomposition (Table 1).

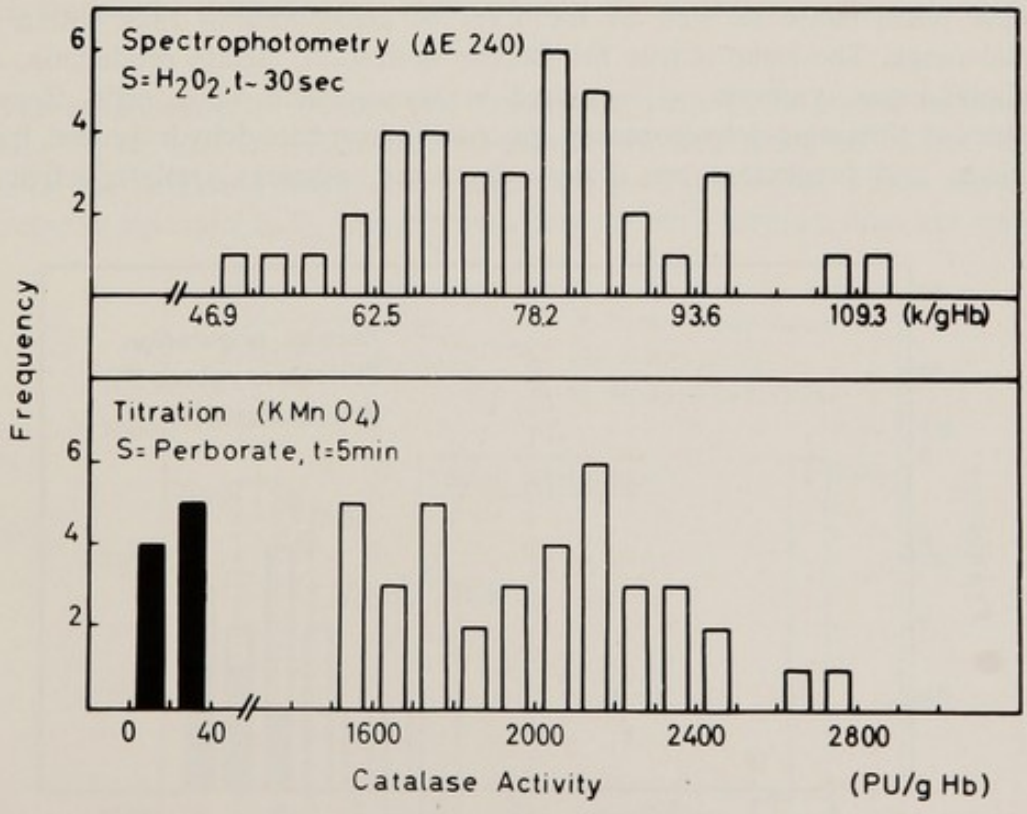


FIG. 1. Frequency distribution of erythrocyte catalase activity in three acatalasia families V., B., and G., as determined by (upper panel) U.V. spectrophotometry (k-value/g Hb) and (lower panel) Feinstein's perborate method (P. U./g Hb). Values for acatalasemic subjects are not given in the upper panel because the U. V. method is unsuitable for the measurement of very low catalase activities in hemolysates.

TABLE 1. *Oxidation of C^{14}-formate in suspension of normal and acatalatic human red cells. Concentration of suspensions: 32-36 mg Hb/system (4 ml) = 8-9 mg Hb/ml.*[5]

Code No.	Substances added:	$C^{14}O_2$-formation from c/min/system		C^{14}-formate μMoles/g Hb/2 h	
normal	—	70	—	—	
red cells	+ 0.02 M Cysteine	2320	2250	25.4	
(H.A.)	+ 0.02 M Cysteamine	3560	3490	39.4	
	+ 0.02 M red. Glutathione	2540	2470	30.9	(= 100 %)
	+ 0.02 M ox. Glutathione	75	5*	0	
acatalatic	—	60	—	—	
red cells	+ 0.02 M red. Glutathione	175	115	1.3	(= 4.2 %)
(F.V.)	+ 0.02 M red. Glutathione + 1 mg Catalase	1230	1170	13.2	

* Not significantly different from zero.

Analysis of Enzymes in Blood Other than Catalase

In a limited number of cases, measurements of activity of serum, white cell, and red cell enzymes have been made. The propositi in families V. and B. as well as 4 normal members of each family were investigated. The results, published in detail previously,[2,8] indicate that in the serum of acatalatic individuals, the activity of amylase, cholinesterase, glutamate-oxaloacetate-transaminase, alkaline phosphatase as well as the level of ceruloplasmin falls within the normal range. The same is true for glucose utilization, lactate production, and porphobilinogen synthesis, as measured in homogenized white cells. Enzyme activities of glutamate-dehydrogenase, glucose-6-phosphate-dehydrogenase, transaminases, and peroxidase are slightly decreased, whereas catalase activity is

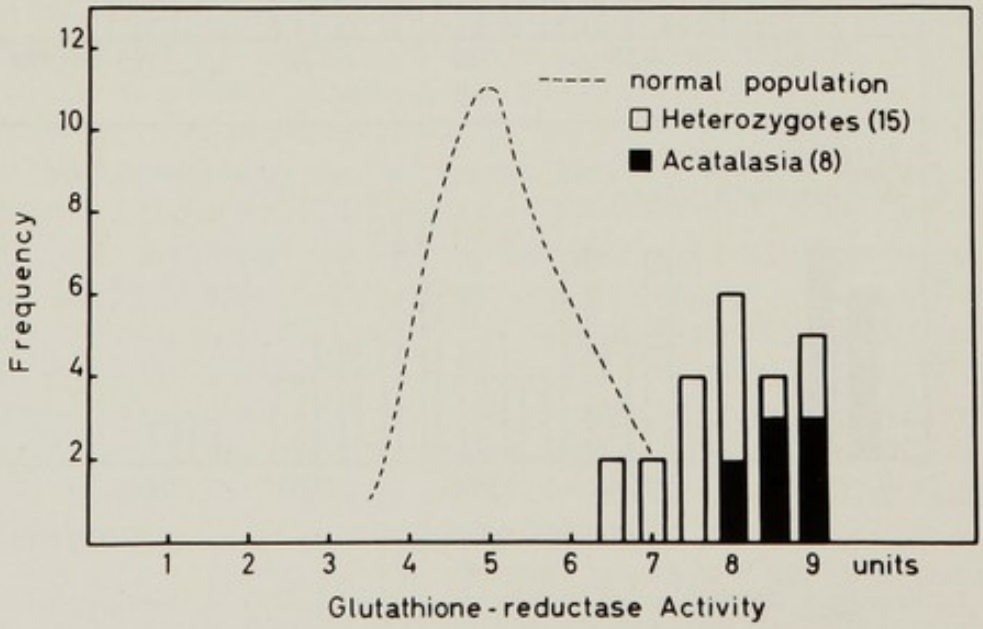


FIG. 2. Glutathione reductase activity in heterozygous and homozygous cases of acatalasia (normal mean 5.2 units, standard deviation 0.87 units). Measurements made by Dr. H. R. Marti (Hemoglobin Laboratory of the Medical Policlinic, University of Basel).

lacking completely. Furthermore the activity of the following red cell enzymes has been found to be normal: glucose-6-phosphate dehydrogenase, hexokinase, lactate dehydrogenase, and aldolase. However, glutathione reductase and glutathione peroxidase activity is slightly elevated to above normal levels. This seems to be the case not only in the homozygotes but also in the other members of the acatalasemic families (Figure 2).

The most impressive observation made in this respect has been reported by Jacob et al.[24] They found that human acatalatic red cells, when incubated in vitro with 1-C^{14}-glucose, produce $C^{14}O_2$ about three times as fast as normal red cells. If ascorbate (5 mM) is added as a peroxide generator, glucose metabolism via hexose-monophosphate shunt is increased twelvefold. Despite this drastic enhancement of shunt activity, formation of lactate by these cells is unaltered (Figure 3). Since NADPH regeneration is a prerequisite for several protective mechanisms in the red cell, this increase may be regarded as evidence of a compensatory stimulation of the hexose-monophosphate shunt. In this connection it may be of interest that in red cells of the duck, which is physiologically acatalasemic, GSH-concentration is about 3 times, and glutathione-reductase activity five times as high as it is in normal human red cells.[28] Therefore it appears that the protective action of catalase has been taken over by some other mechanism, whenever, in evolution, a mutation led to a block in the synthesis of erythrocyte catalase.

Hemoglobin Pattern and Hemoglobin Stability in Acatalas(em)ic Red Cells

The examination of red cell and hemoglobin patterns in several members of family V., B., and G. has revealed a completely normal red cell status, except that in one acatalatic family (V.) there are several subjects with a significant increase in hemoglobin F. This elevated hemoglobin F-content does not coincide

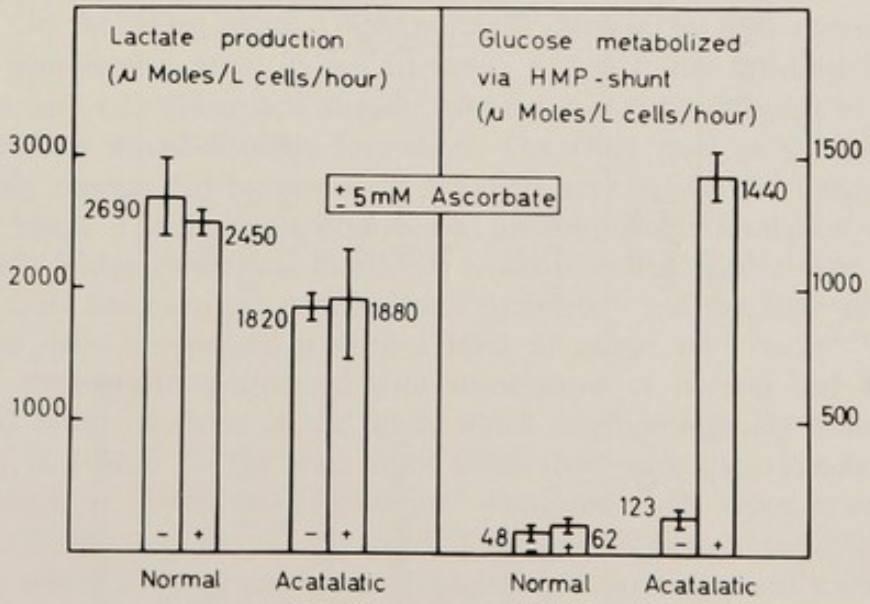


FIG. 3. Activity of HMP-shunt in normal and acatalatic erythrocytes. The height of each bar indicates the mean value. The line indicates 1 standard deviation. Data taken from Jacob et al.[24]

TABLE 2. *Data on red cell and hemoglobin pattern.*

	Normal range	Family V. V_6	V_7	*V_{24}	V_{25}	*V_{26}	*V_{27}
hemoglobin (g %)	13.5-17	16.7	13.7	15.0	14.6	13.8	13.7
red cell count (10^6 mm^3)	4.5-5	4.8	4.0	4.7	4.2	5.0	4.4
hematocrit	38-46	46	39	42	42	41	41
MCH (10^{-12}g Hb/cell)	30-34	35	34	32	35	28	31
MCV (μ^3)	78-94	96	97	89	100	82	93
MCHC (g %)	32-36	36	35	36	35	35	33
reticulocytes (%)	0.5-1.5	0.9	0.5	1.1	0.7	1.4	0.7
hemoglobin F (%)	0.25-0.75	0.92	1.38	1.64	1.16	0.49	0.79
hemoglobin A_2 (%)	1.0-3.0	2.3	2.4	2.0	2.2	2.0	2.0
hemoglobin-F cells		+	+	++	+	—	(+)

* = Acatalatic subjects[5]

with hypo- or acatalasia (Table 2). For example, Marianne (V_{26}) is acatalatic, but is one of the few members of the family with a normal value for hemoglobin F. In addition, all blood samples mentioned in Table 2 were analyzed for methemoglobin. None was detectable in fresh ACD blood samples; after storage at 4° for 9 days, the methemoglobin content varied between 0.2 and 1.1%. In the Heinz body test with acetylphenylhydrazine, there was the same rate of Heinz body formation in acatalatic as in normal blood samples.

In order to examine the postulated role of catalase in protecting hemoglobin against oxidative degradation, a number of in vitro experiments has been made using normal and acatalatic red cells. First the sensitivity of acatalatic erythrocytes against reagent hydrogen peroxide, which serves as a useful tool in screening, has been studied under more physiological conditions. The rate of methemoglobin formation was studied, using glucose-oxidase and glucose as a hydrogen peroxide-generating system. There is a relationship between the percentage of peroxide required for hemoglobin oxidation and the catalase content of the cells; the lower the catalase activity, the higher the cellular sensitivity to enzymatically-produced peroxide.[6] This is also true for peroxides or radicals produced in auto-oxidation of certain drugs (e.g., methylhydrazines).[10,11] The same correlation can be seen in irradiation experiments. Acatalatic red cells are very sensitive to X-rays.[45] Since their sensitivity can be reduced to normal by adding pure catalase or a small fraction of normal red cells to the suspension, it is very likely that—at least under in vitro conditions—this radiation effect is largely due to hydrogen peroxide.[7] Furthermore, this may be regarded as a manifestation of the protective action of catalase against oxidizing agents. There is as yet no answer to the question of whether this protective effect of catalase is also exerted in other cells or even in the organism as a whole.

Besides catalase, there are other mechanisms in the red cell that protect hemoglobin and other SH-proteins against oxidation. On one hand, catalase competes directly with hemoglobin or other oxidizable molecules for hydrogen peroxide. On the other hand, GSH, normally present in high concentration, serves as a protective agent in several ways: (1) It is the substrate for GSH peroxidase; and (2) SH-groups already oxidized to the disulfide can be restored by intermediate mixed-disulfide formation. The GSH pool in the red cell is continuously regenerated by means of NADPH supplied by the hexose-monophosphate shunt. The lag phase observed in methemoglobin formation in acatalatic red cells is due to the fact that H_2O_2 reacts with hemoglobin only after the supply of GSH has been exhausted almost completely and the H_2O_2-concentration in the cells has passed a critical level of about 10^{-6} molar[6,17] Results of typical experiments performed with suspensions of normal and acatalatic human red cells and those of the duck, which is physiologically acatalasemic, are shown in Figure 4. The data from these short-term experiments confirm earlier findings of Cohen and Hochstein,[17] who used H_2O_2 vapor as an oxidizing agent. Similar results are obtained if Ibenzmethyzin (a methylhydrazine derivative, which is widely used as a cytostatic: Natulan®) is used together with Fe-EDTA as a peroxide-generating system.[11] However, there are quantitative differences in the yield of the over-all reaction. As can be seen from the data in Table 3, H_2O_2 generated enzymatically is used (under suitable experimental

conditions) almost quantitatively for conversion of hemoglobin to methemoglobin (~92%). In this respect, acatalatic red cells are about 80 times more sensitive than normal human red cells. A ratio of about 60 is obtained if the rate of GSH oxidation is taken as an indicator.

There is still controversy regarding the question of whether GSH peroxidase or catalase is more important as a protective enzyme in the normal red cell. Cohen and Hochstein,[17] as a result of studies on duck erythrocytes, have come to the conclusion that catalase plays no role at all. Nicholls,[32] however, has recently given additional evidence for the protective role of red cell catalase under physiological conditions. Both systems are most likely involved, the relative contribution depending on the enzyme pattern of the cells and on experimental conditions. This view is in full agreement with the conclusions drawn by Jacob et al.[24] from their observation of HMP-stimulation in acatalatic human red cells. They state: "It seems clear from these studies that catalase in erythrocytes is a first line of defense against H_2O_2, but that under most circumstances it is dispensable. In its absence, H_2O_2 is dissipated primarily by the oxidation of GSH through the action of glutathione peroxidase, and in response to this, the metabolism of glucose through the HMP is necessarily increased."

Cellular Distribution of Catalase Activity in Blood of Heterozygotes and Homozygotes

Heterozygous individuals in acatalasemic families have an intermediate catalase activity in red cells and other tissues, varying chiefly between 60 and 85% of the normal level. This may be due either to the presence of two cell populations, one of them being virtually free of catalase, or to a single cell population of intermediate enzyme activity. In order to decide between these possibilities, two types of experiments have been performed. Both make use

TABLE 3. *Methemoglobin formation and GSH oxidation in normal and acatalatic erythrocytes: A comparison of data obtained in three different systems. The percentage figures indicate the yield of methemoglobin formed (M) and of GSH oxidized (G) in relation to the theoretical production. In all systems the concentration of the red cell suspension was 1.5 mg Hb/ml (additional data see Aebi and Suter[11]).*

	Rate of peroxide-production (Moles · $ml^{-1} \cdot min^{-1}$)	Normal erythrocytes A	Acatalatic erythrocytes B	Ratio B/A
H_2O_2 generated enzymatically (Glucose oxidase + Glucose)	1.4×10^{-9}	M: 1.2%	92.0%	~ 80:1
		G: 0.7%	46.0%	~ 60:1
Peroxide generated catalytically (Ibenzmethyzin + Fe-EDTA)	1.3×10^{-8}	M: < 0.2%	1.8%	> 10:1
		G: 0.2%	1.0%	~ 5:1
Irradiation by 60 kV X-rays (400 r/min) Assumption: G + H_2O_2 = 3.5	1.4×10^{-9}	M: 0.6%	8.5%	~ 12:1
		G: ca. 1%	ca. 1%	~ 1:1

of the fact that sensitivity of red cells towards H_2O_2 largely depends on their catalase content.

First a simple technique for the distinction of normal and acatalatic red cells in blood smears had to be devised.[4] A suspension (1.5 mg Hb/ml) of washed red cells is incubated in an isotonic, buffered medium containing glucose (5 mg/ml) and glucose oxidase (30 μg Deoxin "Nagase"/ml) at 25° C for 30 min. Under these conditions, i.e., at an H_2O_2-generation rate of 3.0×10^{-9} Moles H_2O_2 min^{-1} ml^{-1}, normal human red cells remain unaltered, whereas hemoglobin is completely oxidized to methemoglobin by H_2O_2 in acatalatic cells. After centrifugation of the suspension, the cells are washed, smeared and stained as described by Kleihauer and Betke.[25,26] This differential staining technique is based on the fact that oxyhemoglobin, but not cyanmethemoglobin, is active as a peroxidase. In smears obtained by this procedure, erythrocytes of approximately normal catalase activity (normal human blood) are stained red; and erythrocytes virtually free of catalase (acatalatic human blood) are eluted and appear as ghosts (Figure 5). Erythrocytes from heterozygotes show essentially the same result as normal human red cells. As a control, experiments have been made with a mixture consisting of equal parts of acatalatic and normal blood (imitating mosaicism). By this technique, heterogeneity with respect to catalase activity can be detected (Figure 5a). Evidently this is not the case in blood of heterozygotes (Figure 5c, 5d).

Additional evidence for even distribution of catalase in blood of heterozygotes

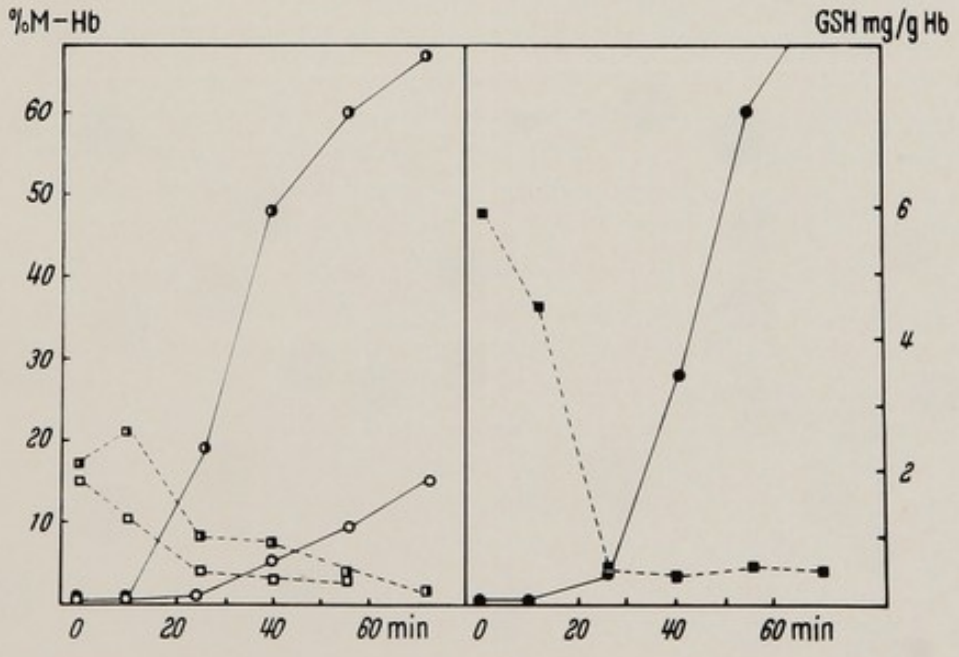


FIG. 4. Methemoglobin formation and GSH oxidation in erythrocytes of man (left) and of the duck (right). The cell suspensions (1.5 mg Hb/ml) were incubated at 25° C in presence of glucose oxidase and glucose.

Scale of ordinates: relative methemoglobin content (circles) and GSH-concentration (squares). The symbols ○, □ refer to normal blood (~ 2500 P.U./g Hb) and a H_2O_2-generation rate of 1.3×10^{-8} Moles $H_2O_2 \cdot ml^{-1} \cdot min^{-1}$.

◑, ◨ = blood of acatalasia case M.V. (~ 20 P.U./g Hb); rate of H_2O_2 production $2.6 \cdot 10^{-9}$ Moles $\cdot ml^{-1} \cdot min^{-1}$.

●, ■ = duck blood (~ 3 P.U./g Hb); rate of H_2O_2 production $1.3 \cdot 10^{-9}$ Moles $\cdot ml^{-1} \cdot min^{-1}$ (for details see Aebi and Suter.[11])

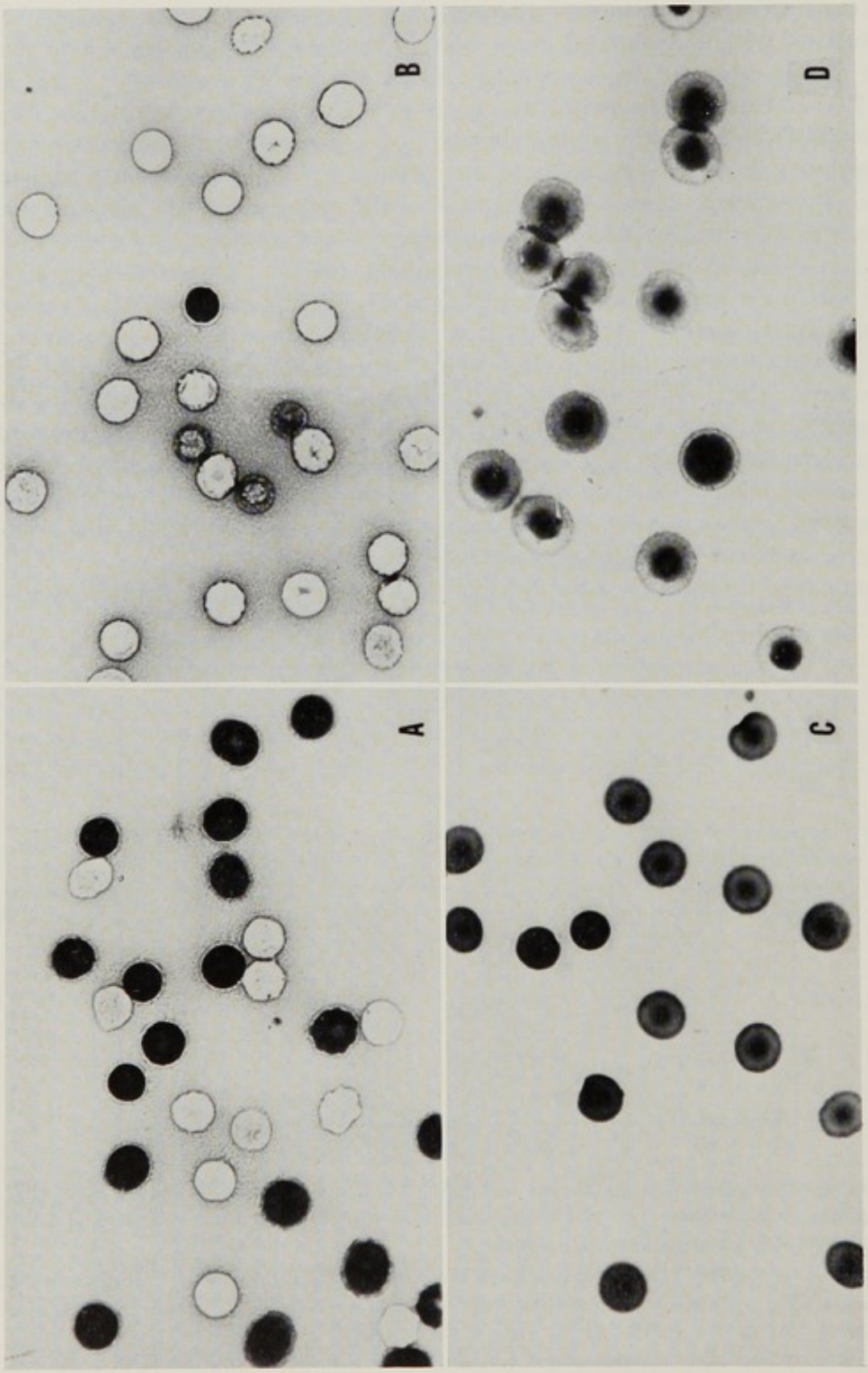


FIG. 5. See legend, facing page.

has been obtained by chemical measurements of methemoglobin formation at varied rates of H_2O_2 generation. In suspensions of acatalatic red cells, H_2O_2 is used almost quantitatively for hemoglobin oxidation; but if catalase is present in the system, only a small fraction of hemoglobin is oxidized. Therefore the amount of methemoglobin formed at a given H_2O_2-production rate is a function of catalase activity. By plotting methemoglobin formation against the rate of H_2O_2 generation, curves of different slope and shape are obtained (Figure 6). The difference between curves 2 and 3 is in agreement with the finding mentioned above that in blood smears of heterozygotes no evidence could be obtained for the existence of two cell populations differing in catalase activity.

The apparently homogeneous distribution of catalase in erythrocytes of heterozygous individuals from all three Swiss acatalasemic families is in agreement with the concept that mosaicism in heterozygotes has been found so far only in enzyme defects, which are inherited by way of the X-chromosomes. On the other hand, these experiments clearly demonstrate, that the principle used by Beutler et al.[15] for the demonstration of mosaicism in blood of female individuals heterozygous for G-6-PD deficiency may be applied to the study of any problem of this kind.

Cellular distribution of residual catalase activity in blood of individuals homozygous for acatalasia has been studied in the same way. As shown in Figure 5c, catalase activity is not evenly distributed in acatalatic blood, but rather is localized in a small fraction of red cells. Under suitable experimental conditions, a pseudomosaicism can be observed inasmuch as one out of 100 to 150 red cells behaves as if it contained approximately the same amount of catalase as a normal erythrocyte. The average percentage of apparently normal red cells detectable in blood smears of acatalatic Swiss individuals is 0.6-1.0% and corresponds roughly to the level of residual catalase activity as measured by means of the perborate method (0.1-1.3%). These figures also fall within the range of reticulocyte counts (0.3-1.0%). In this respect it is of interest that in acatalasia, the life-span of red cells is not markedly diminished[24] and that reticulocyte counts generally fall within the normal range (Schmid, unpublished data).

Fractionation of erythrocytes with regard to density and age can be achieved by centrifugation of red cell suspensions in a 20% solution of human serum albumin and subsequent separation of different layers.[37] On analysis of these fractions it has been observed that there is a direct proportionality between catalase activity and the number of reticulocytes. The least dense fraction contains up to 4% reticulocytes and has a catalase activity of up to 110 P.U./g Hb (Figure 7). In three homozygotes, catalase activity in reticulocytes and

FIG. 5. Blood smears stained according to *Kleihauer and Betke*[26] after preincubation of the red cell suspension with glucose oxidase and glucose. (A) Artificial mixture consisting of equal parts of normal and acatalatic erythrocytes. H_2O_2-production rate: $3 \cdot 10^{-9}$ Moles $\cdot$ ml^{-1} $\cdot$ min^{-1}. Normal cells are stained red; acatalatic cells are completely eluted and appear as ghosts. (B) Erythrocytes of A.B., homozygous for acatalasia, H_2O_2-production rate: $1.1 \cdot 10^{-9}$ Moles $\cdot$ ml^{-1} min^{-1}. (C) and (D) Erythrocytes of P.V., heterozygous for acatalasia, at two different levels of H_2O_2-production ($3 \cdot 10^{-9}$ and 10^{-8} Moles $\cdot$ ml^{-1} $\cdot$ min^{-1}). In (C) elution is just starting, whereas in (D) it has reached an advanced stage in all cells.[3]

erythrocytes, as calculated by extrapolation, may differ by a ratio of up to 300:1. It must be pointed out, however, that it has not been possible so far, to decide whether the catalase-positive cells visible in the smears treated according to Kleihauer and Betke[26] are identical with reticulocytes or not. If samples of normal blood are treated in the same manner, the fractions exert a similar distribution pattern as far as the reticulocyte count is concerned, but there is no significant difference in catalase activity.[4] This finding is in agreement with earlier observations of Sass[36] and Thorup et al.[44] that catalase belongs to those enzymes that normally do not manifest a significant alteration in activity level during the life-span of the red cell. The apparently drastic decrease in catalase activity during the life-span of acatalatic red cells can be explained in various ways; e.g., the instability of a variant enzyme or an accelerated inactivation of the normal enzyme. Whatever the cause may be, it is important to note that even in the homozygous acatalatic, there is a small, but distinct amount of catalase in blood, notably in juvenile red cells.

In order to obtain information on the distribution of catalase within the single erythrocyte, ghosts of normal and of acatalatic red cells were prepared. This was done by repeated washings of the cells in hypotonic salt solution of stepwise decreasing osmolarity, according to the method of Blomfield et al.[16] Normal and acatalatic erythrocytes lose most of their catalase activity when treated in this manner (Figure 8). However, ghosts of normal erythrocytes,

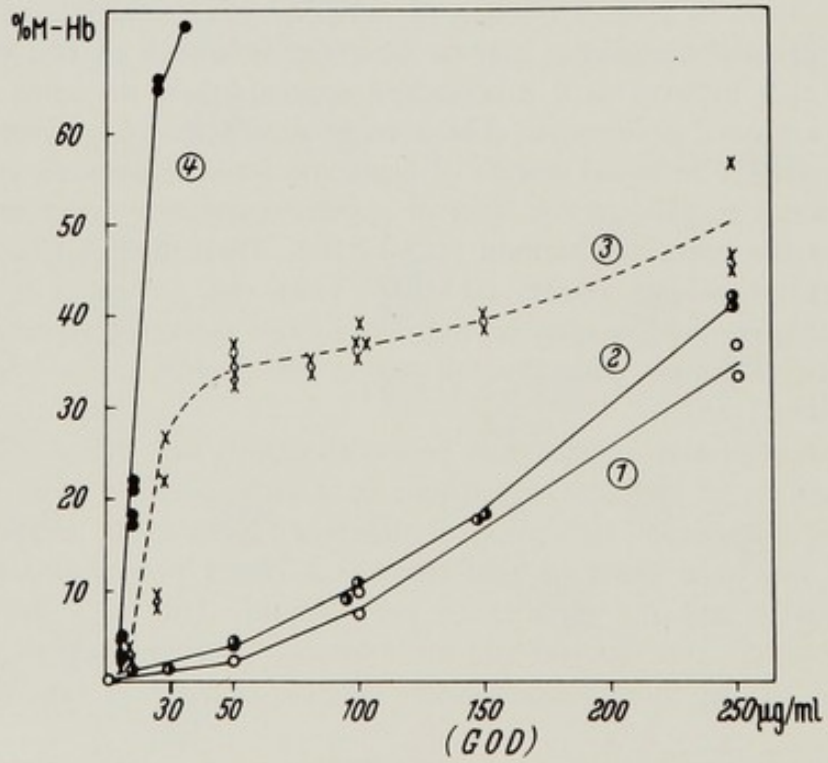


FIG. 6. Methemoglobin formation as a function of the rate of H_2O_2-production. Cell suspensions (approx. 1.5 mg Hb/ml) incubated for 30 min at 25°C. For spectrophotometric estimation of methemoglobin the method of Fleisch[21] has been used. (1) Blood of H.A. (normal), (2) blood of P.V. (heterozygote = hypocatalasemia; mother of M.V.), (3) 1 : 1 = mixture of 1 and 4, (4) blood of M.V. (homozygous acatalasia). The arrow indicates the experimental condition used in the standard procedure (i.e., 30 µg/ml glucose oxidase = $3 \cdot 10^{-9}$ Moles $H_2O_2 \cdot ml^{-1} \cdot min^{-1}$.

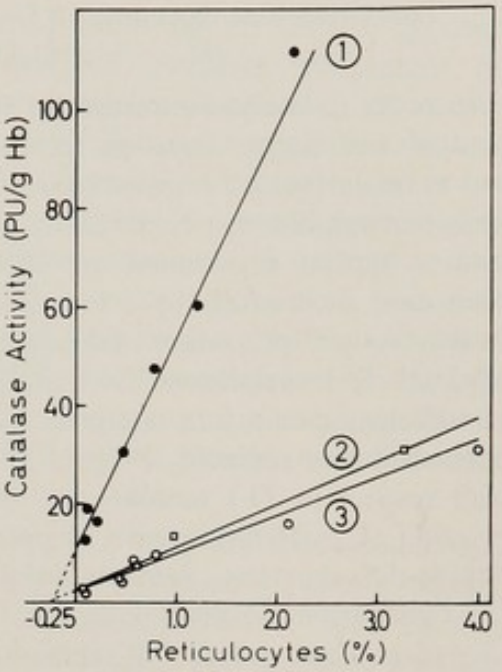


FIG. 7. Correlation between catalase activity and reticulocyte count in fractions of acatalatic blood obtained by the separation technique of Sass et al.[37] Blood donors: (1) A.B. (28 P.U./g Hb); (2) L.G. (6 P.U./g Hb); (3) P.G. (4 P.U./g Hb).

even if completely free of hemoglobin, still retain a small, but significant fraction of catalase, which amounts to 0.5 – 1.5% of the original total activity. This can be measured after solubilization of the ghosts by dilute desoxycholate solution (5 mM). No definite statement can be made whether the smaller figures obtained for relative catalase activity in ghosts of "acatalatic" red cells (0.03-0.2%) differ significantly from those of normal ghosts.

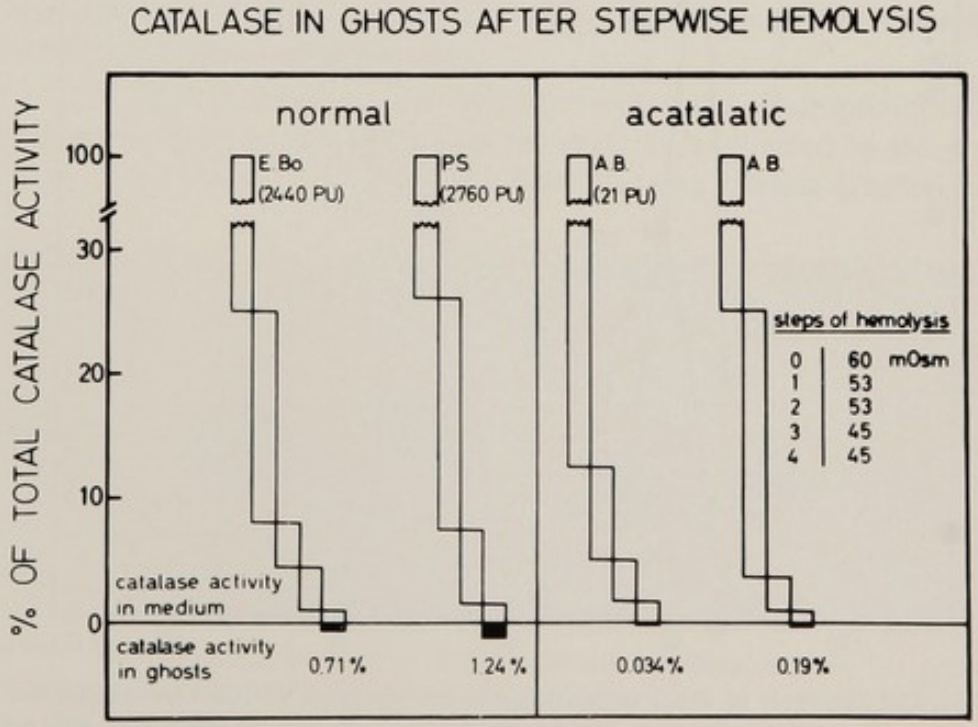


FIG. 8. Catalase activity in solution and in red cell-ghosts prepared according to Blomfield et al.[16] □ Catalase found in the eluate at subsequent washings; ■ Catalase detectable after solubilization of the ghosts in desoxycholate solution. Total catalase activity in the batch taken as 100%.

The Chemical Nature of Catalase in Blood of Heterozygotes and Homozygotes

In order to analyze the traces of residual catalase in "acatalatic" erythrocytes, a rapid and simple isolation technique, avoiding any use of organic solvents, had to be devised.[9] Complete separation of catalase (and other red cell enzymes) from hemoglobin was achieved by gel filtration with Sephadex G-100. Findings from a typical experiment are shown in Figure 9. This technique has also been used successfully by a Japanese group for the same purpose.[34] In contrast to the "rough" procedure of Herbert and Pinsent,[23a] the risk of denaturation is minimal. Thus, catalase-active material from an "acatalatic" subject was obtained in sufficient quantity to compare its properties with those of the enzyme from normal human red cells. Preparations from either source proved to be identical with respect to (1) mobility on the Sephadex G-100 column, (2) sensitivity to azide ($I_{50} \sim 10^{-6}$M), and (3) precipitation by anticatalase in the Ouchterlony double-diffusion test. Semiquantitative evaluation of the results obtained with the Ouchterlony method indicates that the concentration of antigenic protein, i.e., of catalase present in acatalatic blood, differs from that of normal blood by a factor of about 200. The ratio is of the same order as the difference in catalase activity in acatalatic and normal red cells.[31] This was taken as evidence for antigenic identity of catalase-active material in acatalatic blood and of catalase in blood of normal subjects. Consequently it was assumed that acatalasia (at least in the Swiss cases) may be considered as a controller gene mutation.[2]

While these studies were being carried out, the heterogeneity of erythrocyte

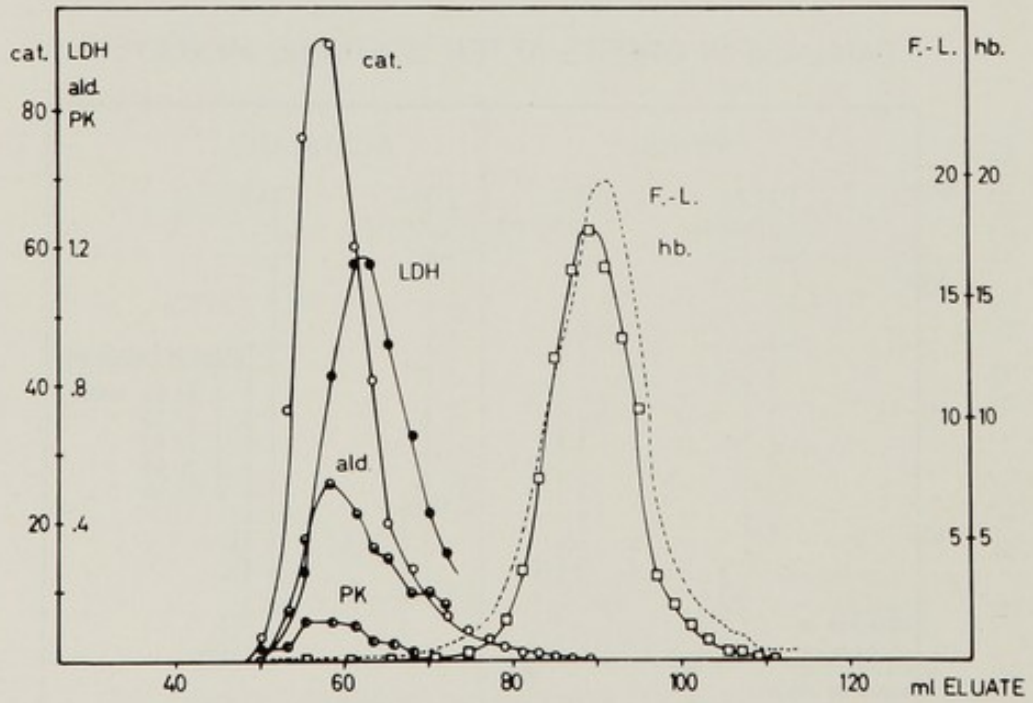


FIG. 9. Gel filtration of 3 ml of hemolysate on Sephadex G-100 (for details see Aebi et al.[9]); ○——○ catalase (cat.) as milli-equivalents of perborate used in 5 min per ml; ●——● lactate dehydrogenase (LDH) as international enzyme units per ml; ◒——◒ aldolase (ald.) as international enzyme units per 10 ml; ◐——◐ pyruvate kinase (PK) as international enzyme units per 10 ml: □——□ hemoglobin (Hb) as mg per ml; - - - - - Folin-Lowry color (F.-L.) as optical density at 750 $m\mu$ (= 1 cm) in the eluate.

catalase was described.[43] Therefore, it seemed promising to analyze catalase-active material from normal, hypocatalatic, and acatalatic individuals for heterogeneity. For this study, requiring large quantities of material, an alternative technique was devised by Matsubara et al.[29,30] By means of column chromatography on calcium phosphate-DEAE-cellulose complex gel, hemoglobin can be separated from catalase in a single step. In addition, two fractions of catalase can be obtained by subsequent extraction of the column with 0.15 M sodium chloride solution (fraction 1) and with 0.15 M secondary sodium phosphate solution, pH 8.2 (fraction 2). This separation method, a "batch technique" suitable for 100–250-ml. quantities of blood, has been applied to samples of normal human blood and blood from a heterozygous and a homozygous case of acatalasia. Thus, 6 different preparations of purified human red cell catalase were obtained by this procedure.

These catalase fractions have unequal mobilities if polyacrylamide-starch-agar mixed gel is used as a carrier medium. From a comparison of all six catalase fractions, it can be seen that both fractions obtained from normal blood (N_1,N_2) and one of the fractions from hypocatalatic blood (H_1) had the same mobility. On the other hand the fractions obtained from blood samples of an "acatalatic" individual (A_1A_2) as well as one fraction of a heterozygote (H_2) migrated considerably more slowly (Figure (10). Therefore, among the six fractions, two types of catalase, differing in electrophoretic mobility, can be distinguished ($N_1=N_2=H_1 > H_2=A_1=A_2$). From this observation it follows that the mobility of both fractions isolated from normal human red cells is the same. However, from the blood of a heterozygous individual, 2 fractions of different electrophoretic mobility can be obtained ($H_1 > H_2$); one fraction (H_1) resembling catalase of normal cells; the other (H_2) resembling catalase-active material isolated from acatalatic cells.

If fractions are combined and the mixture is analyzed electrophoretically, the mobility of the single components does not seem to be affected by the presence of the other fraction (Figure 11). The faster moving catalase fraction may be

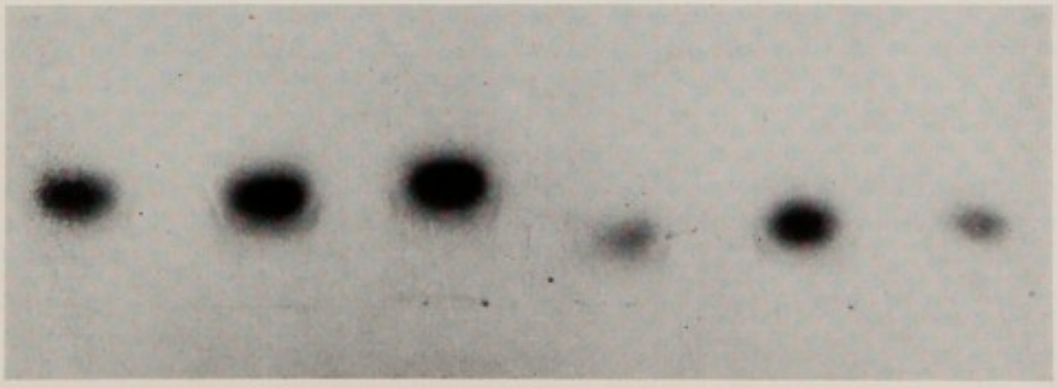


FIG. 10. Electrophoretic mobility of erythrocyte catalase isolated from (a) normal human blood, fractions N_1 and N_2, (b) blood of heterozygous case of acatalasia ("hypo-catalasemia"), P.V., fractions H_1 and H_2, (c) a homozygous case of acatalasia Th.V., fractions A_1 and A_2. From left to right: Single fractions N_1, N_2, H_1, H_2, A_1, A_2. Solutions analyzed: approx. 10 μl containing 0.1-0.5 μg protein. Electrophoresis was run at pH 9.0 (0.114 M. Tris-buffer) at 4°C for 8 hours (300 V; 60 mA; plate 8.5 x 10 cm). Staining of spots with amidoschwarz 10 B.[30]



FIG. 11. Electrophoretic mobility of catalase fractions, single and combined; from left to right: N_1, N_2, H_1, H_2, $N_1 + N_2$, $H_1 + H_2$. The nomenclature of the fractions refers to legend in Fig. 10 and is given in the text. Visualization of catalase active spots according to Thorup et al.[43] Other experimental conditions as described for Fig. 10.

termed I and the slower moving, II. Thus, the formula of catalase in blood of normal individuals may be expressed as I–I, of heterozygotes as I–II, and of homozygous acatalasia cases as II–II.

It is known from studies of other enzymes, that the degree of heat stability reflects minor variations in protein structure. This is also true for the variant enzymes of catalase found in blood and tissues of several strains of acatalasemic mice.[20] A comparison between normal human red cell catalase (catalase I) and the enzymes isolated from acatalatic blood (catalase II) shows that the latter is considerably more labile at 55° C.[30]

Heterogeneity of erythrocyte catalase has also been investigated by means of the fractionation technique of Thorup et al.[43] For this study whole blood as well as reticulocyte-enriched fractions has been used. There is no gross difference between the elution patterns of whole blood and those of the reticulocyte fraction (Figure 12). However, position and shape of peak I (fractions A and B of Thorup) are different for catalase from normal and acatalatic blood. Whereas the ratio between total catalase activity in peaks I and II, is fairly constant in

TABLE 4. *Column chromatography of catalase from whole blood and reticulocyte-enriched fractions of normal and acatalatic humans. All catalase activities are expressed in k-value figures per mg protein (measured as* $\triangle$ *E 215-225m*μ*)*

	Specific activity in hemolysate	Specific activity in main fractions of peak I (eluted by 12 mM phosphate)	of peak II eluted by 50 mM phosphate)	Ratio of total activity in peak I and peak II
Normal blood	0.443	97.0	72.5	2.1
Normal blood (25% reticulocytes)	0.440	84.6	79.5	2.0
Acatalatic blood	0.002	0.66	0.72	0.8
Acatalatic blood (13% reticulocytes)	0.016	2.47	0.56	5.3

normal blood, it varies considerably in specimens from acatalatic subjects (Table 4). The higher the reticulocyte percentage in the sample, the more the catalase activity shifts from peak II to peak I. These findings, although preliminary, permit us to conclude that there is not only a decrease in the activity level in acatalasia, but also a qualitative alteration of the chromatographic pattern of the residual enzyme. To what extent this heterogeneity is due to real isoenzymes or to enzyme conformers, i.e., reversible interconversions between labile alternative forms, remains to be elucidated.

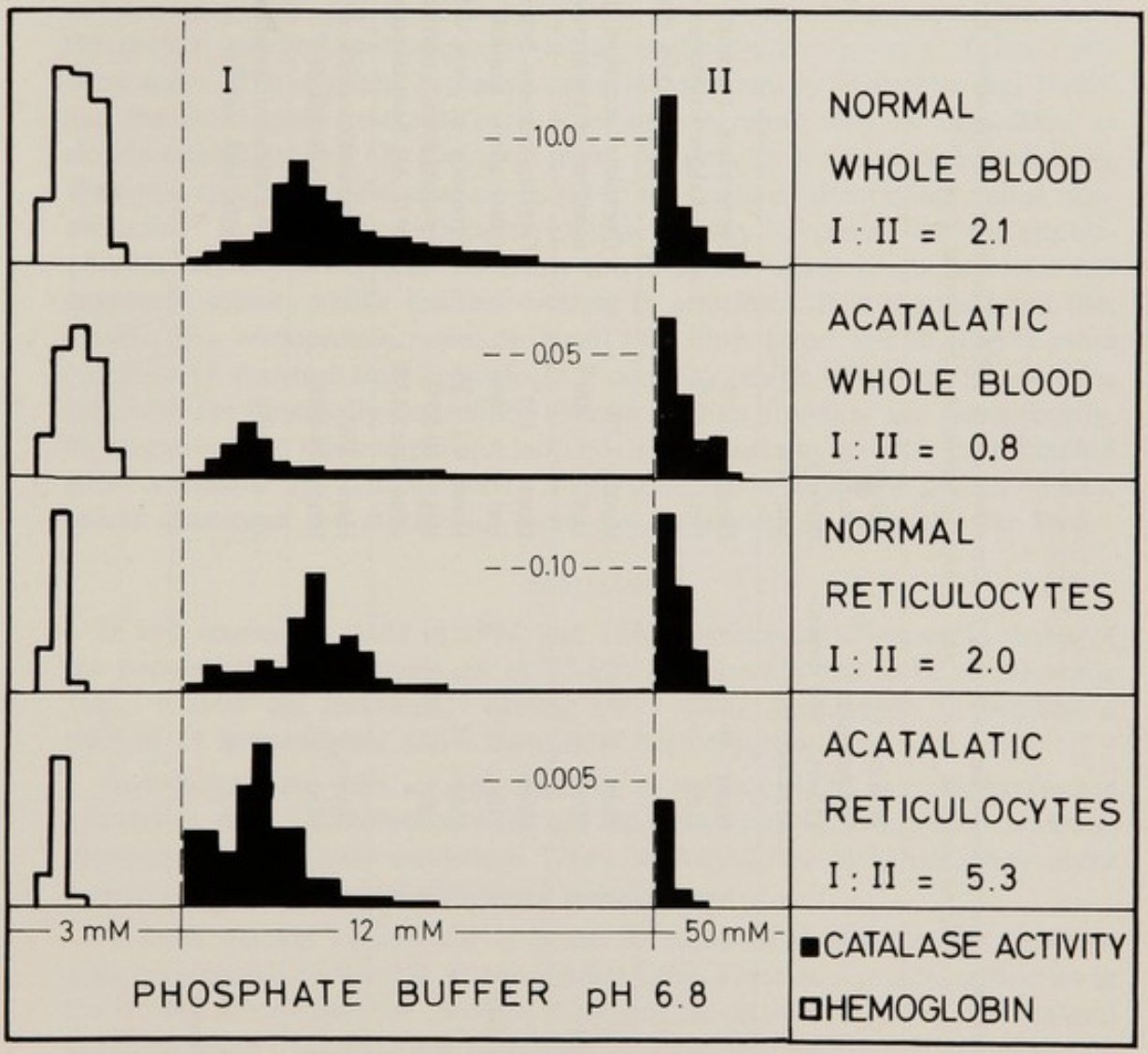


FIG. 12. Chromatographic fractionation on DEAE-cellulose of hemolysates from normal and acatalatic blood. Hemoglobin content of material analyzed (top to bottom):

(a) normal whole blood : 4.54 g Hb
(b) acatalatic whole blood : 4.88 g Hb
(c) normal reticulocyte-enriched cell suspension (25% reticulocytes) : 34 mg Hb
(d) acatalatic reticulocyte-enriched cell suspension (13% reticulocytes) : 52 mg Hb

Preparation of (c) and (d) by centrifugation of a layer of whole blood over a phthalate-ester mixture ($d^{20} = 1.086$) according to Danon and Marikovsky.[35] Dimension of columns: 1.6 x 23.0 cm; flow rate 60 ml/hour. The ratio I : II refers to the proportion of total catalase activity in peaks I and II. Note: For presentation of the data on catalase activity different scales are used (numbers indicate k-value/ml eluate).

TABLE 5. *Acatalas(em)ia cases and related anomalies reported in literature up to 1966. Tentative classification: Frequency, relative amount of residual catalase activity and clinical observations in various types.*[2,22,46]

Type (year of detection)	Origin (No. of families)	Number of homozygotes (Hom.) and heterozygotes (Het.)	Relative catalase activity (normal = 100%)	Remarks
I (1947)	Japan (31) Korea (1)	Hom: 66 Het: > 100	Hom: 0-3.2% Het: 37-56%	Incomplete recessive inheritance. Oral gangrene (Takahara's disease) in ~ 50% of homozygotes. Activity: trimodal distribution curve; no overlap.
II (1959)	Japan (1) Family 13 MI	Hom: 1 (♂) Het: —	Hom: 3.2% Het: ~ 100%	Complete recessive inheritance (involvement of modifier or suppressor genes?)[33].
III a. (1962)	Japan (1) kindred 29 OHH	Hom: 3 (Het: 17)	Hom: 0 (?) Het: > 56%	Overlap between heterozygous carrier and normals (dual allelic control ?)
III b. (1961)	Switzerland (3) Families V., B. and G.	Hom: 11 Het: ~ 30	Hom: 0.1-1.3% Het: 60-85% (one case: 15%)	Synthesis of two different types of catalase in heterozygotes (normal catalase + unstable variant). All homozygotes in good state of health.
IV (1963)	Israel (1) Iranian born	Hom: 1 (♂) Het: 15	Hom: 8% Het: 49-67%	Combination with deficiency of G-6-PD. Intolerance to fungicide.[39]
V (1963)	USA (1) Scandinavian and British extraction	Hom: 0 Het: 6	all ~ 100%	Allocatalasia: Synthesis of a variant catalase; activity and stability as normal catalase.[12]

The study of this enzyme deficiency condition has shown that lack of an enzyme is not necessarily due to repression or any other alteration within the controller gene system. It may, as well, be due to the synthesis of a variant enzyme of reduced activity or reduced stability, which seems to be the case in the Swiss type of acatalasia. However, the assumption that a catalase molecule of slightly altered structure is synthesized in some cases, cannot be extrapolated to all subjects with acatalasemia. The fact that there is a level of residual enzyme activity in homozygotes and in heterozygotes, as well as the fact that the symptomatology is not the same in all families, suggests that acatalasia is not a single entity, but must be considered as a group of anomalies. Based on the present state of knowledge, the tentative classification given in Table 5 has been made. The atypical Japanese cases investigated by Hamilton and Neel[22] and the Swiss cases resemble each other and therefore may be considered as closely-related groups. On the other hand, the findings we have presented bring the Swiss type of acatalasia much closer to the catalase abnormality called allo-catalasia.[12] In this type, erythrocyte catalase activity is normal, but the electrophoretic velocity is atypical. Evidently this enzyme variant seems to retain full enzymatic activity unlike residual catalase in acatalasia. If it is considered that catalase is a hemoprotein molecule, about four times larger and of slightly more complicated structure than hemoglobin,[38] one may realize how many possibilities may exist for genetically-determined alterations in its structure and consequently, its function. With this comparison in mind, it is realistic to consider each kindred with acatalatic (or acatalasemic) individuals as a separate genetic entity, unless identity of the mutational event can be proved at the molecular level.

Summary

In two screenings made in 1961 and 1965 covering all 19-year-old males of the population, 3 individuals out of 73,661 have been found to be acatalasemic (i.e., $\sim$ 0.04 per thousand). Among the 3 Swiss acatalas(em)ic families, a total of 11 homozygotes and a number of heterozygotes have been found.

Acatalasemic red cells are very sensitive to reagent H_2O_2, peroxide-producing cytostatics, and X-irradiation. They are unable to perform coupled peroxidatic reactions, e.g., formate oxidation. When stimulated by ascorbate, they show a twelve-fold increase in HMP-shunt activity.

A small amount of catalase is found in blood of acatalas(em)ic subjects. This corresponds to 0.5-2% of the normal level. There is a pseudomosaicism in the cellular distribution of catalase, inasmuch as catalase seems to be localized in only a small fraction of the total red cell population (0.5-1%). Fractionation by density and age has shown that there is a linear correlation between catalase activity and reticulocyte count. Thus in acatalas(em)ic individuals in contrast with normal ones, catalase activity decreases considerably during the life-span of the cells.

Catalase preparations, isolated from blood of normal and acatalas(em)ic subjects, in spite of their enzymatic and antigenic identity, differ in electrophoretic mobility in polyacrylamide gel and in heat stability.

It is suggested that in the Swiss type of acatalas(em)ia, a less stable enzyme variant is synthesized. Since considerable differences exist between the acatalas-

(em)ia kindreds in Japan, Israel, and Switzerland—indicating heterogeneity—each of the five types must be regarded as a different genetic entity.

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DISCUSSION

JAFFÉ, CHAIRMAN, NEW YORK: Dr. Aebi's and Dr. Takahara's papers are now open for discussion. It is interesting that this pattern of a series of mutations with alterations in structure may well be applicable to more and more enzyme deficiencies. If anything starts out to appear to be very simple, as more people study the problem, it becomes quite complicated.

STAMATOYANNOPOULOS, SEATTLE: I wonder about the validity of the classification of these several genetic types of acatalasemia, which is based only on quantitative data in heterozygotes. The differences between the heterozygotes for the Japanese and the Swiss type could be attributed to differences in techniques. I would like to ask Dr. Aebi and Dr. Takahara whether they have exchanged samples from heterozygotes from Japan and Switzerland.

If the differences in catalase levels between the Swiss and the Japanese heterozygotes really represented differences in the expression of acatalasia genes, we would expect to detect such differences in the homozygotes also. However, both Swiss and Japanese homozygotes have similarly low catalase levels (between 0 and 3%). On the other hand, the differences in the heterozygotes could be attributed not to the acatalasia genes but to the normal catalase present in the Japanese and the Swiss populations.

AEBI, BERNE: Dr. Takahara and I wish that some day we shall have the opportunity to visit each other and to examine each other's cases. So far, this has been done only by mail. I know that Dr. Takahara has examined catalase activity by our technique, and we have done it the other way round. The results are the same. Even if there might have been minor differences in the technique for estimating the level of residual enzyme activity, these could not explain the clear-cut difference in the activity level of the heterozygote. As shown by Hamilton and Neel in the heterozygote type III, the activity level is between 60 and 80%, whereas in the classical type I (Dr. Takahara's disease) this is almost exactly 50%. Therefore, in my opinion, the difference in the level of the heterozygote is another indication that we have to consider every family as a separate genetic entity unless we can prove identity at the molecular level. For this, I contacted Dr. Schroeder in Pasadena, who I think ranks first in the determina-

TABLE 1. *Catalase activity in normal hypocatalasemic, and acatalasemic red blood cells.*

		MANOMETRY warburg's Q_{O_2}/mgHb	TITRATON with $KMnO_4$			
			Peroxide (H_2O_2)		Perborate ($NaBO_3 4H_2O$)	
			ABCC & Okayama's	Inoue's	Feinstein's PU/gHb	
Normal	A		4.71		3200	(Aebi's case)
	B		4.67		3245	
	C		5.34	20 persons	3344	12 children
	D	7232	5.00		3409	
	E		5.29		3722	
	m+6	7232	5.00 ± 0.3	7.4 (4.3~9.5)	3384 ± 184	3142 ± 393
Hypocat.	Fuk	4462	2.22		1116	10 persons
	Fuj	5200	2.41		1114	
	m+6	4831 ± 654	2.32 ± 0.2		1115 ± 2	1955 ± 2
Acat.	Nat	12.5	under analytical sensitivity		4.1 ***	7.5
	Fuk	13.3			5.5 ***	12.0
	Nai			9 persons	5.4 **	4.0
	Fui				5.9 *	4.0
	Gio				6.6 *	5.0
	m+6	12.9 ± 0.4		(0~0.1)	5.5 ± 0.8	5.9 ± 3.1

Lancet Report (1952) : Q_{O_2} 8 ±1.8 in Acatalasemia
4500 in Normal

Oral Disease : * mild, ** moderate, *** severe

tion of the primary structure of catalase. He is not yet ready to approach the problem of acatalasemia at the molecular level although he has described the sequence of many peptides of beef liver catalase as well as of human blood catalase. So far, we have to restrict ourselves to chromatography, electrophoresis, and to heat stability tests.

TAKAHARA, OKAYAMA: I should like to describe the techniques that Prof. Aebi and I are using for the estimation of catalase values. As shown in Table 1, we are routinely using the potassium permanganate titration method, slightly modified from von Euler's, and, occasionally, particularly for the tissues, Warburg's manometric method. Prof. Aebi uses the perborate method devised by Dr. Feinstein. This is also a kind of titration method using potassium permanganate. The units obtained by these methods are quite different, so I rechecked some of our cases, using Dr. Aebi's technique, and the results are shown in the table. Both values agree very closely except for the difference in the average value for hypocatalasemics. From these data, it seems to me that there is a close similarity between the Japanese and Swiss cases. The only difference is that Swiss "patients" have not shown any oral lesions up to date. So, at the moment I do not have any idea about the difference between the cases of Switzerland and Japan.

I would like to add a few words about the catalase values of hypocatalasemia. The newly-made distribution curve from the rechecked data on 13 families and their siblings was shown in Figure 2 of my paper. This shows a clear-cut difference between the heterozygote and homozygote groups. These relationships are also substantiated by the data shown in Figures 1 and 2. The discriminate limit of heterozygotes was estimated by the electronic computer using the above data. The upper limit was 3.6 and the lower limit 1.0 by the titration method.

MORRISON, DUARTE: I would like to direct my first question to both the speakers. I wonder if it is possible that any discrepancy might be attributable to cells other than red cells. Secondly, I would like to ask Dr. Takahara whether he has explored the possibility that the catalase in the tissue of the acatalasemic individuals might arise from the blood in these tissues. Thirdly, as you are aware, there is evidence for two types of catalase; and I wonder if the catalase might not be localized in two different subcellular particulates. There are conflict-

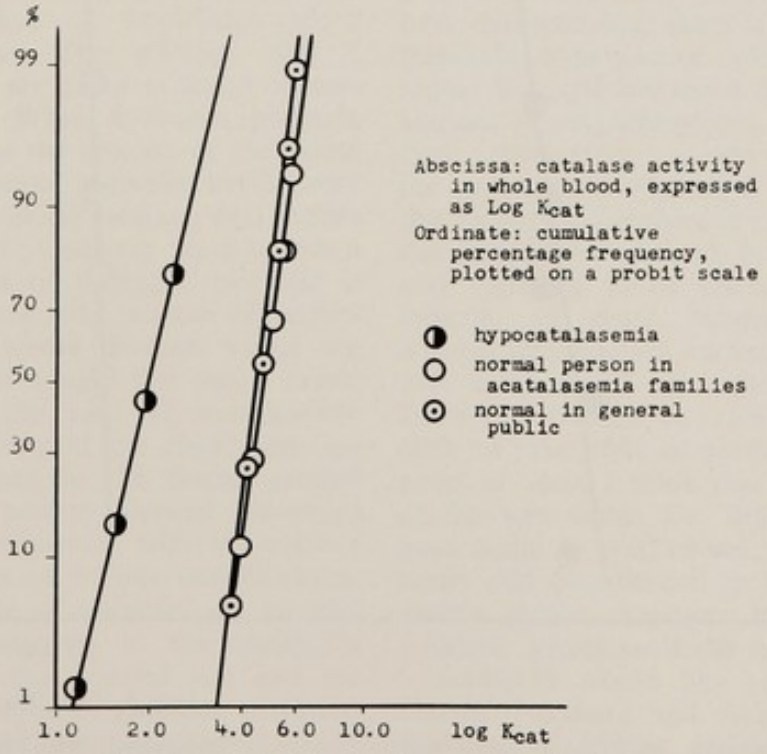


FIG. 1. Log-probit plot of distribution frequency of blood catalase activity.

ing data as to the localization of catalase in the cell. Is it possible, for example, that a liver cell contains two types of catalase, each being localized in different regions of the cell and under separate genetic control?

Lastly, I would like to make a comment regarding the etiology of oral lesions in acatalasemia. We've recently demonstrated the presence of an enzyme, lactoperoxidase, in the salivary, lacrimal, and mammary glands and in only these tissues. This enzyme is a well-characterized hemoprotein peroxidase, which has been shown to inhibit the growth of certain microorganisms. I would like to suggest the possibility that the gingivitis observed in acatalasemia may be due primarily to the result of oxidation of the tissue catalyzed by this enzyme. In the presence of catalase, the substrate for the peroxidase, hydrogen peroxide, is destroyed; hence, no oxidation and no gingivitis in the normal individual.

AEBI: The level of tissue catalase in Japanese and Swiss cases is different. This has been shown by Dr. Krooth (Ann Arbor) on analysis of fibroblasts from each group. He obtained skin biopsy specimens from Japan as well as from Switzerland. He has reported that the residual catalase activity in fibroblasts of A. B. (Swiss-type acatalasemia) is 3-5% of the normal level. In the tissue cultivated under identical conditions from a Japanese case, however, no catalase activity could be detected. This discrepancy is just what makes the story interesting.

The catalase level is rather low in connective tissues. Therefore, the exact level is probably very critical, and I wouldn't be astonished if Takahara's disease would occur only in those cases in which the tissue catalase level is lower than in cases that are not affected by this dis-

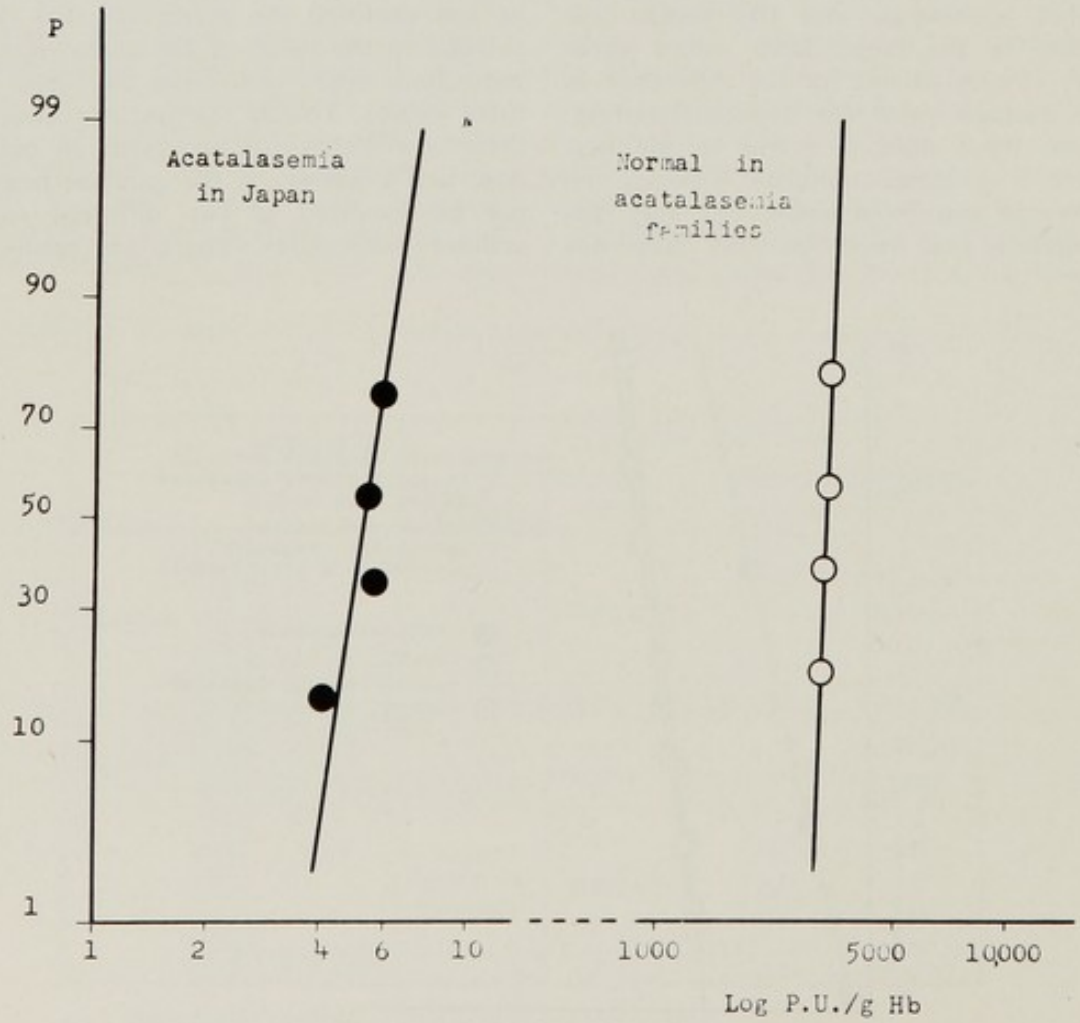


FIG. 2. Catalase activity in acatalasemic subjects compared with that in normal members of acatalasemic families.

ease. So far the data are too scarce to permit any conclusions.

Catalase in liver is mainly localized in a special organelle recently named "Peroxisome." For further information regarding its function and localization, I would like to refer to a review article by Dr. DeDuve in Physiological Reviews published in April, 1966.

MORRISON: Although the studies of Dr. DeDuve and his coworkers are very nice, indeed, they do not completely answer the question. In this type of study there is always destruction of the enzyme in the particulate fractions. Although most of the enzyme is clearly localized in the microbodies or peroxisome, a relatively small percentage may be in mitochondria or some other subcellular particulate.

TAKAHARA: The human tissues, other than blood, that we could examine are pharyngeal mucosa, nasal mucosa, nasal polyp, tonsils, skin, muscle, and bone marrow obtained by sternal puncture. None of these showed any catalase activity. A sample of liver tissue obtained by liver puncture was examined in Japan by Dr. Kaziro and it had no catalase. Tissue from internal organs other than liver has not been examined yet.

I cannot say whether your idea about the pathogenesis of gingivitis is possible because I have no knowledge about lactoperoxidase. The etiology that I mentioned in my paper is based on several findings. When hydrogen peroxide is injected into the mucosa of the palate of ducks or geese, normally having very low values of blood catalase, oral lesions appear readily. Contrary to this, when the same dose of hydrogen peroxide is injected into the oral mucosa of rabbits or chickens, whose catalase values are in the normal range, the lesion rarely appears. In the case of acatalasemic fowl, the activity of the respiratory enzymes contained in the tissues around the injected portion showed decreased activity in comparison with the activity seen in tissues of rabbits and chickens. In the absence of catalase, the circulation of erythrocytes in the capillaries stops around the injected site and the cells are destroyed by peroxide produced by proliferating microorganisms. These factors and pathologic findings in tissue suggest that this type of oral lesion is mainly caused by the lack of catalase. Hygienic and nutritional states as well as other enzymes should be considered in future studies.

MASOUREDIS, SAN FRANCISCO: I would like to ask Professor Aebi to comment on the use of a fluorescent-labelling antibody technique to measure the quantity of catalase in red cells. One of the difficulties is that if catalase is intracellular, just how does the antibody find it to react with it? I wonder if you might just make some general comments about these results.

AEBI: First we tried the fluorescent antibody technique together with Dr. Micheli at Lausanne, but without success. We then modified the technique of Kleihauer and Betke for our purpose. I think this technique is simpler, and more straightforward; therefore, I prefer this method to the antibody technique. However, one has to admit that the antibody technique, according to the specificity of the antiserum used, might lead to a different result.

BEUTLER, DUARTE: The Kleihauer and Betke technique is good for demonstrating that either a great deal of methemoglobin is present or that methemoglobin is virtually absent. But I think that, as both Dr. Stamatoyannopoulos and I have demonstrated, there is some sort of threshold phenomenon with this technique. If a cell has more than a certain amount of oxyhemoglobin, it has sufficient peroxydatic activity to stabilize the hemoglobin, while if it has less than this amount, then there is a tendency for the hemoglobin not to be peroxidated and therefore to be eluted. It is very difficult to draw conclusions about whether the catalase content of the 1 or 2% cells which you see is actually 100% of normal or 75% of normal, 50% or even 30% of normal. From this point of view, I think that if something of the sort which Dr. Masouredis suggests could be worked out, it would certainly add quantitation to the very suggestive results that you have obtained with the Kleihauer-Betke technique.

JAFFÉ: I would like to thank Drs. Harris, Takahara and Aebi, and all of the discussants, for providing such an interesting beginning for this symposium.

Genetic Variation in Red Cell Galactose-1-Phosphate Uridyl Transferase

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THE RED BLOOD CELL would appear to be ideal for the study of inborn errors of metabolism of man because of its easy availability. Unhappily, it has often proven to be inadequate for this purpose. Sometimes, as in the case of phenylketonuria, the gene under investigation is not active in the hematopoietic tissues. In other instances, the red cell has lost, with its nucleus and mitochondria, its capacity to reflect the metabolic activities of most other tissues.

It is particularly fortunate that the red cell is well-suited to the study of galactosemia, for early diagnosis of this disorder is of great importance to the young infant. Galactosemia is caused by hereditary absence of activity of galactose-1-phosphate uridyl transferase, the enzyme catalyzing, together with epimerase, the conversion of galactose-1-phosphate to glucose-1-phosphate.[22] Galactose and its metabolic products, galactose-1-phosphate and dulcitol, accumulate in the tissues when galactose-1-phosphate uridyl transferase activity is absent. The afflicted infant takes food poorly, develops hepatomegaly, jaundice, cataracts, aminoaciduria, and mental retardation. The development of the child is normal, however, if dietary galactose is restricted.[14]

Because of the importance of early diagnosis, we have designed a screening test for galactosemia, drawing upon the lessons learned in designing screening procedures for glucose-6-phosphate dehydrogenase deficiency.[28,36] The application of these screening tests to large numbers of normal individuals has revealed the presence of an additional gene in the population, a gene that leads to the production of an abnormal enzyme and results in a decrease in red cell enzyme activity.[7] It is our purpose to describe recent studies of the metabolism of galactose by human red cells, to discuss some of the screening procedures that may be used to detect galactosemia, and to show how the application of these procedures has resulted in the detection of the new gene. We will describe what has been done to clarify the biochemical genetics of this new abnormality and speculate regarding the possible reasons for the maintenance of apparently "neutral" polymorphisms in the population.

THE METABOLISM OF GALACTOSE

Galactose is a monosaccharide differing from glucose only in the position of the hydrogen and the hydroxyl group at the 4-position. The enzymatic mech-

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anisms of the body have the capacity to distinguish between the two sugars although they are almost identical in structure, and epimerization of galactose-phosphate to glucose-phosphate is necessary before galactose can be metabolized further.[25]

Unlike glucose, fructose, and mannose, which in the presence of ATP are phosphorylated at the 6-position, α-D-galactose undergoes phosphorylation at the 1-position. This process is mediated by the enzyme galactokinase.

(1) α-D-galactose + ATP→ α -D-galactose-1-phosphate + ADP.

Some of the properties of this enzyme from various sources are summarized in Table 1.

After galactose-1-phosphate (Gal-1-P) has been formed, it is transformed to glucose-1-phosphate (G-1-P) by the combined action of Gal-1-P uridyl transferase and epimerase.[25]

$$\text{(2) } \alpha\text{-D-Gal-1-P} + \text{UDPG} \xrightarrow{\text{transferase}} \alpha\ \text{G-1-P} + \text{UDP Gal.}$$

$$\text{(3) UDPG} \xrightarrow{\text{epimerase}} \text{UDP Gal.}$$

G-1-P is rapidly converted to glucose-6-phosphate (G-6-P) through the mediation of phosphoglucomutase (PGM) in the presence of glucose 1-6-diphosphate (G-1-6-P).

$$\text{(4.) } \alpha\ \text{G-1-P} \xrightarrow[\text{G-1-6-P}]{\text{PGM}} \alpha\ \text{G-6-P}$$

Both galactose and galactose-1-phosphate accumulate in the blood and tissues when galactose-1-phosphate uridyl transferase is absent. The reduction of galactose to dulcitol has been implicated in the formation of galactose cataracts.[23] In addition, the accumulation of Gal-1-P serves as a useful means of appraising the adequacy of dietary control in galactosemia.[15] It has not been possible to confirm the finding that Gal-1-P inhibits G-6-PD.[9,24] Yet, it is possible that accumulation of this phosphate may interfere with the metabolism of the cell in some other way.

Unexpected, and Partially Unexplained, Findings in Metabolism of Galactose by Erythrocytes

When glucose serves as a substrate for methemoglobin reduction, maximal methemoglobin reduction rates are observed at levels of approximately 1 millimolar. The rate of methemoglobin reduction in the presence of glucose is greatly accelerated by the addition of methylene blue, toluidine blue, Nile blue sulfate, or other dyes with an appropriate redox potential. This occurs because these dyes have the capacity to link TPNH with the reduction of methemoglobin through TPNH diaphorase. The rate of methemoglobin reduction is greatly accelerated without an appreciable increase in the rate of glucose utilization, since the glucose is recycled through the hexose monophosphate pathway, reducing more methemoglobin than would be reduced were the glucose metabolized only through the Embden-Meyerhoff pathway. The established pathways of galactose metabolism would lead one to suppose that the maximum rate of methemoglobin reduction would also be achieved when the limiting enzyme,

TABLE 1. *Characteristics of galactokinase from various sources.*

Authors	Tissue	K_m Galactose	K_m Galactose in presence of Gal-1-P	K_m ATP	Gal-1-P (product) inhibition
Cuatrecasas and Segal[13]	Adult rat liver	1.5×10^{-4}M	0.8×10^{-4}M (1mM)* 0.8×10^{-4}M (5mM)	1.2×10^{-4}M	15% (1mM) 50% (5mM)
	Newborn rat liver	6.5×10^{-4}M	6.5×10^{-4}M (1mM) 6.5×10^{-4}M (5mM)		50% (1mM) 60% (5mM)
	Fetal rat liver	9.1×10^{-4}M			
Ballard[1]	Pig liver	$1\text{-}3 \times 10^{-4}$M		2×10^{-4}M	
Sherman and Adler[21]	E. Coli	7×10^{-4}M		1×10^{-4}M	
Heinrich[20]	Yeast	5.3×10^{-4}M for galactose			
Mathai and Beutler (unpublished)	Adult blood	1.35×10^{-4}M	0.89×10^{-4}M (1mM) 0.84×10^{-4}M (5mM)		
	Fetal blood	2.2×10^{-4}M	1.64×10^{-4}M (1mM) 1.11×10^{-4}M (5mM)		

*Concentration of Gal-1-P

galactokinase, was saturated with galactose and that similar degrees of acceleration of methemoglobin reduction would occur when methylene blue was added (Figure 1). In point of fact, the rate of methemoglobin reduction continues to increase until very high levels of galactose are present, levels which approximate 3700 times the Michaelis constant of galactokinase. Furthermore, little, or no acceleration of methemoglobin reduction is observed when methylene blue is added (Figure 2).

We believe that we have been able to find a satisfactory explanation for the failure of methylene blue to accelerate methemoglobin reduction when galactose serves as a substrate. In the transformation of glucose-1-phosphate to glucose-6-phosphate by phosphoglucomutase, the metabolism of galactose appears to merge with that of glucose. The merger is, in point of fact, incomplete. Glucose-6-phosphate may exist in two forms, α and β G-6-P. The product of α and β-D-glucose phosphorylation is a mixture of α and β G-6-P; in contrast, the action of PGM on α G-1-P forms only α G-6-P. This anomer serves as an adequate substrate for phosphohexose isomerase, but not for glucose-6-phosphate dehydrogenase (G-6-PD).[32] Some interconversion of the two anomeric forms undoubtedly occurs. The limited amount of the β anomer available, however, may be sufficient to consume all of the TPN available for the action of glucose-6-phosphate dehydrogenase. Hence, the addition of methylene blue to red cells metabolizing galactose fails to increase significantly either oxygen consumption,[2] methemoglobin reduction[9] (Figure 3), or GSH stability[10] (Figure 4).

The fact that increasing concentrations of galactose resulted in increasing

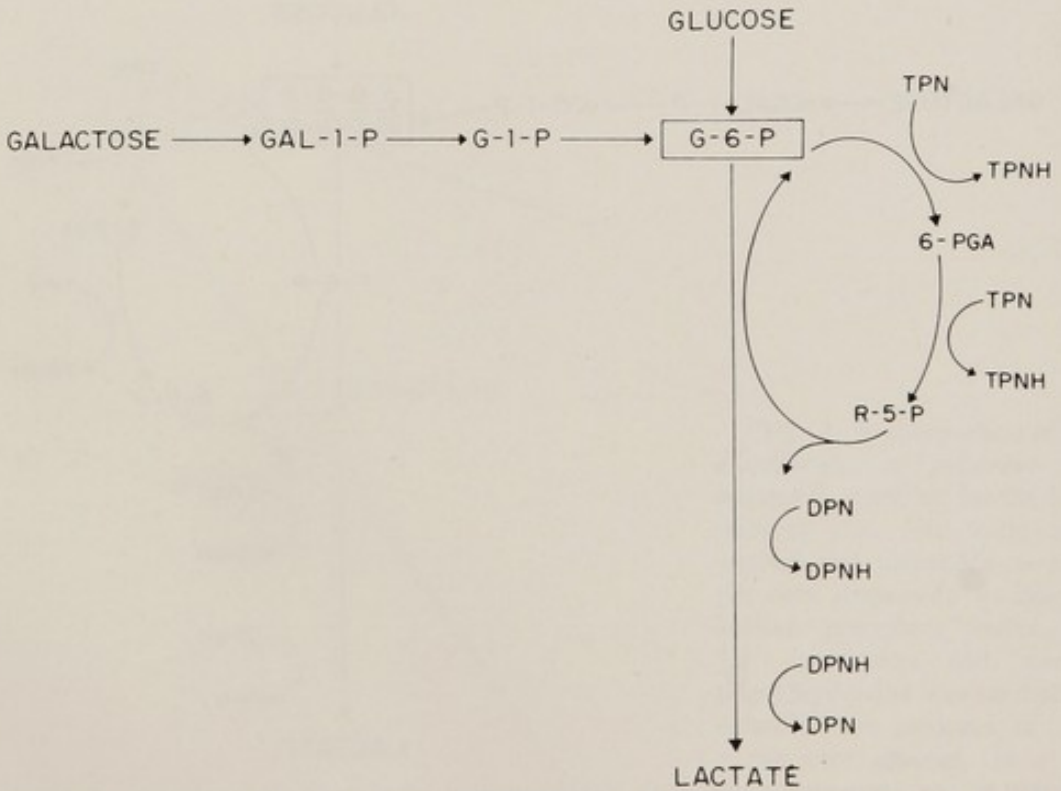


FIG. 1. An abbreviated schematic representation of galactose and glucose metabolism in the erythrocyte. It appears that the metabolism of these two monosaccharides merge at the glucose-6-phosphate step.

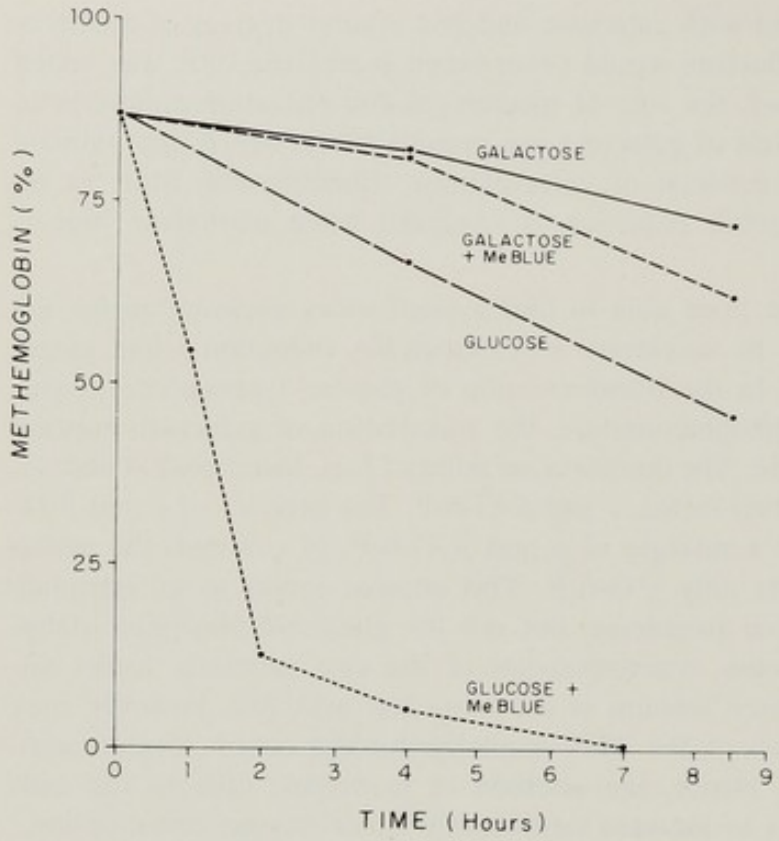


FIG. 2. The effect of methylene blue on methemoglobin reduction with galactose and glucose substrates (based on data of Beutler and Collins[6]). Methylene blue results in marked acceleration of the rate of methemoglobin reduction when glucose serves as substrate, but there is little or no acceleration when galactose is substrate.

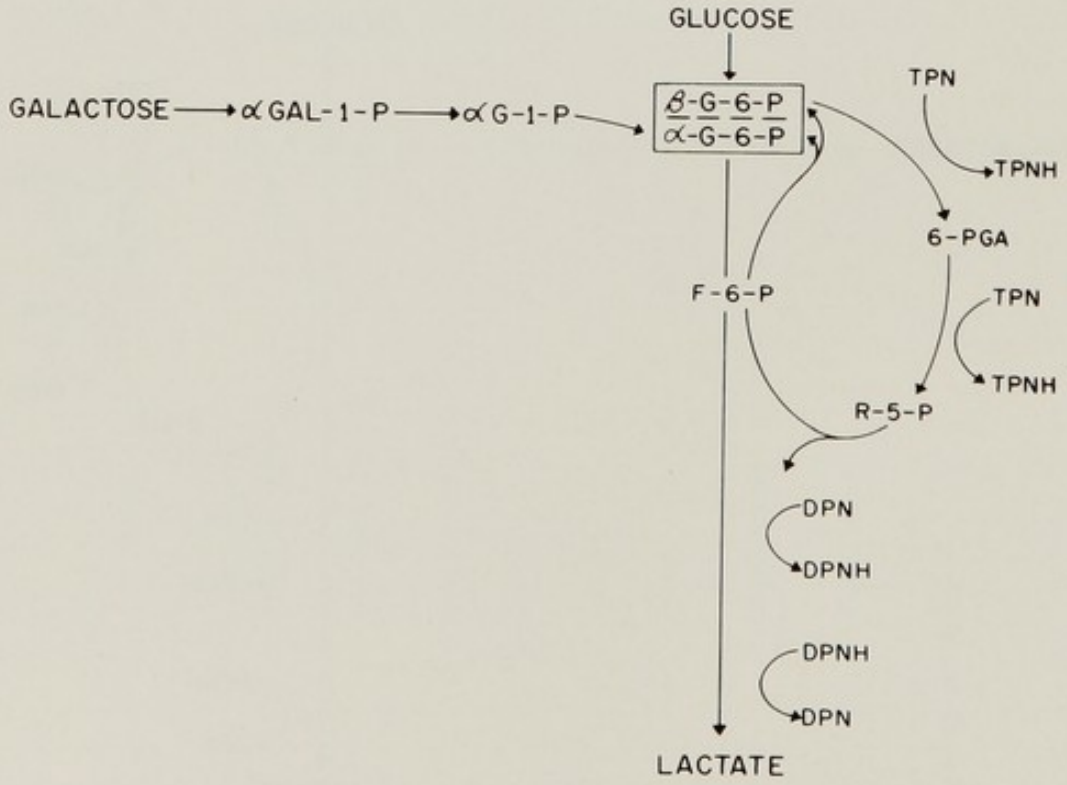


FIG. 3. An abbreviated schematic representation of glucose and galactose metabolism by the erythrocyte. Galactose is converted into the α anomeric form of galactose-1-phosphate, glucose-1-phosphate and glucose-6-phosphate. α glucose-6-phosphate must be converted to β glucose-6-phosphate before it can pass through the shunt pathway.

rates of reduction of methemoglobin suggests that another pathway of galactose metabolism may be present, at least in the erythrocyte. Hjelm and de Verdier[21] have shown that the utilization of galactose by human red cells increases markedly as the concentration of galactose is increased.

We have been able to demonstrate that at high galactose concentrations methemoglobin reduction is as rapid in galactosemic red cells as in normal red cells. Thus, the pathway which is operative at high galactose concentration does not appear to involve glucose-1-phosphate uridyl transferase. One such possible pathway might consist of phosphorylation of galactose at the 6-position to galactose-6-phosphate, its transformation to tagatose-6-phosphate by phosphohexose isomerase and its subsequent phosphorylation by phosphofructokinase to tagatose-1-6-diphosphate, the galactose-like analog of fructose-1-6-diphosphate. Subsequent splitting of this ester between the 3- and 4-carbon by aldolase would be expected to result in the formation of dihydroxy-acetone phosphate and of glyceraldehyde-3-phosphate, the same products as are formed by the splitting of fructose-1-6-diphosphate.

We have been unable to demonstrate any phosphorylation of galactose at the 6-position by human hemolysates although this has been reported for rat brain galactokinase, with low efficiency and a high Michaelis constant.[34] Chromatographic examination of trichloracetic acid extracts of human red cells and hemolysates incubated with and without fluoride fails to reveal the presence of any detectable quantities of galactose-6-phosphate, using the galactose oxidase system after hydrolysis with alkaline phosphatase.

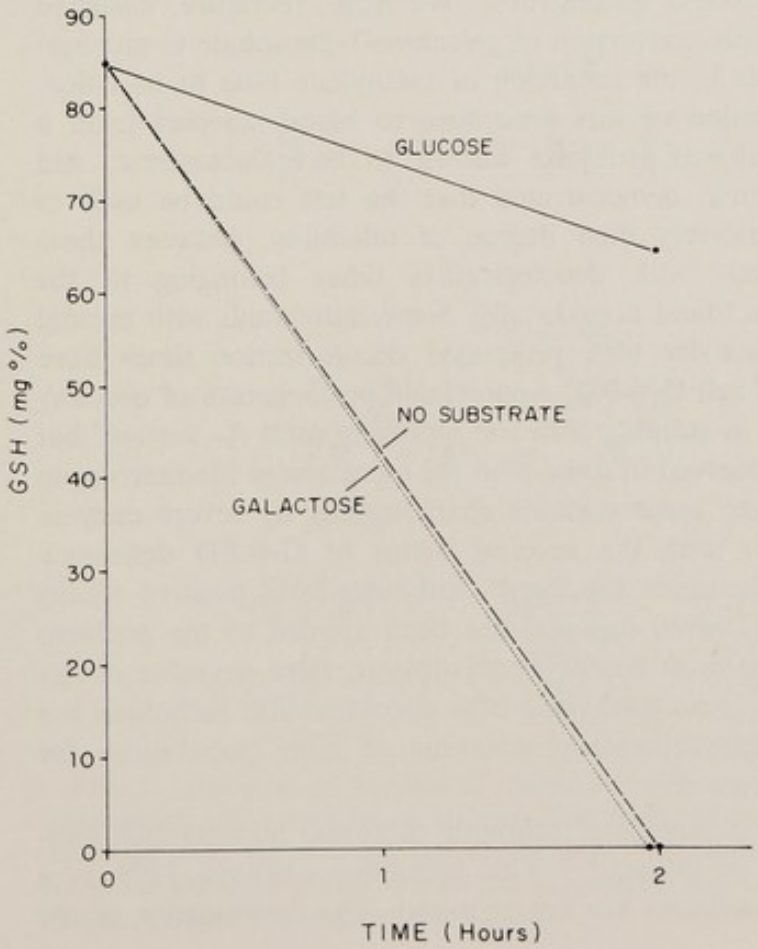


FIG. 4. A comparison of the effectiveness of galactose and glucose as substrate for the GHS stability test. Red cells were washed and suspended as a 50 per cent suspension in isotonic sodium phosphate buffer, pH 7.4. Incubation with acetylphenylhydrazine was carried out either in the presence of .250 g per cent glucose, 10 g per cent galactose or in the absence of substrate.

Although these studies do not rule out the possibility that low concentrations of galactose-6-phosphate are formed, but are not detected by the system used, it is necessary also to search for other possible pathways of galactose metabolism in the erythrocyte.

Screening for Galactosemia

Some screening tests for galactosemia depend upon the fact that in the presence of dietary galactose and occasionally even prenatally-acquired galactose, galactose accumulates in the blood and may be spilled in the urine. Thus, one of the oldest methods of screening for galactosemia is the examination of the urine for reducing sugar. If sugar is found, differentiation from glucose may be achieved by a variety of means. More recently, a microbiological inhibition assay for galactosemia has been devised in which the galactose and galactose-1-phosphate content of erythrocytes is estimated.[18] All of these methods, however, depend upon dietary ingestion of galactose. Since galactosemic infants often do not take feedings well, it is entirely possible for such tests to give false negative results.

It is therefore preferable to examine the patient for the defect which is basic to the disorder. This can most readily be accomplished by study of the erythrocyte. Two techniques employing radioactive galactose have been devised. One depends upon trapping radioactive carbon dioxide,[26] and the other upon chromatographic separation of intermediates.[29] No large-scale field trial of either technique has been reported, and it appeared to us that for the widest application, nonisotopic techniques would be desirable. We have, therefore, designed a system in which the enzymatic conversion of galactose-1-phosphate to glucose-1-phosphate eventually results in the reduction of methylene blue to its colorless leuko-derivative. Application of this procedure to blood samples from a large number of normal subjects, subjects known to be galactosemic, and proven carriers of galactosemia, demonstrated that the test could be used to differentiate, with a comparatively high degree of reliability, between these groups.[6,12] Normal individuals with decolorization times belonging to the heterozygous range have been found occasionally. Some individuals with normal Gal-1-P uridyl transferase activity with prolonged decolorization times have been found to be lacking red cell G-6-PD. Appreciable prolongation of decolorization times does not occur in subjects with the relatively mild A- variant, but mild prolongation has been observed in some with the more severe Mediterranean variant, and in some with the rarer variants characterized by severe enzyme deficiency. Blood of patients with the severest forms of G-6-PD deficiency occasionally even fails to decolorize the dye.[16] Although false positive results may occasionally be observed when this test has been applied to the problem of detecting heterozygous and even homozygous subjects, false negative results have not been observed. Thus, the methylene blue decolorization technique has proved to be quite useful in preliminary screening of large populations for enzyme deficiency.

We have devised a separate screening technique to reveal homozygous individuals in large populations of infants.[5,12] This technique employs TPN as a receptor. Thus, anaerobic conditions are not required. The fluorescence of the

reduced coenzyme indicates whether or not Gal-1-P uridyl transferase is present. This technique is less quantitative than the dye reduction technique because of the relatively subjective nature of the appraisal of fluorescence on filter paper and is not as suitable for heterozygote detection. Quantitation can be achieved, however, by shortening the incubation period to 30 minutes, making a 1:200 dilution in .01 M phosphate buffer, pH 7.4, and reading TPNH fluorescence in a photofluorometer.

Frequency of the Gene for Galactosemia in the Population

The methylene blue-linked screening procedure has been carried out on 2,172 subjects, chiefly normal blood donors but some normal hospital personnel and mental patients as well.[8,11] Uridine diphosphoglucose (UDPG) consumption assays[4] were carried out on the red cells of many subjects that had a prolonged decolorization time. Thirty of the subjects were found to have transferase activities below 12.5 units, the range of activity found in approximately 83 per cent of subjects heterozygous for galactosemia. Whenever possible, family studies were carried out on galactosemic individuals revealed by this survey. As shown in Figure 5, most of the pedigrees were found to be quite consistent with the transmission of a single gene, amorphic with respect to

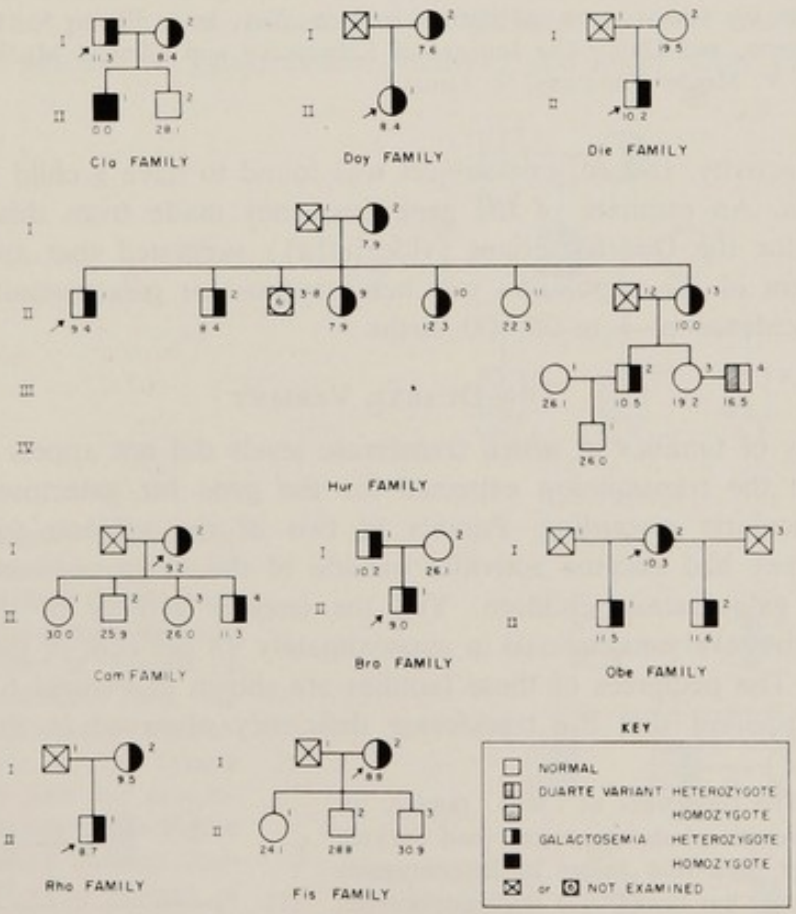


FIG. 5. Pedigrees of families of individuals with approximately half-normal galactose-1-phosphate uridyl transferase activity. The mode of transmission in these families was consistent with the transmission of the gene for galactosemia. (From Beutler, Baluda, Sturgeon, and Day[8]: The Journal of Laboratory and Clinical Medicine 68: 646, 1966, The C. V. Mosby Company, St. Louis.)

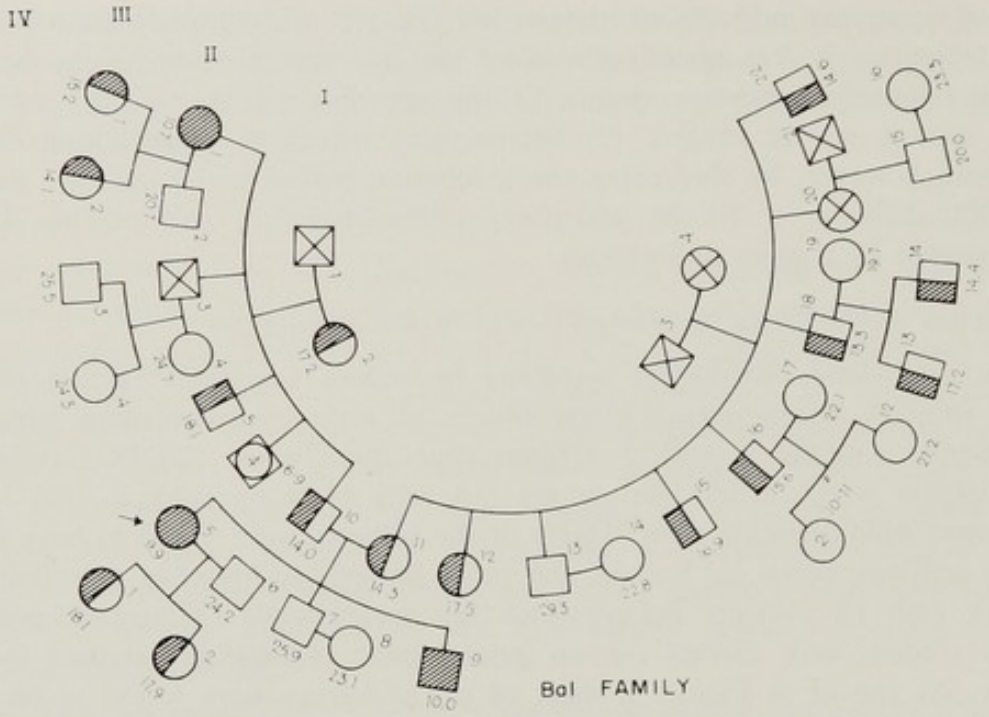


FIG. 6. The pedigree of the Bal family, in which both parents of the propositus had enzyme activity higher than that found in heterozygotes for galactosemia but lower than that encountered in the vast majority of normal subjects. Key, as in Figure 5. (From Beutler, Baluda, Sturgeon, and Day[5]: The Journal of Laboratory and Clinical Medicine 68: 646, 1966, The C. V. Mosby Company, St. Louis.)

transferase activity. Indeed, one subject was found to have a child with known galactosemia. An estimate of the gene frequency made from this population (corrected for the Duarte variant [vide infra]) suggested that approximately 1.25 per cent of the population was heterozygous for galactosemia, giving an expected incidence of 1 in 25,600 births.

The Duarte Variant

The study of families in which transferase levels did not appear to be compatible with the transmission expected for the gene for galactosemia proved to be particularly rewarding. Parents of two of the subjects found in the original survey had enzyme activities outside of the range encountered in the parents of galactosemic children. Yet, the enzyme activity of the red cells was below the level encountered in approximately 98 per cent of the unselected population. The pedigrees of these families are shown in Figures 6 and 7.

It was apparent that the transferase deficiency observed in these families

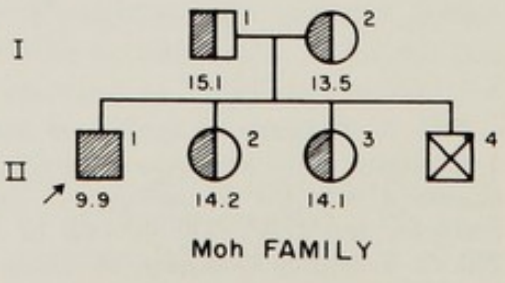
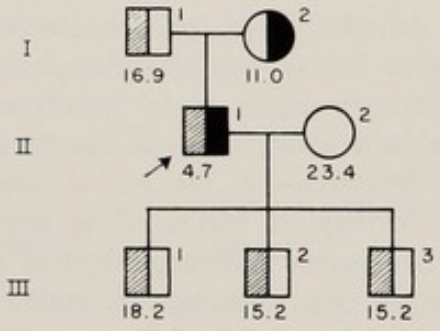


FIG. 7. The pedigree of Moh family, in which both parents of the propositus had enzyme activity higher than that found in heterozygotes for galactosemia, but lower than that encountered in the vast majority of normal subjects. Key, as in Figure 5. (From Beutler, Baluda, Sturgeon, and Day[5]: The Journal of Laboratory and Clinical Medicine 68: 646, 1966, The C. V. Mosby Company, St. Louis.)

was, indeed, genetically-determined, yet it remained unclear whether only a single gene pair was involved. The possibility that unlinked modifiers of transferase activity were involved appeared to be real, and the pedigree of the Bal famiiy (Figure 6) proved very helpful in this respect. Two of the individuals (II, 1; III, 5) with transferase activity as low as those of subjects heterozygous with galactosemia had borne children (III, 1 and 2; IV, 1 and 2). All four of the children had activities in the same range as that of the parents of the propositi: neither as low as that of heterozygotes for galactosemia, yet well below the usual normal range. These findings suggested strongly that the propositi carried allelic or closely-linked genes (in repulsion).

The relationship of this gene, which we named the Duarte variant (Gt^D), to the gene for galactosemia (gt) also required elucidation. To resolve this question on a genetic basis, it seemed necessary to find individuals who carried not only the gene for the Duarte variant but also the gene for galactosemia. We reviewed red cell transferase levels of the 30 subjects detected in our survey and found that there were two individuals whose enzyme activity was well below that of the others, and well below the range encountered ordinarily in the parents of galactosemic children. Family studies of these two individuals are presented in Figure 8. All parents or children of these individuals that were studied had enzyme activities that were either half-normal (the range found in parents of galactosemic children) or three-fourths-normal (higher than parents of galactosemic children, yet lower than the general



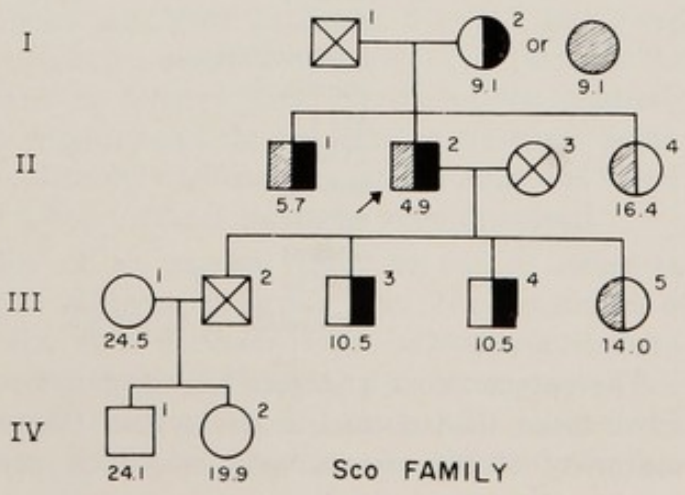


FIG. 8. Pedigrees of the Dic and Sco families. The red cells of the propositi in these families had less enzyme activity than found in the large majority of parents of galactosemic children. They are considered to be doubly anomalous for the gene for the Duarte variant and the gene for galactosemia (Gt^D/gt). Key, as in Figure 5. (From Beutler, Baluda, Sturgeon, and Day[5]: The Journal of Laboratory and Clinical Medicine 68: 646, 1966, The C. V. Mosby Company, St. Louis.)

population). Assuming that the propositi of these two studies did, indeed, carry both the gene for galactosemia and the gene for the Duarte variant, the absence of parents or children with normal activity, and the absence of parents or children with activities as low as those of the propositi indicate that the gene for the Duarte variant is allelic with the gene for galactosemia. As we shall see, subsequent studies bear out this interpretation.

Frequency of the Gene for the Duarte Variant in the Population

Estimates of the gene frequency of the gene for the Duarte variant have been based on the following data: (1) The prevalence in the population of individuals with between 12.5 and 18.5 units of transferase activity. Such subjects have enzyme activity in the range found in heterozygotes for the Duarte variant. (2) The prevalence of individuals doubly anomalous for the gene for the Duarte variant and galactosemia (Gt^D/gt) as compared with those heterozygous for galactosemia (Gt^+/gt). (3) The frequency in the general population of homozygotes for the Duarte variant, as established by family studies. All of these methods give estimated gene frequencies between .05 and .07, suggesting that 10-14 per cent of the population is heterozygous for this gene (Gt^+/Gt^D).[8]

Electrophoretic Studies of the Duarte Variant

Electrophoretic characterization has been very useful in helping us to elucidate polymorphisms of many red cell enzymes. Electrophoretic variants of glucose-6-phosphate dehydrogenase, phosphogluconate dehydrogenase, phosphoglucomutase, adenylate kinase, and acid phosphatase, have been described.[19] The activity of galactose-1-phosphate uridyl transferase, however, is normally only 1/5 of that of the least active of these enzymes, even when measured under optimal conditions. Furthermore, the use of sulfhydryl agents, necessary for maximal transferase activity, is precluded in tetrazolium-linked methods because of a rapid and nonspecific reducing effect of these compounds on the receptor dye. Nonetheless, it was possible to stain, faintly, galactose-1-phosphate uridyl transferase in a tetrazolium-linked reaction mixture comprised of the following reagents:

UDPG	5 mg
Gal-1-P	10 mg
TPN	1 mg
methosulfate phenozine	1 mg
MTT	1 mg
PGM	4 E.U. in .01 ml
G-6-PD	5 E.U. in .01 ml
6-PGD	.025 E.U. in .01 ml
catalase	2 mg
$MgCl_2$, .1 M	0.2 ml
tris-acetate .1 M pH 8.0	4.8 ml

The enzyme from a subject homozygous for the Duarte variant appeared to move faster than normal in this system (Figure 9). Yet, because of the low sensitivity of the system, long incubation periods were required, and there

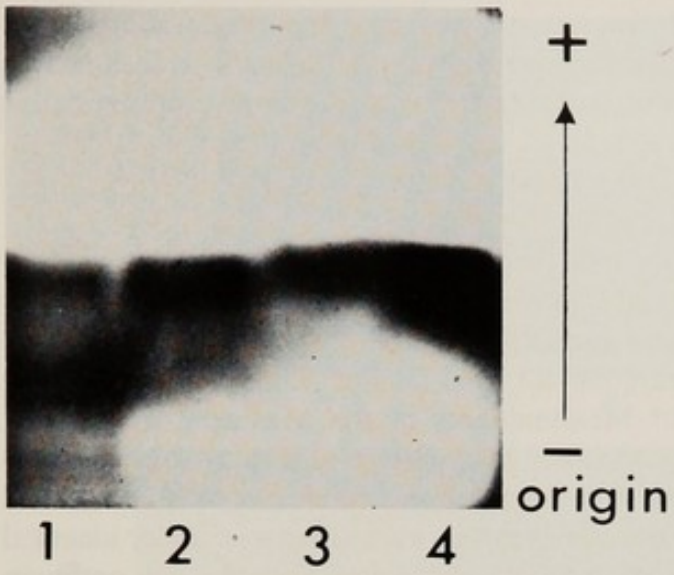


FIG. 9. Starch gel electrophoresis of galactose-1-phosphate uridyl transferase using the tetrazolium-linked method given in the text. Channel 1 and 4 contained hemolysate from one normal subject; channel 2, hemolysate from a second normal subject; and channel 3, from a homozygote for the Duarte variant.

was considerable nonspecific darkening of the gel, even in the presence of catalase, which retards nonspecific discoloration.

Considerably more consistent and satisfactory results could be obtained in a system in which cysteine was added as an activator of the enzyme and substrate quantities of TPN were used rather than tetrazolium. The following mixture was employed[27]:

UDPG	5 mg
Gal-1-p	10 mg
TPN	5 mg
cysteine	7.5 mg
$MgCl_2$, 0.1 M	.4 ml
G-6-PD	5 E.U. in .01 ml
PGM	4 E.U. in .01 ml
6-P-GD	.025 E.U. in .01 ml
tris-acetate .1 M pH 8.0	4.6 ml

Even with relatively dilute enzyme, it was now possible to visualize reduced TPN on the gel by virtue of its fluorescence when activated with long-wave ultraviolet light. The specificity of these methods has been confirmed by the finding that galactosemic samples failed to result in staining of the gel (Figure 10). Using this system, it has been possible to show that both homozygotes for the Duarte variant, whose pedigrees are shown in Figure 6 and 7, had a red cell enzyme with rapid electrophoretic mobility (Figure 11). Two distinct enzyme bands could not be seen in heterozygotes because of the relatively small distance between the two enzymes and the breadths of the band. Sometimes a broadened band with a relatively weak-staining leading edge was found. Similar findings were observed when artificial mixtures were examined.

Rapid electrophoretic mobility of an enzyme might be due to either an increased electric charge or to a different molecular size. The separation of molecules with different molecular sizes increases when increasing concentrations of starch are used in preparing the gel.[33] Accordingly, electrophoresis was carried out using starch gel concentrations of 10, 13, 16, and 20 per cent. No change in the degree of separation of the two enzymes was observed. Further-

more, the molecular weight of the enzyme samples has been estimated by filtration of Sephadex gel G-200. The results of gel filtration are shown in Figure 12. The molecular weight of both enzymes was the same, approximating 85,000.

Kinetic Properties

Differences in kinetic properties have been found to be very useful in characterizing certain enzymatic variants, particularly those of G-6-PD. We have therefore compared the enzymatic properties of galactose-1-phosphate uridyl transferase from subjects homozygous for the Duarte variant with enzymes obtained from normal red cells.[3] Measurements of the Michaelis constant of the enzymes are complicated by the fact that each of the two substrates influences the affinity of the enzyme for the other substrate. No difference could be demonstrated, however, when the two enzymes were compared under identical conditions. The Michaelis constant for both substrates and the pH optimum curve were identical. The heat of activation of the normal and Duarte variant enzymes appear to be identical. In addition, thermal stability and inhibition of the enzyme by chloride ion was found to be identical for both enzymes. Thus it appears that although enzyme obtained from subjects with Duarte variant is abnormal in structure, the abnormality does not appreciably influence the active site of the enzyme.

The Effect of the Duarte Variant on Fitness

No obvious clinical effects have been associated with the homozygous state of the Duarte variant. One of the propositi (MOH) was mentally retarded.

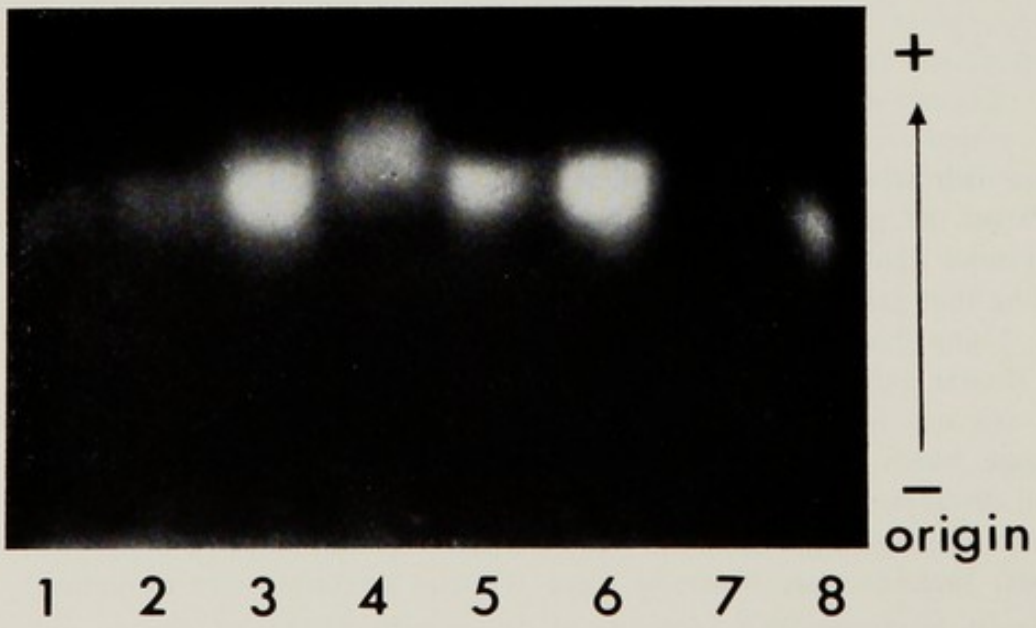


FIG. 10: Starch gel electrophoresis of galactose-1-phosphate uridyl transferase using the fluorescent staining method described in the text. Channels 1 and 2, a mixture of galactosemic and normal hemolysate; channel 3, a mixture of hemolysate from a homozygote for the Duarte variant and a normal subject; channel 4, hemolysate from a homozygote for the Duarte variant; channel 5, hemolysate from a heterozygote for galactosemia; channel 6 and 8 normal hemolysate; channel 7, galactosemic hemolysate.

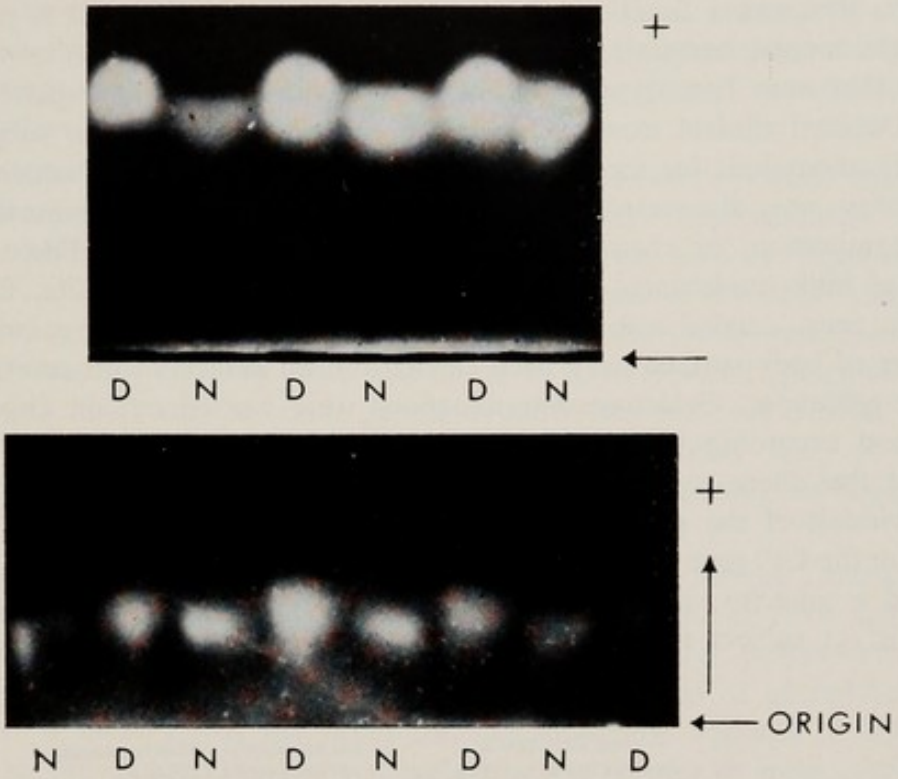


FIG. 11. Starch gel electrophoresis of normal hemolysate and hemolysate from a homozygote for the Duarte variant. Normal hemolysates are labeled N; Duarte variant hemolysate is labeled D.

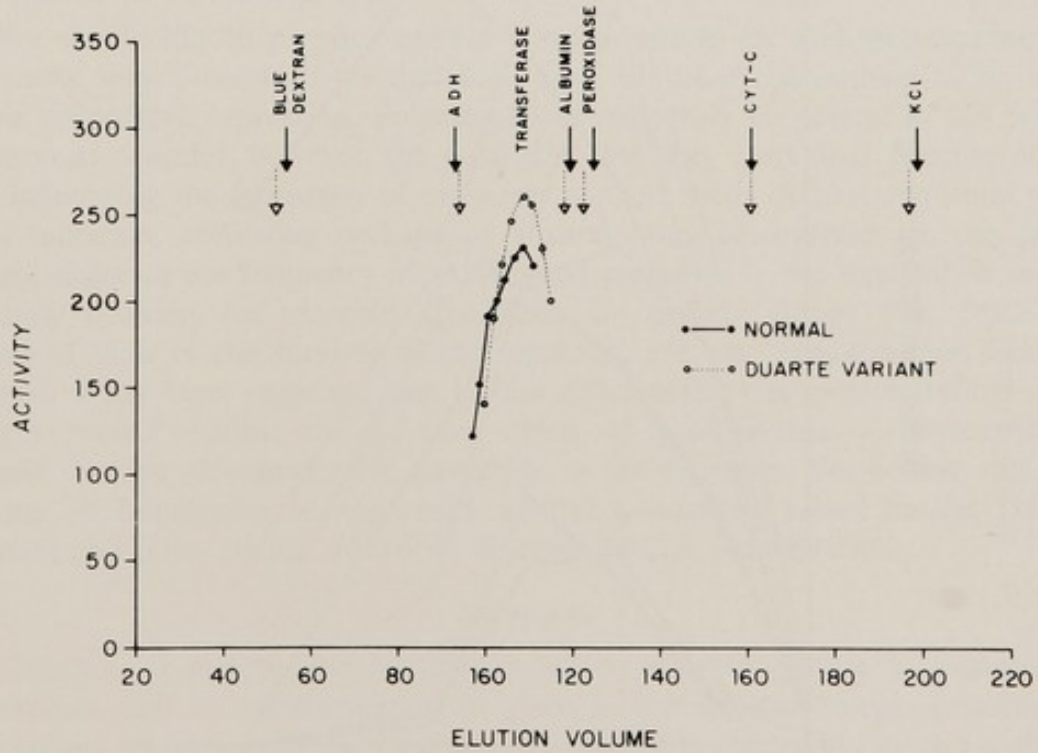


FIG. 12. Sephadex G-200 gel filtration of galactose-1-phosphate uridyl transferase and marker enzymes. When the logarithm of the molecular weight of the marker enzymes was plotted against the K_d value, a straight line was obtained and the K_d value of transferase suggested that the enzyme obtained both from a normal subject and from a homozygote for the Duarte variant had a molecular weight of 85,000.

However, this was a function of ascertainment, since MOH was a patient in one of the mental hospitals screened in this study.[11] At least two of the other subjects that were homozygous for the Duarte variant were of superior intelligence. Detailed clinical examination has been carried out on one subject who is doubly anomalous for the Duarte variant and the gene for galactosemia. No abnormality was discovered in this individual by physical examination, slit lamp examination, or chemical examination of liver function. There was no history of milk intolerance during infancy, childhood, or adult life. Galactose tolerance tests, carried out by oral administration of 35 g of galactose per sq. meter of body surface have been carried out on subjects with several of the possible genotypes. Galactose determinations were carried out on finger puncture blood according to the galactose oxidase method of Roth et al.[30] It is apparent that there is great variability in the galactose tolerance curve, even on individuals of the same phenotype. However, the findings suggest that the effect that the Gt^D gene exerts is not very great. The curves of the Gt^D/gt subjects indicated a capacity to metabolize galactose which was as great as that of the single Gt^+/gt subject tested. The curve of the Gt^D/Gt^D subject did not differ

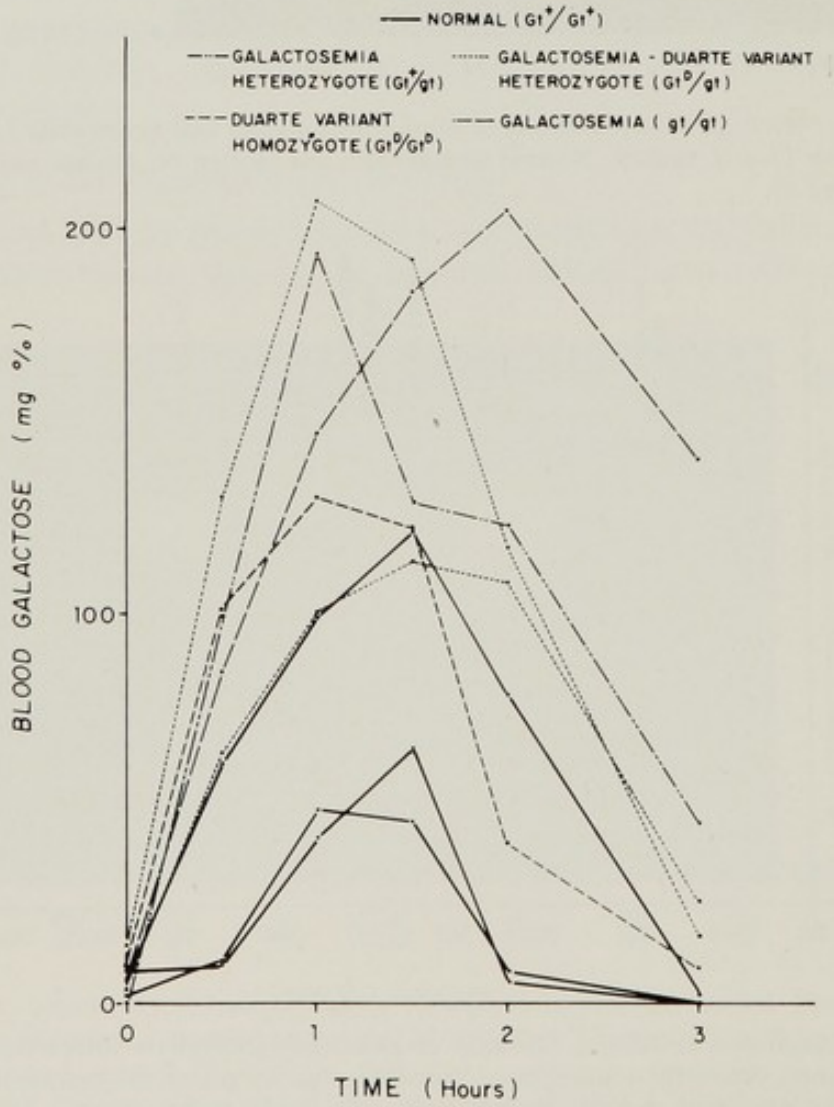


FIG. 13. Galactose tolerance tests on subjects with various transferase variants. Each subject was given 35 g galactose per sq. meter body surface.

greatly from that of one of the Gt^+/Gt^+ subjects. Thus, although the number of individuals homozygous for the Duarte variant or carrying the gene for both abnormalities that we have studied has been limited, no obvious effect on fitness has been observed.

Gitzelmann[17] detected a newborn infant with one-fourth normal enzyme activity. The father had 9.3 units of enzyme activity (approximately one-half normal) while the mother had 15 units of enzyme activity (approximately three-fourths normal). Electrophoresis of the enzyme of the child showed the same rapid mobility which we found in our two subjects who were homozygous for the Duarte variant. The child was found to have elevated galactose-1-phosphate levels (16.7 mg per cent) in the erythrocytes and was treated, at least initially, with a galactose-free diet.[17] Thus, the possibility that, at least in some instances, clinical symptoms could arise from the doubly-anomalous state (Gt^D/gt) must be borne in mind.

It is interesting to speculate why a gene such as that for the Duarte variant should have reached such a high frequency in the population. The same question may be raised with respect to other, apparently neutral, polymorphic genes. Why, for example, are genes for electrophoretic variants of phosphoglucomutase, type A G-6-PD, or the genes for electrophoretic variants of acid phosphatase so prevalent? Obviously, these genes may confer or have conferred in the remote past some phenotypic advantage that is not at all apparent to us. It is also possible, that the gene is a disadvantageous one, and is being displaced from the population by a new variant, which is now so prevalent that it is considered to be the wild type.

We should like to propose another possible reason for such polymorphisms. It seems very possible to us that their survival value has nothing to do with their phenotypic expression. Polymorphisms may truly be neutral in this sense. One must consider, however, the possibility that they exert their selective effect by influencing the frequency of crossover at their locus. A phenotypically neutral mutation, consisting perhaps of a small area of inversion in the gene, might decrease the frequency of pairing and crossover in the segment. It seems entirely possible, for example, that there are linkage groups that, from the point of view of the survival of the organism, are best kept together. For instance, it has been suggested that lactase deficiency in the gastrointestinal tract might protect against the adverse effects of galactosemia.[35] Heterozygosity would then be of considerable advantage to the organism. We believe that the matter of frequencies of apparently neutral mutuations raised by the Duarte variant and other similar mutations deserves further consideration.

Summary

Galactose is metabolized by the red cell by phosphorylation to galactose-1-phosphate and its transformation to glucose-1-phosphate through galactose-1-phosphate uridyl transferase. Glucose-1-phosphate is changed to glucose-6-phosphate by phosphoglucomutase. When galactose serves as substrate, the rate of methemoglobin reduction is strongly substrate-dependent and is accelerated only slightly or not at all by the addition of methylene blue. The failure of methylene blue to accelerate methemoglobin reduction, when galactose serves

as substrate, appears to be due to the fact that galactose is converted to α-glucose-6-phosphate, which cannot serve as substrate for glucose-6-phosphate dehydrogenase. The substrate-dependency of methemoglobin reduction at relatively high levels of galactose implies the existence of another pathway of galactose metabolism in the erythrocyte. This pathway does not involve transferase, since it is present in galactosemic red cells.

Screening for galactosemia has revealed the presence of a prevalent mutation involving the locus for galactose-1-phosphate uridyl transferase. This gene, which has been designated as the Duarte variant, results in reduction of enzyme activity to approximately one-half of normal in homozygotes, and approximately three-fourths of normal in heterozygotes. The gene appears to be allelic with the gene for galactosemia and results in the production of an enzyme which is kinetically normal, has a normal molecular weight, but has a rapid electrophoretic mobility. The preliminary data suggest that the mutation exerts no strong effect on fitness.

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DISCUSSION

BERGREN, LOS ANGELES, CHAIRMAN: Dr. Beutler has suggested that I add a few comments concerning studies on galactose pathway enzymes in hemolysates by Dr. Won Ng in association with Dr. George Donnell and myself. We have shown galactokinase activity to be markedly elevated in hemolysates from newborn as compared with those from adults, and the activity of galactose-1-phosphate uridyl transferase for the newborn to be about the same, or only slightly elevated. In the case of UDPGal-4-epimerase, the third of the galactose pathway enzymes, we found that hemolysate epimerase activity is markedly elevated in the newborn. Further, it was surprising, in view of the established need for addition of NAD in hemolysate epimerase assays, to note that the epimerase activity in the newborn is independent of exogenous NAD. The apparent NAD-independent

epimerase activity was found to be lost when hemolysates from newborn were preincubated with hemolysates or red cell stroma from adults. Further study led to recognition of the absence, or virtual absence of NADase activity in the red cells of newborn. It was found that with increasing age, NADase activity increases, until levels comparable to those of adults are found by the end of the first year of life. At the same time, the apparent NAD-independent epimerase activity decreases. We then recalled that some time ago, in a publication on an enzymatic assay for galactose-1-phosphate, Dr. H. N. Kirkman had noted that the requirement for exogenous NAD, when assaying epimerase, can be eliminated if the hemolysate is prepared in the presence of an NADase inhibitor such as nicotinamide. By employing nicotinamide in this way, we were able to show NAD-independent epimerase activity in hemolysates of adults. In effect, our observation of NAD-independent epimerase activity in hemolysates of newborn was an artifact that resulted in demonstrating the lack of NADase activity in the erythrocytes of the newborn. In further studies, we have carried out starch gel electrophoresis of epimerase, employing a system somewhat similar to that which Dr. Beutler has described for transferase. The substrate for demonstrating the location of epimerase is UDPGal. The reaction product is UDPG, which is detected by coupling to NAD in the presence of UDPG dehydrogenase. Two different forms of epimerase can be seen (Figure 1). The hatched areas correspond to the location of the hemoglobin of the hemolysates after migration, while the dark bands represent the epimerase positions.

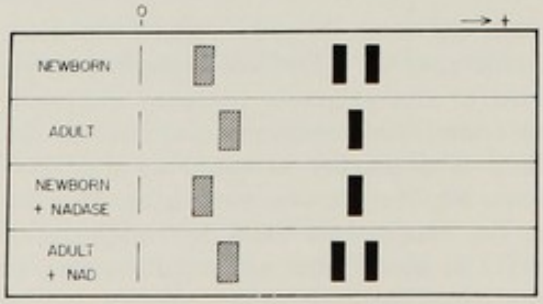


FIG. 1. Electrophoretic study of epimerase in which UPDGal is the substrate.

The separate epimerase bands are very distinct but difficult to photograph, since the NADH fluorescence is very faint. The diagram has been prepared to scale, and the results shown are representative of what is seen in the gels. For newborn hemolysates there are two epimerase bands; and for the adult, there is only one band intermediate in position between the two for the newborn. If hemolysate from a newborn is preincubated prior to electrophoresis with washed red cell stroma from an adult as a source of NADase, the newborn epimerase form (2 bands) is converted to the adult form (1 band). Conversely, if we start with an adult hemolysate and preincubate it with NAD, the two-banded newborn epimerase form is formed. The importance of NAD as a determinant of the two electrophoretic forms of epimerase is evident. We consider that the relationship has to do more with NAD-binding to the enzyme than to intrinsic NAD levels within the cell or in a hemolysate.

SIMON, ALBUQUERQUE: Dr. Beutler, I wish to ask about the purity of the galactose used in your methemoglobin reduction studies, using galactose as substrate. Formerly, when glucose-contaminated galactose was used, methylene blue accelerated the reduction, in contrast to the results that you have presented. However, at the very high concentrations of galactose which you used, about 0.5 molar, a very small contamination by, for example, glucose, might still account for the methemoglobin reduction rate that you observed. This would obviate the necessity for postulating a new route of metabolism to account for your results.

BEUTLER, DUARTE: I think that's an excellent point, and one which we have considered fairly carefully. The galactose that was used for these studies is Sigma's, containing virtually undetectable amounts of glucose. This, of course, does not prove that no glucose is present. However, when one uses galactose that is not glucose-free, one obtains a two-component curve with methylene blue. As the glucose substrate is exhausted, a change of rate of methemoglobin reduction is seen. In other words, if there were just a trace of glucose contaminating the galactose, this would be used

up to reduce methemoglobin in the first 30 minutes or hour of the experiment. If one does the assay without the methylene blue, then the curve is a little more difficult to interpret.

There is another very important contaminant that we have recently discovered in galactose—one which I think recently has caused a tremendous confusion in literature. Many of you have been acquainted with a series of articles published by Cuatrecasas and Segal* on a new liver enzyme they have called "galactose dehydrogenase". We were able to confirm their findings that when liver hemogenates are incubated with DPN and Sigma's glucose-free galactose, there is, indeed, an increase in optical density at 340 mμ. The rate of change of optical density which we observed was virtually identical to the rate of change reported by these investigators. The shape of the curve, however, was that of a substrate exhaustion curve.

We have also been able to confirm that upon electrophoresis of liver homogenate, staining with a mixture containing galactose, DPN, phenozine methosulfate, and nitro-BT or MTT, a band appears which moves cathodally, even at an alkaline pH. We were struck by the resemblance of this band to the "nothing dehydrogenase" that has been described by other investigators. Therefore, we stained slices of a gel with and without addition of galactose to the staining mixture. The same band was present when galactose was left out of the mixture as when galactose was present. Therefore, the band which Cuatrecasas and Segal have described clearly represents "nothing dehydrogenase." Although the term "nothing dehydrogenase" implies that there is no substrate, some substrate must be present to serve as a donor for electrons to be passed to the receptor dye. We therefore examined commercially-available starch (Connaught) as a substrate for the reduction of DPN by liver homogenate supernatant, and were able to demonstrate that either boiled or unboiled starch contained ample substrate for DPN reduction. Further analysis showed that this substrate could be extracted with cold water and that it could be removed on drying of the washings. Shaw and Koen† had suggested that "nothing dehydrogenase" might, in point of fact, be alcohol dehydrogenase. Indeed, we found that the washings of starch were quite rich in substrate for purified yeast or horse liver alcohol dehydrogenase.

Since the reaction of liver supernatant with galactose had exhibited the properties of a substrate exhaustion curve, it occurred to us that alcohols might also be present in the highly purified galactose used in assays for "galactose dehydrogenase." Indeed, we were able to demonstrate that the lot of Sigma's glucose-free galactose, which we were using, contained .6 mMole of substrate for alcohol dehydrogenase per mole of galactose. When galactose dehydrogenase activity of liver is assayed with this type of galactose, a substrate exhaustion curve is found. However, when the galactose solution used is simply dried on an evaporating dish at 80° and is then redissolved in the same volume, there is no change in optical density when liver supernatant and DPN are added. There is, however, no destruction of galactose as measured with galactose oxidase. Furthermore, the activity of crystalline horse liver enzyme and of the enzyme that we found in rat liver is the same in all stages of purification, using alcohol substrate, on one hand, or galactose (obviously contaminated with alcohol), on the other. It is also apparent that the purification procedure reported by Cautrecasas and Segal for "galactose dehy-

*Cuatrecasas, P. and Segal, S.: Galactose Conversion to D-Xylulose: An alternate route of galactose metabolism. Science 153: 549-551, 1966.

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†Shaw, C. R. and Koen, A. L.: On the identity of "nothing dehydrogenase." J. Histochem. & Cytochem. 13: 431-433, 1965.

drogenase" closely resembles the classical procedure for the preparation of crystalline horse liver alcohol dehydrogenase. Although these investigators have reported the isolation of radioactive galactonic acid from radioactive galactose incubated with "galactose dehydrogenase," we cannot confirm this critical observation.

So it is certainly true that when one is dealing with high concentrations of these reagents, one must be very careful about contamination. We have taken the precaution of determining whether ethanol would act as a substrate for methemoglobin reduction after our discovery of this, and we find that it does not. We therefore believe that this is really a galactose effect. I might say that the work of Hjelm and de Verdier† also showed increased utilization of galactose at high galactose concentrations when the galactose oxidase method was used. These investigators found that galactose was utilized with two K_m's; one apparently high K_m utilization, the other low.

KEITT, BOSTON: I thought you might be interested in some findings that I made last year which make it difficult for me to interpret the relation between galactose and methemoglobin reduction. This occurred in studying the effects of fluoride on methemoglobin reduction. I wanted to reconfirm Gibson's work that fluoride did not inhibit the methemoglobin reduction rate with glucose. It was observed that fluoride actually increased the rate of methemoglobin reduction, and on looking at this further, it was apparent that with high doses of fluoride, which completely inhibited glucose consumption, this effect was still present. I wondered about the substrate for this methemoglobin reduction in the absence of any glucose utilization and, therefore, tried to see whether the effect would be apparent if glucose was absent. Significant reduction (4-5 μMole/ml cells) occurred without glucose with fluoride as the only additive, and continued for 6-8 hours before levelling off. Although I don't know exactly what the substrate was, I think it is probable that it was the ribose from adenine nucleotide that would be catabolized very rapidly in the presence of fluoride. Therefore, you have to be cautious in attributing methemoglobin reduction to an exogenous substrate, because there is some endogenous substrate available for methemoglobin reduction.

BEUTLER, DUARTE: We have always included a control without added substrate, and find no significant methemoglobin reduction in our system in the absence of substrate.

LOOS, AMSTERDAM: I would like to ask Dr. Beutler if this transferase deficiency has any influence on the metabolism of the red cells. I remember a remark of Dr. Prankerd in his book, The Red Cell, suggesting that the ATP-content is likely to decrease in galactosemic cells, when these cells are incubated with high concentrations of galactose because the formed galactose-1-phosphate in these cells cannot be used for generation of energy-rich phosphate bonds. Have you had any opportunity to test this?

BEUTLER: No, we haven't made any studies of that sort. We have observed that galactose is a very poor substrate for maintenance of ATP levels, even in normal cells. How does Prankerd suggest that this interferes with the utilization of glucose which, after all, is present in the circulation?

LOOS: He suggests that galactose-1-phosphate piles up in the red cells of galactosemic patients after galactose-rich meals and that this causes a loss of ATP. Do you think that these cells are able to stabilize their ATP-level by normal glycolysis?

BEUTLER: Yes, I suppose that is possible, although the activity of galactokinase is very low and what one would anticipate is that the cell would deplete its ATP only very, very slowly and certainly be able to rephosphorylate ADP with normal glycolysis. We have had no experience with this situation, Dr. Loos.

BERGREN: We know that galactose-1-phosphate can pile up in the erythrocyte. In the galactosemia patient *in vivo* this may be as much as 20 mg per cent after a tolerance dose of galactose.

†Hjelm, M. and de Verdier, C.-H.: Über die Bestimmung des Galaktose-Verbrauchs in menschlichen Erythrozyten mit Galaktose-Oxydase. Folia Haemat. 83:251-256, 1966.

Methemoglobin Reductases

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IN ORDER TO accomplish its primary physiological mission of transporting oxygen from the lungs to peripheral tissues, the erythrocyte has been designed structurally and functionally to provide protection for hemoglobin, the agent responsible for the reversible oxygen-binding. Thus, the cellular membrane shields hemoglobin from destructive enzymes, and most of the enzyme systems in the mature red cell appear to be present for the purpose of maintaining the membrane. Despite these elaborate safeguards, hemoglobin is slowly oxidized to methemoglobin at a rate estimated to be one or two per cent of the total per day; the biological oxidant, or oxidants, for the unwanted reaction is still unknown.[15]

Since methemoglobin lacks the capacity to carry oxygen, it is clear that erythrocyte function requires some device for the continuous regeneration of reduced hemoglobin. This might be accomplished by some of the chemical reductants present in the cell, but an enzymatic system would seem, a priori, to be much more suitable for this purpose. Attempts to find such an enzymatic mechanism began over 30 years ago with some definitive observations made on whole cells. Erythrocytes were deliberately made methemoglobinemic by treatment with nitrite, and it was observed that the addition of substrates, such as lactate and glucose, greatly accelerated the conversion of methemoglobin to hemoglobin. Data illustrating this effect have been given by Jaffe.[13] Further work with methemoglobinemic hemolysates, however, showed that these substrates merely served as a device for generating the reduced pyridine nucleotides (DPNH or TPNH) and that the latter were very probably the actual reductants for methemoglobin.*[5,17] These studies led to the concept that the red cell contained DPNH- and TPNH-dependent methemoglobin reductases, perhaps similar to the pyridine nucleotide-dependent cytochrome *c* reductases isolated from other tissues.[6,20] In retrospect, we know that the analogy was somewhat unfortunate since, as will be discussed subsequently, the cytochrome *c* reducing capacity of the latter enzymes is now believed to be an artifact. The existence of separate methemoglobin reductases was further strengthened by Scott's

*It is conceivable that the methemoglobin-reducing system may also serve a reciprocal purpose, namely as a regulator of the level of reduced pyridine nucleotides. If the level of reduced pyridine nucleotides becomes too high, a small amount of hemoglobin might be converted to methemoglobin in order for the latter to serve, in turn, as the oxidant for the reduced pyridine nucleotides.

observation[25] that patients with congenital methemoglobinemia appear to lack the DPNH-dependent, rather than the TPNH-dependent, activity.

There is a fascinating, and still not entirely explained, effect of dyes upon methemoglobin reduction in erythrocytes. Early observations by Harrop and Barron,[7] Michaelis and Salomon[21] and Warburg et al.[30] demonstrated that the slow rate of glucose oxidation in red cells could be stimulated considerably by the addition of such dyes as methylene blue. Methylene blue likewise accelerated the substrate-dependent reduction of methemoglobin in whole cells (see Jaffe[14] for typical data) as well as its reduction by reduced pyridine nucleotides in hemolysates.[3,5,17] Although some caution must be exercised in considering nitrite-treated cells as "physiological," these findings suggest that some rate-limiting step in the methemoglobin-reducing system in red cells is by-passed upon addition of the dye.

From the above considerations, it is clear that attempts to isolate pyridine nucleotide-dependent methemoglobin-reducing enzymes from erythrocytes must be prepared to cope with the possibility that multiple oxidation-reduction groups, perhaps carried by separate proteins, may intervene between the reduced pyridine nucleotide and methemoglobin and that the requirement for methylene blue as an electron carrier may introduce an element of artificiality into the system. These complexities, which are inherent in the isolation of electron transport *sequences,* such as the DPNH dehydrogenases from mitochondria, are not ordinarily encountered in other erythrocyte enzymes. However, in spite of these difficulties, several methemoglobin reductases have been partially purified and characterized. Kiese and his colleagues[17,18] first described a TPNH-methemoglobin reductase that utilized methylene blue as an added carrier, and other preparations of the TPNH-dependent activity were reported subsequently by our group,[11,12] by Shrago and Falcone[29] and by Scott et al.[27] A DPNH-methemoglobin reductase* has been purified by Scott's group.[26,27,28]

Rationale for the Present Study of Methemoglobin Reductases

Several years ago, we decided to reinvestigate both the DPNH- and the TPNH-linked methemoglobin reductase activities using, as a background, our experience with the mitochondrial DPNH dehydrogenase.[19,23] We began by establishing *optimal* assay conditions for each of these activities in red cell hemolysates.[16] Optimal concentrations of the substrates (reduced pyridine nucleotides), the acceptor (methemoglobin), and the oxido-reduction dye (methylene blue) were determined for the maximal rate of reduction of methemoglobin. It should be noted that the concentrations selected in this empirical manner actually turned out to be well above the respective Michaelis constants (as determined subsequently with the purified enzyme preparation) but not high enough to cause inhibition. Although a number of dyes have been reported to stimulate methemoglobin reductase activity,[17] we decided to use methylene blue since it appeared to be among the more effective ones. In addition to substrate, carrier, and acceptor, it was found that phosphate and bovine serum albumin

*This enzyme has often been referred to as a "DPNH diaphorase"[26,28] since it is routinely assayed with an indophenol dye, rather than methemoglobin, as the acceptor. Jaffé[15] has discussed the use of the term "diaphorase" for this enzyme.

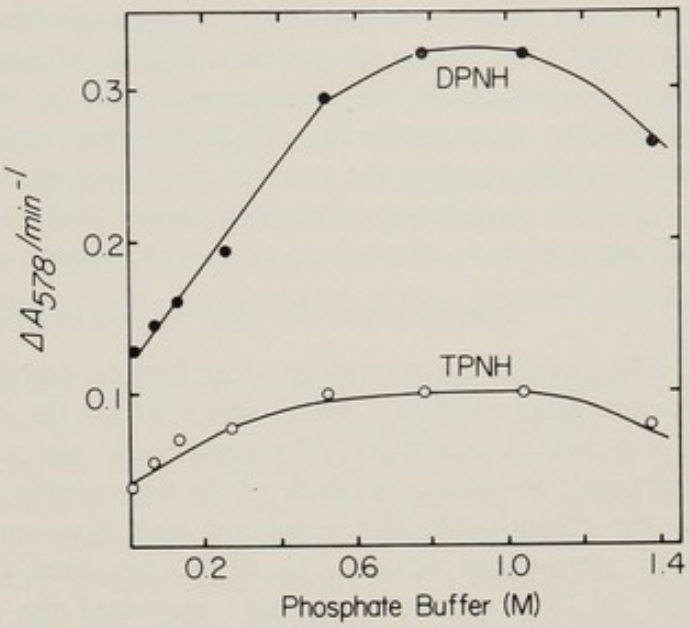


FIG. 1. Effect of phosphate concentration on methemoglobin reductase assays. Standard assay system (see legend for Fig. 2) with phosphate concentration as indicated.

were required for maximal activity. As shown in Figure 1, phosphate in the concentration range of 0.8 to 1.0 M produced optimal stimulation of both the DPNH and TPNH activities; the former was more sensitive to this activator. Phosphate stimulation has been encountered with a number of other electron transport enzymes, e.g., xanthine oxidase and DPNH dehydrogenase. Bovine serum albumin increased both activities by approximately three-fold.

The data in Figure 2 illustrate that under optimal assay conditions the rates of both DPNH- and TPNH-dependent methemoglobin reduction were linear with respect to the amount of enzyme. The present assay permits both activities to be measured at approximately a ten-fold greater sensitivity than in our previous study.[11] Under these conditions, the specific activity (defined in the

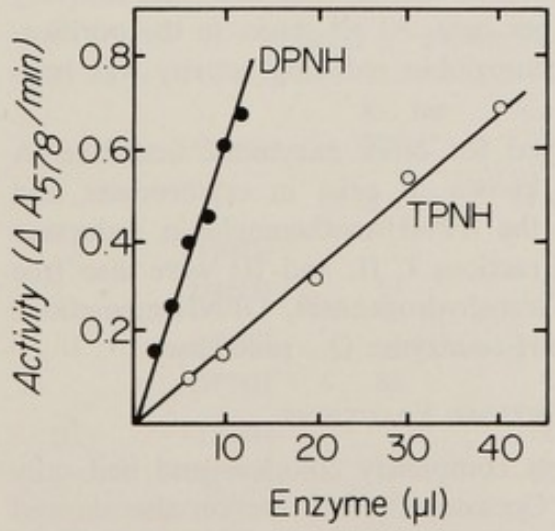


FIG. 2. Effect of hemolysate concentration on DPNH- and TPNH-methemoglobin reductase activities. A standard assay mixture was prepared by combining 4.5 ml of 1 mM methemoglobin, 8 ml of 3 M potassium phosphate buffer, pH 7.5, 2 ml of 5 per cent bovine serum albumin and 10.5 ml of distilled water. To 0.84 ml of this solution in a 1-ml cuvette having a 1-cm light path, 0.1 ml of 1.5 mM TPNH or 3 mM DPNH (each freshly prepared in 0.06 M phosphate buffer, pH 6.5) and the amounts of hemolysate indicated on the abscissa were added. The reaction was initiated by the addition of 3 µl of methylene blue (1 mM for the TPNH system or 5 mM for the DPNH system). The change in absorbancy at 578 mµ was followed at room temperature with the Cary recording spectrophotometer, Model 14, against a control that contained all components except the enzyme and the reduced pyridine nucleotide. A 0-1.0 slide wire was used routinely unless methylene blue was omitted; in the latter case, the 0-0.1 slide wire was substituted. The reaction velocity (v) was expressed as the absorbancy change at 578 mµ per minute ($\triangle A_{578}$/min).

legend for Fig. 2) of the TPNH-methemoglobin reductase in hemolysates was increased from 0.4 to greater than 6. It is, of course, an open question whether these optimal conditions, determined under in vitro conditions, are relevant to events that occur within the cell. In the intact erythrocyte many complex factors undoubtedly regulate the level of these two methemoglobin reducing activities, but for the purification envisioned, we wished to use assay conditions that gave full expression to whatever activity was present in a given fraction.

Purification of the Methemoglobin Reductases

The purification procedure, described in detail elsewhere,[16] is a modification of our earlier method,[11] which was used for the isolation of a TPNH-methemoglobin reductase activity. The hemolysate was treated batchwise with DEAE-cellulose[10] to remove the bulk of the hemoglobin; this appeared to be preferable to the ethanol-chloroform method used previously.[11] Subsequent fractionation with ammonium sulfate (55 to 70 per cent precipitate) and with acetone (50 to 75 per cent precipitate) increased the specific activities several-fold, although there was some loss in total activity during the latter procedure.

At this stage, chromatography of the preparation on DEAE-cellulose resolved the enzymatic activity into three distinct fractions. Residual free hemoglobin appeared first when the column was washed with 3 mM phosphate buffer, pH 7.0. Three fractions (called hereafter I, II, and III) were eluted from the column by using a gradient of increasing buffer strength of 3 to 30 mM. Each of these fractions displayed *both* DPNH- and TPNH-methemoglobin reductase activities. Rechromatography of each fraction on DEAE-cellulose, employing the original gradient elution conditions, yielded single and reasonably symmetrical protein bands at the expected positions in the profile.

After rechromatography on DEAE-cellulose, the specific activities for methemoglobin reduction in fractions I, II, and III had increased approximately one- to two-thousand-fold relative to the original hemolysate. The total recovery of activity in all fractions was about 15 per cent. At all stages in the purification, the ratio of DPNH- to TPNH-methemoglobin reducing activity was relatively constant, i.e., about 4:1.

Each of the three fractions was assayed for other enzymatic activities. A TPNH-dependent glutathione reductase, known to exist in erythrocytes and considered as a possible constituent in the TPNH-methemoglobin reductase system,[12] was absent from all fractions. Fractions I, II, and III were also free of TPNH to DPN and DPNH to TPN transhydrogenases, DPNH-menadione reductase, lipoyl dehydrogenase and DPNH-coenzyme Q_{10} reductase.

Properties of the Enzyme Fractions

(a) *Absorption Spectra.* Fraction I was completely colorless and had only a single absorption maximum at 278 mμ. Occasionally this fraction also showed a small absorbancy at 407 mμ which, if calculated as methemoglobin, corresponded to about 1 per cent of the total protein. Fraction II was pale yellow in appearance and had a sharp peak at 268 mμ* with a smaller peak at 405

*The supernatant fluid obtained by heat denaturation of Fraction II had an absorption maximum at 263 mμ.

mμ. The absorbancy at the latter wavelength corresponded to about 2 per cent methemoglobin. Fraction III was brownish-red and contained a considerable amount of a heme component (ca. 30 per cent when calculated as methemoglobin). The absolute amounts of "methemoglobin" in fractions II and III varied somewhat in different preparations, but III always contained much more of the pigment than I or II. All three fractions were devoid of flavin as judged by their complete lack of absorbancy in the 350 and 450 mμ regions and by the absence of fluorescence after denaturation of the proteins by acid or heat.

(b) *Effect of Pyridine Nucleotide Concentrations on Enzyme Activities.* The effect of DPNH and TPNH concentrations upon the rate of methemoglobin reduction was studied in detail for each fraction. K_m values for TPNH and DPNH were calculated to be 3.6×10^{-6} M and 5.5×10^{-5} M, respectively, for fraction I. Maximal activity was observed above 1.5×10^{-4} M for TPNH or above 3×10^{-4} M for DPNH, while concentrations of the reduced pyridine nucleotides greater than 5×10^{-4} M caused inhibition of both activities. K_m values for TPNH and DPNH with each of the three fractions are summarized in Table I. In all cases, the K_m values for TPNH were about one order of magnitude lower than those for DPNH.* The V_{max} values, however, were much higher with DPNH than with TPNH.

TPN was an effective inhibitor for both the TPNH- and DPNH-methemoglobin reductase activities. The inhibition was competitive as judged by the Lineweaver-Burk plots. On the other hand, DPN was unable to inhibit the TPNH-methemoglobin reductase activity, although it was a competitive inhibitor of the DPNH activity. K_I values for the inhibitory effects of the oxidized pyridine nucleotides are also summarized in Table 1. It is evident that the K_I value of TPN for the TPNH-methemoglobin reductase activity is lower by approximately one order of magnitude than that for DPN for the DPNH-methemoglobin

TABLE 1. *Kinetic constants of enzyme fractions*

Fraction	Substrate	K_m for PNH*	K_m for H_i†	K_m for MB‡	K_I for TPN	K_I for DPN
		M x 10^{-6}	M x 10^{-6}	M x 10^{-7}	M x 10^{-7}	M x 10^{-5}
I	TPNH	3.6	8.3	4.8	48	No inhibition
	DPNH	55	8.3	15	3.4	7.7
II	TPNH	4.4	8.1	4.8	67	No inhibition
	DPNH	83	8.1	16	4.1	18
III	TPNH	2.7	18	6.1	35	No inhibition
	DPNH	68	18	23	4.6	8.8

*Reduced pyridine nucleotide.
†methemoglobin.
‡methylene blue.

*This difference in affinity may be correlated with the level of pyridine nucleotides in human erythrocytes: DPN, 3.0×10^{-6} M; DPNH, 2.2×10^{-6} M; TPN, 1.65×10^{-6} M; and TPNH, 1.25×10^{-6} M[4].

reductase activity. If the K_m values are assumed to be a measure of enzyme-substrate affinities, and hence comparable with K_i values, the affinities of the oxidized and reduced pyridine nucleotides for each fraction fall in the same order: TPNH>TPN>DPNH>DPN.

(c) *Effect of Methemoglobin and Methylene Blue Concentrations on Enzyme Activities.* Both the DPNH- and TPNH-methemoglobin reducing activities were inhibited at high concentrations of methemoglobin but, for any fraction, the K_m value for methemoglobin was the same for either reductase activity (Table 1).

Methylene blue was required for both methemoglobin reductase activities (Fig. 3), but the two curves were quite different. The TPNH-methemoglobin reductase activity was optimal when the dye concentration was about 3×10^{-6} M while for the DPNH-dependent activity it was necessary to use a five-fold higher concentration of methylene blue to achieve maximal stimulation. These data emphasize the importance of testing each activity at its optimal dye concentration. K_m values of the dye with fractions I, II, and III are listed in Table 1. With each fraction, the K_m value in the TPNH-methemoglobin reducing system was lower than that in the DPNH-system.

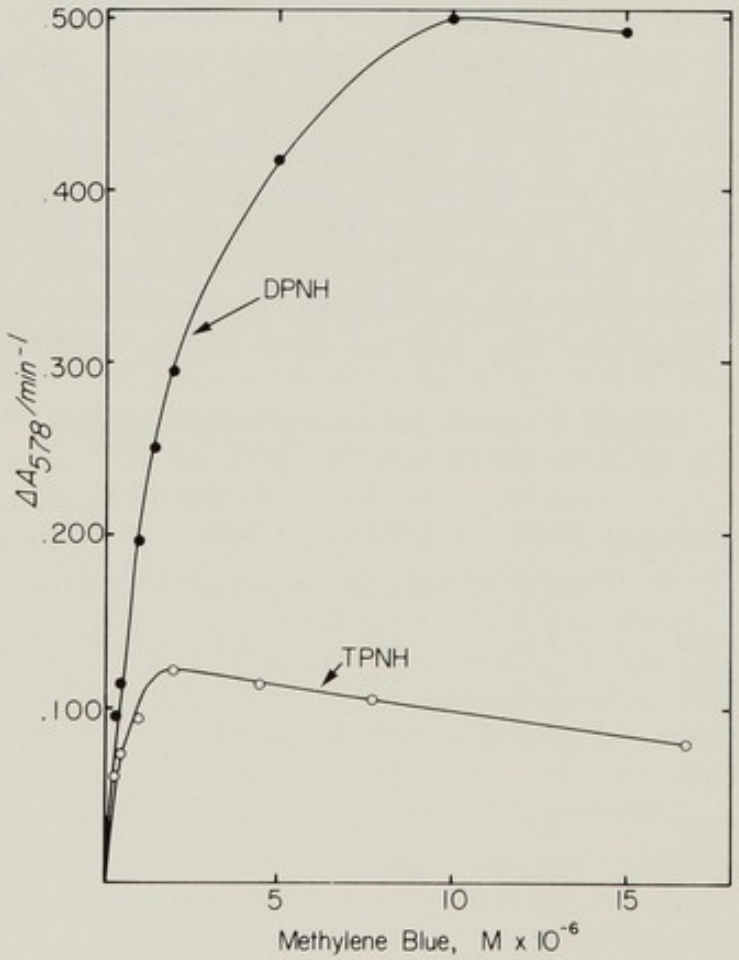


FIG. 3. Effect of methylene blue concentration on the methemoglobin-reducing activities. Standard assay system, as described in Figure 2, with methylene blue concentration varied as indicated. One unit of enzyme activity was defined as that amount producing an absorbancy change of 0.010 at 578 mμ in 10 min. under the above conditions; specific activity was expressed as units per mg protein.

The following substances, when tested at 1 x 10^{-4} M, were unable to substitute for methylene blue: FAD, FMN, toxoflavin, menadione, coenzyme Q_{10}, lipoic acid, lipoamide, *a*-tocopherol, oxidized glutathione, dehydroascorbic acid, and ergothioneine.

(d) *Terminal Electron Acceptors.* In the absence of methylene blue, there was essentially no oxidation of DPNH or TPNH, using cytochrome *c*, methemoglobin, or oxygen as the acceptor. In the presence of the dye, all of these oxidants could serve as the terminal acceptor, which is in agreement with our previous findings.[11] A summary of the comparative rates for the TPNH- and DPNH-dependent reduction of these acceptors with the three fractions is given in Table 2. Acceptor specificity was compared on an equivalent, rather than on a molar, basis. The following equivalents per mole were used for the various acceptors: methemoglobin, 4; 2,6-dichlorophenolindophenol and methylene blue, 2; and cytochrome *c*, 1. Regardless of the fraction tested, the efficiency of the acceptors was as follows: cytochrome *c*>methemoglobin>oxygen. In each instance, the DPNH-acceptor activity was several-fold greater than the corresponding TPNH activity.

Under anaerobic conditions, methylene blue itself could be used as the terminal acceptor. The rate of pyridine nucleotide turnover under these conditions was somewhat less than maximal, however, owing to inhibition by the dye when used at substrate concentrations (5 x 10^{-5} M). When DCIP replaced methylene blue as the electron acceptor, the rates were about 20 times faster. This dye has an additional advantage in that it may be used under aerobic conditions since its reduced form is not reoxidized readily by oxygen. Con-

TABLE 2. *Acceptor specificity of enzyme fractions*

Fraction	Acceptor	Activity* TPNH	Activity* DPNH
I	O_2	582	1350
	methemoglobin	972	3100
	cytochrome *c*	1580	3900
	DCIP	2134	3020
	methylene blue	95	109
II	O_2	596	1550
	methemoglobin	1520	4480
	cytochrome *c*	2230	5900
	DCIP	2524	3056
III	O_2	177	540
	methemoglobin	476	1345
	cytochrome *c*	673	1720
	DCIP	656	782

*mμequivalents/min/mg protein.

Standard assay system with the indicated acceptors; methylene blue was omitted in the indophenol assay. When methylene blue was used as the terminal acceptor, its concentration in the assay was raised to 5 x 10^{-5} M.

versely, the stability of the reduced form makes it unsuitable for use as a carrier when methemoglobin is the terminal acceptor.

(e) *Inhibition of Enzyme Activities by Mercurials.* After incubation with 1.5 x 10^{-4} M PCMS for 5 min. at room temperature, the TPNH-methemoglobin reductase activities of fractions I, II, and III were inhibited 90, 48, and 30 per cent, respectively, while the DPNH-dependent activities were inhibited 91, 41, and 33 per cent, respectively. Thus, the sensitivity of the fractions toward the mercurial is: I>II>III. It is noteworthy that TPNH- and DPNH-methemoglobin reductase activities were inactivated to a similar extent for each fraction. The anaerobic reduction of methylene blue by TPNH and DPNH was also inhibited about 80 per cent by 1.5 x 10^{-4} M PCMS.

(f) *Heat Inactivation of the Enzyme Fractions.* Results similar to those for PCMS inhibition were obtained in experiments involving heat inactivation of the fractions (Fig. 4). Fraction I was more sensitive to heat than either II or III. Again, it should be noted that in any fraction the TPNH- and DPNH-methemoglobin reductase activities showed an identical sensitivity to heat.

Nature of the Enzyme Fractions

Our earlier work[11] on the isolation of a TPNH-methemoglobin reductase activity was carried out without the benefit of chromatography on exchangers such as DEAE-cellulose. Consequently, the existence of separable forms of the methemoglobin reductase was not recognized at that time, and the properties reported for the enzyme were undoubtedly composite values for a mixture of the three fractions. The low activity observed with DPNH (about one-fifth that with TPNH) may have been due to (a) nonoptimal assay conditions for DPNH activity, or (b) selective destruction of the DPNH activity by the chloroform-ethanol step used in the previous purification procedure. Conversely, Scott's isolation of an enzyme that has predominantly DPNH-methemoglobin reductase activity[28] might be due to (a) nonoptimal assay conditions for the TPNH activity; (b) use of an indophenol dye, rather than methemoglobin, as the acceptor; and (c) some step in the purification procedure that selectively inactivates the TPNH activity.

The first indications that methemoglobin reductase might exist in multiple forms came from the work of Shrago and Falcone,[29] who obtained two peaks of TPNH-dependent activity following chromatography on DEAE-cellulose. Later, Scott et al.[27] used chromatography on calcium phosphate to separate four forms with diaphorase activity (TPNH dehydrogenases A and B and DPNH dehydrogenases I and II). Of the four proteins, only DPNH dehydrogenase I showed appreciable methemoglobin-reducing activity. From the present data it appears that three separate proteins, each carrying *both* DPNH- and TPNH-methemoglobin reductase activities, can be isolated from human erythrocytes. Since pooled red cells (generally 10 units of whole blood) were used as the starting material, it is not yet clear whether all three components are present in each person's blood.

Analysis of an individual blood for the three methemoglobin reductase fractions should be possible by electrophoretic methods. Preliminary experiments using electrophoresis on starch gel have revealed that purified I, II, and III

each move toward the positive electrode at pH 7.5 with the same order of mobility (i. e., III>II>I) that would be predicted from their behavior on the DEAE-cellulose columns. In order to examine hemolysates for these fractions, a simple and reliable histochemical method is required for detection of the TPNH- and DPNH-methemoglobin reductase activities on starch gel. Unfortunately, the enzyme cannot couple the reduced pyridine nucleotides to tetrazolium dyes, thereby obviating the technique that has proved to be so useful for locating lactate dehydrogenase activity. Some success has been obtained by spraying the gel surface with DPNH (oxygen being used as the terminal acceptor) and, after a period of time, examining the slab under ultraviolet light. The surface has a bluish-white fluorescent appearance, with dark blue bands marking enzyme activity.* Alternatively, the slabs can be treated with DPNH and indophenol which gives a dark blue surface with white zones corresponding to the enzyme activity. Neither of these methods, however, is ideal because

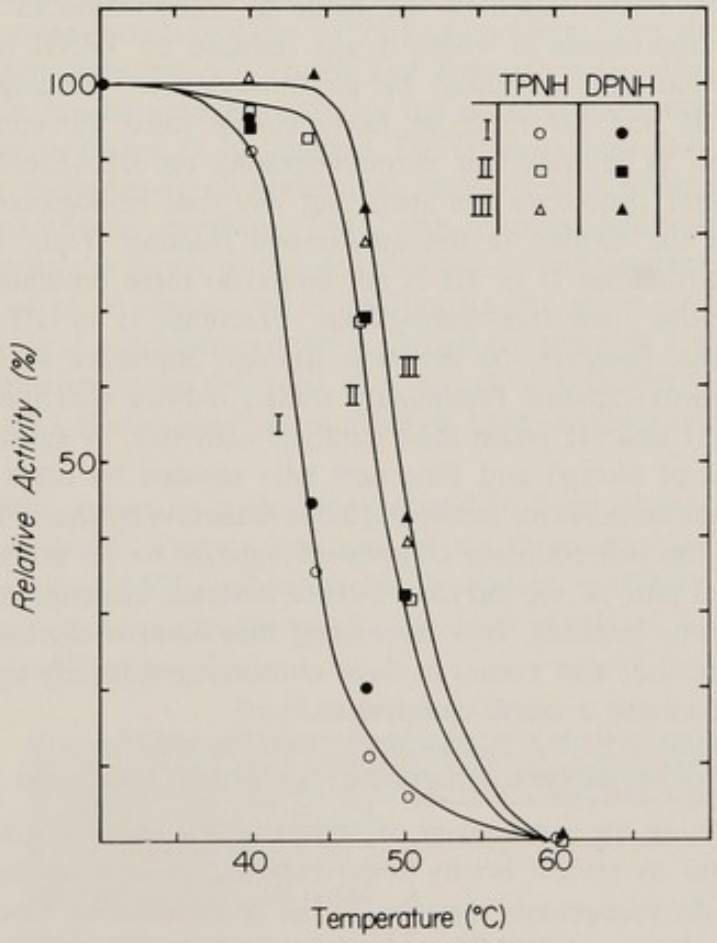


FIG. 4. Heat inactivation of methemoglobin reductase fractions. Two-tenths ml of each fraction was exposed at the designated temperature for 5 min., chilled immediately, centrifuged to remove any precipitate, and assayed by the standard method. ○ and ●, TPNH and DPNH activities of Fraction I; □ and ■, TPNH and DPNH activities of Fraction II; △ and ▲, TPNH and DPNH activities of Fraction III.

*Drs. J. C. Kaplan and E. Beutler (personal communication) have used essentially this same technique for locating methemoglobin reductase activities. See also page 109 of these proceedings.

the enzymatic zones become progressively more diffuse and the effects are transient.

Some caution should be exercised in categorizing I, II, and III as "isozymes" of methemoglobin reductase because of the heme pigment associated with II and III. The presence of this material might well affect the behavior of a protein on DEAE-cellulose and could also alter kinetic constants, susceptibility to inhibitors, and heat stability. In addition, II apparently contains a nucleotide-like component that might affect its properties. The nature and possible function of this 268-mμ absorbing material has not been investigated further, but it should be recalled that bovine erythrocytes contain a nucleotide factor that stimulates the TPNH-methemoglobin reductase activity.[12]

The nature of the heme component and its interaction with II and III is interesting. In our earlier studies, carried out with a mixture of I, II, and III, this component was characterized by its absorption spectra which, in the oxidized, reduced, and reduced-oxygenated states, were very similar to those of hemoglobin.[11] Participation of the heme in methemoglobin reduction was inferred from experiments in which it was reduced by TPNH in the presence of methylene blue and reoxidized by methemoglobin.* Binding of the heme component to II and III must be fairly strong since the complex survives procedures such as dialysis and chromatography on DEAE-cellulose. Conditions of the latter procedure are such that any free hemoglobin emerges immediately from the column in the unadsorbed fraction. Thus, if the pigment that appears in fractions II or III is not bound to these proteins, it must have different properties than free hemoglobin. Fractions II or III do lose their heme component, however, by filtration through Sephadex G-75 or chromatography on hydroxylapatite. Preliminary studies indicate that the "hemoglobin-free" forms of II and III retain their catalytic activities, in agreement with the previous results of Shrago and Falcone[29] who showed by other methods that the heme was not essential for methemoglobin reduction by the TPNH-dependent enzyme. Thus, the heme-protein component appears to be neither a structural nor a functional part of the enzyme, but its intimate association with proteins II and III remains puzzling. It is interesting that another erythrocytic enzyme, glutathione reductase, also occurs in three chromatographically-separable forms, one of which contains a heme component.[1]

Mechanism of Electron Transport in Methemoglobin Reductase

Before discussing the mechanism of action of the methemoglobin reductase, it may be useful to review briefly some relevant aspects of electron transfer in the DPNH dehydrogenase segment of the mitochondrial electron transport system. The oxidation of DPNH and succinate by molecular oxygen, according to the sequence shown in Figure 5, is carried out with high efficiency by an electron transport particle (ETP) derived from mitochondria. It should be noted that DPNH and succinate are served by individual electron transport chains that converge at coenzyme Q; thereafter a common chain leads to oxygen,

*Morrison[22] has described briefly a hemoprotein from erythrocytes that contains a unique iron porphyrin prosthetic group (see also Reed and Hultquist[24]) and can be linked to a TPNH diaphorase.

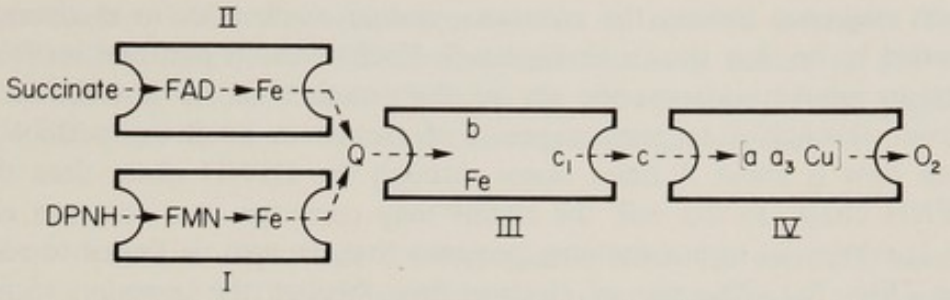


FIG. 5. Sequence of electron transport in mitochondria or ETP.

the terminal acceptor. As shown by Hatefi,[8] ETP can be comminuted into four subparticles (indicated by dashed lines in Fig. 5), each of which represents a functional and structural portion of the original system. The subparticle representing the DPNH chain, i.e., DPNH-Q reductase, can be further resolved by an appropriate treatment into a soluble DPNH dehydrogenase (flavoprotein), an insoluble iron protein, and an insoluble structural protein plus lipid (Fig. 6). Complete reconstitution of the DPNH-Q complex from these components has not yet been achieved, but Hatefi and Stempel[9] have shown recently that the iron protein can be used to reoxidize the reduced flavoprotein as predicted by the sequence in Figure 6. In addition, DPNH dehydrogenase can be coupled to ferricyanide, indophenol, quinones, and cytochrome *c*, although the behavior with these acceptors is complex. Thus, one of these acceptors, ferricyanide, appears to bypass the flavin in the dehydrogenase, and another, cytochrome *c*, functions out of place relative to its position in the sequence of Figure 5. In fact, it appears as if any oxido-reduction carrier can serve as a terminal acceptor for DPNH dehydrogenase provided that it has a sufficiently high oxidation potential and that it can bind to, or interact with, an acceptor region on the enzyme.

Methemoglobin reductase may be examined in light of the above observations. The present data suggest that each of the three protein fractions carries both DPNH- and TPNH-methemoglobin reductase activities; using optimal assay conditions, these activities are in the ratio of about 4:1. The electron

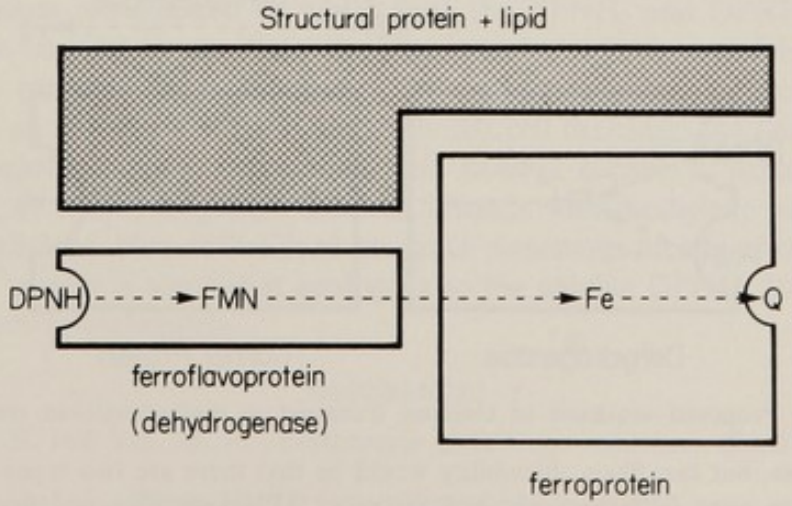


FIG. 6. Components of DPNH-coenzyme Q reductase.

transport sequence linking the reduced pyridine nucleotides to methemoglobin is assumed to be that shown in Figure 7. Each reduced pyridine nucleotide is believed to interact at a specific site on the protein* and to transfer its reducing power through a separate segment of the chain involving a thiol group. Electron flow is about 4 times faster through the DPNH chain than through the TPNH chain. In the cell, the chains may converge at a common electron carrier (perhaps an iron-containing protein) that, in turn, is linked to methemoglobin (Fig. 7a). The rate of electron flow through the common segment is slower than through either of the pyridine nucleotide segments, and the former, therefore, represents the rate-limiting portion of the chain. When methylene blue is added, it displaces the natural carrier in the sense of providing a rapid, but non-enzymatic, bridge between the dehydrogenase protein and methemoglobin. By arbitrarily choosing dye-linked assays to follow the enzyme through purification, investigators have probably insured that only the dehydrogenase portion of the cellular methemoglobin reducing system would be isolated. In support of this hypothesis, one may cite the work of Cormier and Rostorfer[2] who demonstrated that several flavoproteins, such as diaphorases or xanthine oxidase, readily reduce methemoglobin in the presence of methylene blue; the latter portion of the sequence (dye→methemoglobin) is believed to be non-enzymatic.

As stated above, the bound heme component of methemoglobin reductase was shown to be susceptible to reduction under anaerobic conditions by TPNH. This may represent an alternate pathway for reducing methemoglobin in the cell, but once the enzyme is removed from the cellular environment and linked to methemoglobin via a dye, the bound heme may be extraneous. Thus, removal of the heme does not impair the capacity of the enzyme to function in the artificial electron transport system. This is perhaps similar to the occurrence of cytochrome *b* in certain succinic dehydrogenase or succinic-Q reductase preparations;[31] although present in stoichiometric amounts, this cytochrome does not appear to be an intermediate in the main pathway of electron transport.

It is apparent from this brief discussion that there are still a number of problems relevant to the elucidation of enzymology and mechanism of methemo-

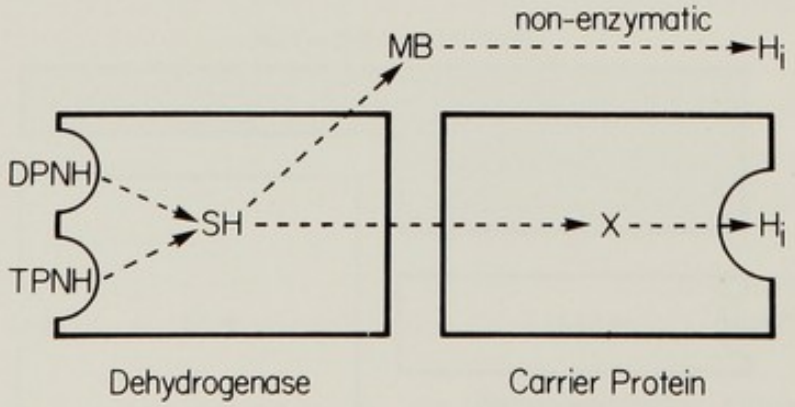


FIG. 7. Proposed sequence of electron transport in methemoglobin reductase.

*An alternate, but less likely, possibility would be that there are two types of methemoglobin reductase (one DPNH-specific and the other TPNH-specific) and that, because of similarities in protein structure, their ratio remains relatively constant during purification.

globin reductase. Until the complete sequence of electron transport between reduced pyridine nucleotides and methemoglobin, as it occurs in the cell, is discovered, it may be difficult to delineate the exact nature of the genetically-controlled enzymic deletion that occurs in methemoglobinemia.

Summary

Pyridine nucleotide-dependent methemoglobin reductase activities have been purified from human red cell hemolysates by a modification of the procedure originally developed in this laboratory. The hemolysate is treated with DEAE-cellulose to remove most of the hemoglobin, and the enzyme activities are futhered purified by means of fractionation with ammonium sulfate and acetone. Chromatography of the preparation on DEAE-cellulose, using a phosphate gradient for elution, yields three protein fractions, possibly isozymes, each of which carries DPNH- and TPNH-methemoglobin reductase activities in a constant ratio of about 4:1.

Fraction I is colorless and has a single absorption maximum at 278 mμ. Fraction II contains a small amount of a heme component and a nucleotide-like material that is characterized by an absorption maximum at 268 mμ. Fraction III contains a relatively large amount (ca. 30 per cent) of a heme component whose spectrum resembles that of methemoglobin. None of the fractions contains any flavin. Michaelis constants have been determined for TPNH and DPNH with I, II, and III. The K_m values for TPNH are about one order of magnitude lower than the corresponding values for DPNH, although the V_{max} values with the latter are about four-fold higher. Both methemoglobin reducing activities require methylene blue as an electron carrier; the K_m value for the dye in the TPNH system is lower than that for DPNH. Michaelis constants for methemoglobin are the same for DPNH and TPNH in each fraction. Each fraction will utilize cytochrome *c*, methemoglobin, and oxygen as terminal electron acceptors provided that methylene blue is present as a carrier. 2,6-Dichlorophenolindophenol can be used as a terminal acceptor in the absence of methylene blue. In each fraction, both the TPNH- and DPNH-methemoglobin reductase activities are inhibited to the same extent by mercurials and inactivated to the same degree by gradual heat denaturation.

A mechanism is suggested by which both the TPNH- and DPNH-dependent activities are carried by separate electron transfer segments on the same dehydrogenase protein. These chains are believed to converge at a common site occupied by an unknown electron carrier in the cell that links the dehydrogenase to methemoglobin. Upon purification, the natural carrier is presumed to be lost, leading to a dehydrogenase that can interact with methylene blue. In turn, reduced methylene blue is believed to react nonenzymatically with methemoglobin. This system is somewhat analogous to the soluble DPNH dehydrogenase obtained from mitochondria.

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DISCUSSION

BERGREN, LOS ANGELES, CHAIRMAN: The discussion is open.

MORRISON, DUARTE: I wonder whether you were indicating that SH actually plays a role as a redox component, or were you suggesting that SH is a necessary part of the structure of the enzyme? If the former is the case, do you have any evidence that SH is reduced and oxidized?

HUENNEKENS, LA JOLLA: I think that the evidence is much stronger in the case of the DPNH dehydrogenase where one (or two) SH groups appear when the enzyme is reduced with DPNH. As a matter of fact, there is a possibility that this might not be due to the cleavage of an SS bridge but something more like a thioketone being reduced to a thioalcohol. I think the evidence is less decisive in the case of the methemoglobin reductase, although, when the enzyme is incubated with the reduced pyridine nucleotides, there does appear to be an increase in thiol groups. Perhaps a better reason for invoking thiol groups in the electron transport sequence is that little else is available to link to the methylene blue. There is no flavin involved as a carrier, and one is at a loss to suggest any other oxidation-reduction carrier.

MORRISON: Have you evaluated the possibility that your enzyme may contain a flavin that is covalently linked, such as in the case with succinic dehydrogenase?

HUENNEKENS: Yes, one always has to bear in mind that the flavin in succinic dehydrogenase is not released with acid or heat treatment. We have tried to digest the methemoglobin reductase with proteolytic enzymes, and nothing emerges. That, coupled with the fact that highly purified preparations of the enzyme have essentially no flavin spectrum, more or less rules out any flavin involvement.

Congenital Methemoglobinemia Due to DPNH-Diaphorase Deficiency

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METHEMOGLOBINEMIA due to enzyme deficiency is a rare but widespread metabolic abnormality with the classic features of an autosomal recessive. Before describing it in detail, I would like to consider the chemistry of hemoglobin.

Oxyhemoglobin is, from a thermodynamic standpoint, a metastable substance. This is apparent from Table 1, where oxidation-reduction potentials of several important susbtances are listed. Obviously the thermodynamically-stable form of hemoglobin in the presence of oxygen is methemoglobin. The reasons for the resistance to oxidation of oxyhemoglobin are not known.

There are three ways in which hemoglobin may be oxidized to methemoglobin. First, there may be direct oxidation. Oxygen is believed to oxidize hemoglobin, although at a very slow rate. Ferricyanide also will oxidize hemoglobin directly as will 2, 6-dichlorophenolindophenol (DCIP). Second, it may be oxidized through an intermediary substance. Methylene blue is such an intermediary. While its oxidation-reduction potential is too low for methylene blue itself to oxidize hemoglobin directly to any appreciable extent, the dye reacts quickly and reversibly with hemoglobin, and also with atmospheric oxygen. Thus, hemoglobin in the presence of methylene blue and air and in the absence of reducing agents will oxidize rapidly to methemoglobin. 2,6-dichlorophenolindophenol does not react with oxygen and is not an intermediary in this type of reaction.

The third way in which a substance may affect oxidation of hemoglobin is by forming a complex that increases the lability of hemoglobin to oxidation. This is the action of nitrite, which forms a complex readily oxidizable by oxygen. It has recently been shown by E. Hegesh (personal communication) that ferrocyanide forms a similar labile complex. It should be noted that a labile form of hemoglobin is not only more readily oxidized, but that the methemoglobin formed should show a similar lability to reduction.

TABLE 1. *Standard oxidation-reduction potentials (E'o) at 25° and pH 7*

	Volts		Volts
$H_2O/\frac{1}{2}O_2$	0.815	Methylene blue	0.01
Ferro/ferricyanide	0.36	Lactate/pyruvate	—0.18
DCIP	0.22	Glutathione	—0.25
Hb_2/MetHb	0.165	Glyceraldehyde-3-P	—0.28
Ascorbic acid	0.08	DPNH/DPN^+	—0.32

A large number of substances will reduce methemoglobin directly. Both DPNH and TPNH in high concentration will nonenzymatically reduce methemoglobin, but at their normal concentration in red cells, no reaction can be measured. Ascorbic acid is a good reductant of methemoglobin and at the concentration present in red cells will reduce it at the rate of about 2 per cent per hour.[11] Glutathione is only about a tenth as effective as ascorbic acid in reducing methemoglobin, but at the usual concentration in cells, will reduce it at a rate of about 1.5 per cent per hour.[11]

The enzymatic mechanisms of methemoglobin reduction are of more interest, and of primary importance is the enzyme that is lacking in congenital methemoglobinemia. That this enzyme is indeed completely lacking was shown by parallel purification of it from normal and methemoglobinemic blood[10] No trace of the normal enzyme was found in methemoglobinemic cells, after the procedure applied had produced a 5400-fold purification of the normal enzyme.

The enzyme has the properties of a diaphorase; it is quite specific with respect to the electron donor, but much less so with respect to electron acceptor. DPNH is the preferred donor; TPNH had only 1.5 per cent as much activity.[14] The acceptor can be oxygen, methemoglobin, cytochrome *c*, ferricyanide, or any of several dyes. The most active of those acceptors studied in detail is 2,6-dichlorophenolindophenol (DCIP). This substrate is the best and easiest for assay of the enzyme in whole hemolyzates, and it is used to establish diagnosis of this type of methemoglobinemia.[9]

Since diaphorase is a trivial name, the enzyme has been also called DPNH dehydrogenase I, to distinguish it from a second similar enzyme in red cells. One of the least active substrates of the enzyme is methemoglobin, which is only 1:9000 as active as DCIP. Because of its demonstrable physiologic role, however, the proper systematic name is DPNH-methemoglobin oxido-reductase. In spite of its poor reactivity with methemoglobin, it can be shown that there is sufficient enzyme in red cells to account for observed rates of methemoglobin reduction in red cells.

The enzyme contains a flavin with the properties of flavin adenine dinucleotide. Like all known flavin enzymes, the kinetics of the reaction are consistent with the successive formation of binary complexes of substrate enzyme. In all probability, DPNH first reduces the flavin and the flavin in turn reduces the other substrate. Kinetic studies showed that the dependence of rate on DPNH concentration was less than its dependence on DCIP concentration; and thus the rate of reduction of dye was more nearly rate-limiting than the reduction of DPNH. Consequently, the reaction is nearly zero order with respect to DPNH but almost first order with respect to dye under usual assay conditions. The rate of reduction of methemoglobin is so much slower that the reaction is zero order with respect to DPNH and first order with respect to methemoglobin. During methemoglobin reduction, the enzyme must consequently be essentially fully reduced.

Above pH 7.5, the rate of enzymatic reduction is independent of pH, but the rate of reaction becomes much faster at lower pH. It is well known that hemoglobin is much more readily oxidized by oxygen at low pH[5]; this lability to oxidation is accompanied by a corresponding lability to reduction.

There is a second, minor, DPNH diaphorase activity in normal cells that is distinct from that we have described. This enzyme was first purified from methemoglobinemic red cells.[10] Methemoglobinemic hemolyzates do show a small amount of DPNH diaphorase activity. Part of this can be accounted for by the system of DPNH-glutathione reductase plus glutathione, since glutathione reduces DCIP rapidly, but part is due to a second DPNH dehydrogenase. This second enzyme is also present in normal cells, and can be separated from the major DPNH dehydrogenase by chromatography.[11]

The principal differences in the two enzymes are shown in Table 2. Besides these considerable differences, DPNH dehydrogenase I is more stable to heat and acid, is less active at low pH, and has a higher temperature coefficient. The ratio of activity with DCIP and with methemoglobin is similar however. The minor DPNH dehydrogenase is unusual among diaphorases because of its ternary kinetics. It appears to consist of a protein that binds DPNH, and the bound form of DPNH is more reactive than the free form.

At least two TPNH dehydrogenases occur in red cells.[11] These are quite similar in properties, but can be separated chromatographically. The properties described here are those of the major component. A TPNH dehydrogenase has been prepared that has only 1 per cent activity with DPNH. This enzyme does not contain flavin, and gives typical binary kinetics. It is strongly activated by high ionic strength. By contrast, the DPNH dehydrogenases are inhibited by salts; even the small amount of TPNH dehydrogenase activity in DPNH dehydrogenase I is so inhibited. The TPNH dehydrogenases are poor methemoglobin reductases being only 1/55000 times as active as with DCIP.

A summary of the relative rates of hemoglobin reduction by the known systems under conditions simulating those in the red cell is shown in Table 3. The sum of these calculated rates is in good agreement with that found in red cells under in vitro conditions. These results, however, do not agree with the situation in vivo.

In normal persons, the amount of methemoglobin is scarcely measurable. Van Slyke et al.[15] have estimated this as about 0.4 per cent of the total hemoglobin. It is commonly believed that the amount of methemoglobin in cells is

TABLE 2. *Differences between DPNH dehydrogenases*

	Dehydrogenase I	Dehydrogenase II
Methemoglobinemia	absent	present
Relative amount in normal cells	90	10
Flavin	yes	no
Relative activity:		
DPNH	100	100
TPNH	1.5	?
Deamino DPNH	97	15
Kinetics:		
Complex	binary	ternary

TABLE 3. *Estimated rates of methemoglobin reduction by various systems*

	Per cent reduced per hour	Per cent of reducing capacity
DPNH dehydrogenase I	8.0	61
Ascorbic acid	2.1	16
Glutathione	1.6	12
TPNH diaphorases	0.7	5
DPNH dehydrogenase II	0.8	6
Total	13.2	100
Reduction in whole cells:		
Normal	10-15	
Methemoglobinemic	2-5	

the result of two opposing processes—a continuing oxidation of hemoglobin balanced by a continuing reduction of methemoglobin. If the rate of oxidation is proportional to the concentration of hemoglobin, and the rate of reduction is proportional to the concentration of methemoglobin, the following relationship holds:

$$\text{Rate of oxidation} = k_{ox}\,[\text{Hb}]$$
$$\text{Rate of reduction} = k_{red}\,[\text{MetHb}]$$
$$\frac{k_{ox}}{k_{red}} = \frac{[\text{MetHb}]}{[\text{Hb}]}$$

These assumptions are worth examining. The oxidation of hemoglobin, if non-enzymatic, would be expected to be first order with respect to hemoglobin. Kiese et al.[4] have shown that this is the case in acid solution. Our studies indicate that the reduction of methemoglobin, both enzymatic and non-enzymatic, is first order with respect to methemoglobin, at least in vitro.[11]

The values of k are rate constants and represent a measure of reducing or oxidizing capacity that is independent of substrate concentration. The consequences of this equation are shown in Table 4. If only 0.4 per cent of the total hemoglobin is methemoglobin, then the reducing capacity of the cell must be 250 times as great as its oxidizing capability. Methemoglobin cells contain

TABLE 4. *Relative capacities of red cells for oxidation and reduction of hemoglobin*

Type of cells	Per cent MetHb	Assumed k_{ox}	Relative k_{red}
Normal	0.4	1	250
MetHb	10.0	1	9
	25.0	1	3
	45.0	1	1.2
Heterozygote	0.8	1	126-130

between 10 and 45 per cent methemoglobin, and the reducing capacity in these cells should be only 1.2 to 9 times the oxidizing capacity. A heterozygote would be expected to have an intermediate reducing capacity, 126 to 130 times the oxidizing capacity. This would result in an increase of methemoglobin to only 0.8 per cent, which is within normal limits. This concept, then, describes exactly the situation found in vivo, where no appreciable increase in methemoglobin is found in heterozygotes. Furthermore, it provides a purely kinetic basis for expecting a genetic trait to be recessive.

An obvious consequence is that DPNH diaphorase must account for over 95 per cent of the total methemoglobin-reducing capacity of the red cell. This is far higher than the per cent found in vitro studies, either in whole cells or hemolyzates. The reason for this discrepancy is unknown.

It is obvious from this equation that in the normal person, small changes in rate of oxidation of hemoglobin, of the order of two- to three-fold, or of amount of DPNH diaphorase will have no important effect on the amount of methemoglobin. This is consistent with clinical observation; methemoglobinemia is found only in complete absences of DPNH diaphorase or in massive intoxication. The heterozygote will not differ greatly from the normal individual in sensitivity to these changes.

This concept is consistent with the known therapeutic effect of ascorbic acid on methemoglobinemia. It has been our experience that high dietary intakes of ascorbic acid can reduce methemoglobin levels by about two-thirds over a period of two weeks, e.g., from 30 to 10 per cent.[12] This requires a three-fold increase in reducing capacity, which would appear unlikely unless the reducing capacity were very low to begin with.

The DPNH-diaphorase-deficient person, however, will be sensitive to small changes in both rate of oxidation and in residual reducing capacity. This is consistent with the variability of per cent of methemoglobin observed in various diaphorase-deficient individuals.

A final consequence of the concept is to reinforce the conclusion previously stated[11]; that the only defects in reducing mechanism that can cause methemoglobinemia are either a deficiency of DPNH diaphorase or a deficiency of DPNH itself. Further, it is evident that if a DPNH deficiency were the cause, it must be a very profound deficiency to produce a measurable increase in methemoglobin. Neither in scurvy, in glutathione deficiency,[7] nor in TPNH diaphorase deficiency[8] has methemoglobinemia been found.

It has been reported that older cells in individuals with methemoglobinemia contain more methemoglobin than young cells, and it was concluded that this segregation is due to a loss of reducing capacity of older cells.[4] This appears easily possible, since small changes in this capacity will readily produce large changes in methemoglobin concentration in methemoglobinemic subjects. A recalculation of these results in Table 5 shows that 87 per cent of the methemoglobin in blood occurs in the 62 per cent of cells classified as "old."

The form of the methemoglobin present in enzyme-deficiency methemoglobinemia has been studied, but no definite conclusions reached.[2] There appear to be only two major components—oxyhemoglobin and half-oxidized hemoglobin when a hemolyzate is subjected to starch gel electrophoresis.

The pattern of inheritance of methemoglobinemia is easily established since use of the dye assay for diaphorase permits clear identification of heterozygotes. Measurement of the rate of methemoglobin reduction has also been suggested for this purpose, but never proved very successful in our hands.

Two problems in genetics still remain. First, it would be interesting to determine whether the gene involved is a "control" gene or whether an amino acid substitution has occurred. This problem could perhaps be solved by immunoelectrophoretic means, although the amount of DPNH diaphorase present in red cells (less than 0.002 per cent of total protein) may be too small for successful use of this technique.

The second problem is one of locating the gene on the human chromosomes. This can be done if linkage can be established with other polymorphisms. So far, no linkage has been found. We have already reported no close linkage with the blood groups ABO, MNS, P, Rh, Duffy and Kidd, and with haptoglobins.[13] More recently we have found no linkage with the red cell enzymes acid phosphatase and phosphoglucomutase, nor with the Gc factor and the bisalbuminemia recently described by Melartin and Blumberg[6] in Alaskan and Canadian Indians.

Another way of determining location on the chromosomes is by study of methemoglobinemic patients with chromosomal abnormalities. A significant number of methemoglobinemic persons are also mentally retarded or otherwise grossly abnormal. One of these was examined and no chromosomal abnormality found,[3] but if the aberration were small it might not be readily detectable. The possibility still remains that among mentally-retarded patients with methemoglobinemia, a significant chromosomal defect may yet be found.

It has been proposed that the combination of mental retardation and methemoglobinemia may be due to an interaction of factors.[1] It is difficult to see how DPNH diaphorase deficiency or excess methemoglobin could interact with maternal or other genetic factors; the relative anoxia that could occur during fetal development seems more likely to be involved in such an interaction.

Hereditary methemoglobinemia is believed to be benign, and its chief clinical importance is as a possible cause for what can be a striking cyanosis. Scientifically, it serves as a model of a human genetic metabolic defect, and deserves further study.

Summary

Methemoglobinemia due to enzyme deficiency in all cases studied to date has been found to be due to absence of a DPNH diaphorase. This enzyme has been purified from normal cells and distinguished from other similar enzymes. Both actual measurements and kinetic considerations show this enzyme to be

TABLE 5. *Segregation of methemoglobin in methemoglobinemic red cells*

	Per cent of total cells	Per cent MetHb of total	Per cent of total MetHb
Young cells	38	7.4	17.5
Old cells	62	21.3	82.5

the principal means by which hemoglobin is maintained in the reduced form. The nature of the gene change, whether structural or a "control" gene, is unknown. The significant number of mentally-retarded methemoglobinemic children deserves further study.

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DISCUSSION

BERGREN, LOS ANGELES, CHAIRMAN: We are ready for discussion.

WALLER, TUEBINGEN: Dr. Löhr and I have found that the methemoglobin level is higher in older red blood cells than in younger ones. The methemoglobin concentration in the 80 to 90-day-old red cell population is about 7 per cent as determined on red blood cells separated by differential agglutination in vivo. The cause is a decrease of enzymatic methemoglobin reduction in older red cells. For some patients with methemoglobin reductase deficiency, the velocity of methemoglobin reduction with glucose as substrate is normal, but with lactate as substrate it is sharply decreased. I have no explanation for this observa-

tion. Is it possible that the activity of the TPNH-dependent reaction is higher? Dr. Mathies in Madeberg has made similar observations in another patient. He found that methemoglobin reduction in the enzyme-deficient red cells was normal with glucose in the absence of methylene blue, but was greatly decreased with lactate.

SCOTT, ANCHORAGE: In all our cases of methemoglobin reductase deficiency, the TPNH diaphorase activity is normal; in one or two it seemed a little high, but still in the normal range.

WALLER: I am also interested in the metabolism of methemoglobin reductase-deficient cells. We have seen, as has Dr. Jaffé, that the ratio of lactate to pyruvate in nitrited normal cells with high methemoglobin concentration is decreased, but that this ratio is high in methemoglobin reductase-deficient cells. Theoretically, this seems to be clear, but have you also observed this?

SCOTT: I have never done anything with this.

KAPLAN, DUARTE: I would like to mention some very recent results that I obtained in Dr. Beutler's laboratory concerning electrophoretic studies of methemoglobin reductases. I could stain these diaphorases by linking them to a tetrazolium salt (MTT) through dichlorophenolindophenol, thus obtaining a positive stain. The bands that were seen with DPNH or TPNH were in good agreement with the location of defluorescence when examined under UV light. Several bands were obtained; at least four (Figure 1): the two slower bands were strongest and the two faster were much weaker. The two main slow bands appear to differ in their intensity according to the reduced coenzyme which is used as an electron donor. One band is much stronger with DPNH, the other being weak; the reverse is observed with TPNH without any change of mobility. We believe that these bands do represent a diaphorase activity since they are not obtained if DPNH or TPNH is omitted. We do not know yet whether they represent two different enzymes without complete specificity towards the coenzyme or two or more isozymic forms of the same enzyme whose reactivity with DPNH and TPNH is different.

SCOTT: The little gel electrophoresis we did gave results not too different from yours. I missed the fast bands with DPNH. The slowest band is the strong band. The second slowest is the one that is missing in DPNH diaphorase deficiency. I don't know what the slowest band is. It's there. With TPNH we got

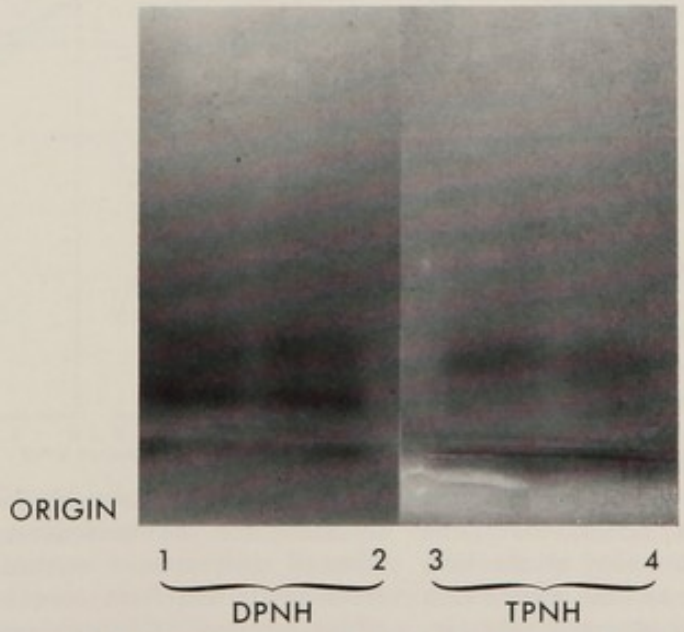


FIG. 1. Starch gel electrophoresis of RBC diaphorase at pH 7.5 (crude hemolysate). Two very weak bands, more anodic, are only barely visible on this picture. Hemoglobin is on the starting line.

the slow strong band again, and a series of more rapid bands. To do this, we just used the DPNH or TPNH and 2-6 dichlorophenolindophenol and looked for the white spots.

JAFFÉ, NEW YORK: I disagree on one minor point with Dr. Scott. I think that we have been able to show that it is possible to show a decreased capacity to reduce methemoglobin in the intact cell of heterozygous subjects. I also think, however, that this technique is not as neat and tidy as doing the actual diaphorase assay in hemolysates. Under very carefully controlled conditions, it does work. If one oxidizes the hemoglobin to methemoglobin with nitrite, washes the cells thoroughly and then incubates them, as in this case, with glucose or with inosine or lactate, a difference in the rate of reduction can be observed between the heterozygote- and the homozygote-deficient, confirmed by diaphorase assay, and the normal, especially after 22 hours (Figure 2). One of the problems has been that most investigators have tended to stop the incubations after six or, possibly eight hours. I think that you will agree that the difference that one would see at this point (six to 8 hours) might well be attributed to normal variation. Also, I would make the proviso that a normal specimen always be run at the same time because of variation in different buffer systems and under different renditions. With this system, however, one can differentiate, even with the intact cell, the heterozygote, in which erythrocytes have a decreased capacity to reduce methemoglobin and whose DPNH methemoglobin reductase activity, when assayed in hemolysates, is decreased. Clinically, however, the heterozygote has no difficulty. We had a theory at one time that those individuals who were more susceptible to toxic methemoglobinemia might, in fact, be heterozygotes. Ordinarily, they would have no difficulty, but when ex-

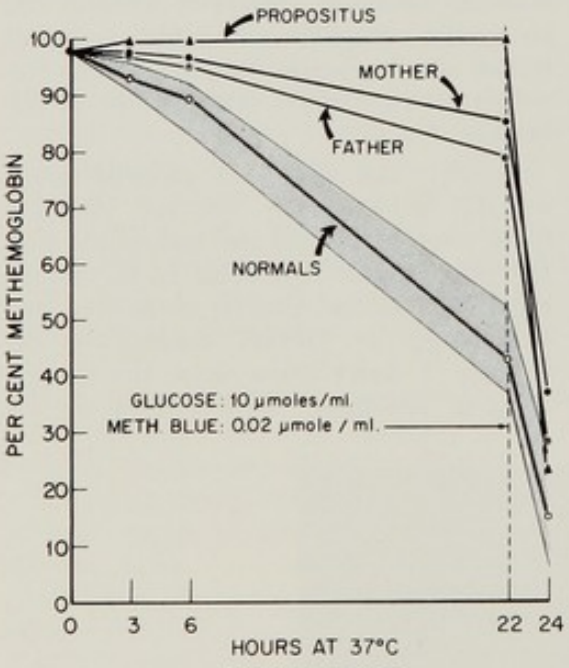


FIG. 2. Reduction of methemoglobin to hemoglobin during incubation of suspensions of nitrite-treated, washed erythrocytes from a child with hereditary methemoglobinemia associated with deficiency in the activity of NADH-methemoglobin reductase. Intermediate levels of activity of NADH-methemoglobin reductase were observed with hemolysates of erythrocytes from the acyanotic mother and father. Methylene blue (meth. blue) was added at the time indicated by the arrow, and the shaded area represents the variation observed with erythrocytes from 14 unrelated controls. (Reprinted from the American Journal of Medicine, 41: 42, 1966).

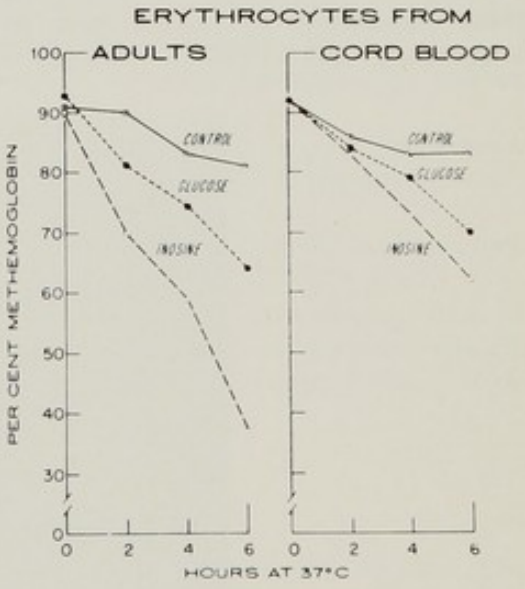


FIG. 3. Reduction of methemoglobin to hemoglobin during incubation of suspensions of nitrite-treated, washed erythrocytes from adults and from cord blood without added substrate (Control) and with 6.7 x 10^{-2} M glucose or inosine. (Reprinted from Proceedings of the Society for Experimental Biology and Medicine, 124: 214, 1967).

posed to a drug that increases the rate of oxidation of hemoglobin, they might have more methemoglobinemia. We recently had an opportunity to investigate a patient who had, for lack of a better name, enterogenous cyanosis, a toxic methemoglobinemia associated with large bowel ulcerative disease. Her erythrocytes had perfectly normal or slightly elevated DPNH methemoglobin reductase activity. The rate of reduction of methemoglobin in intact cells was markedly elevated. Unfortunately, at the time we were able to study her cells, she had a hemolytic episode as well. There is, I think, a slight increase in DPNH methemoglobin reductase activity in young erythrocytes.

Another situation in which there is good evidence for a decrease in the capacity to reduce methemoglobin has been recorded by many investigators. Erythrocytes of the newborn infant show a decreased capacity to reduce methemoglobin (Figure 3); this observation goes back to the German investigators, Künzer and Betke.* Both with glucose and with inosine as substrates, erythrocytes obtained from cord blood show a decreased capacity to reduce methemo-

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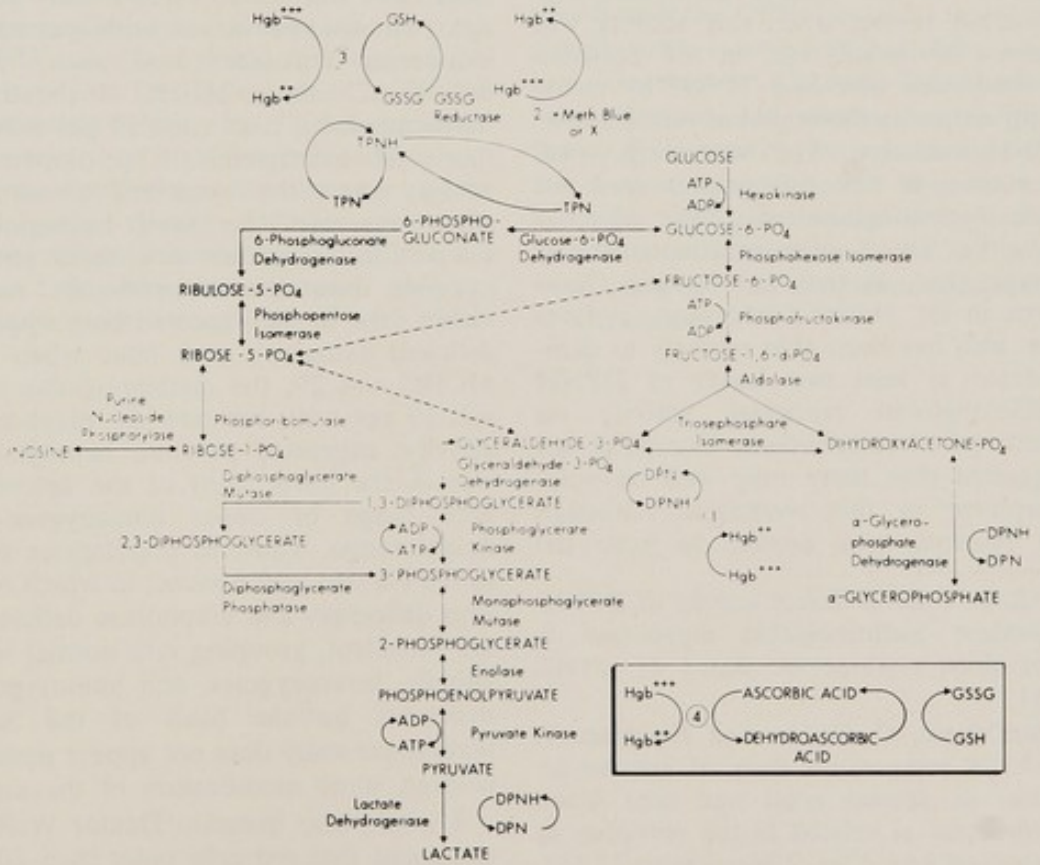


FIG. 4. Diagram of the relationship between the pathways of glucose and purine nucleoside metabolism and the reduction of methemoglobin (Hgb^{+++}) to hemoglobin (Hgb^{++}) in human erythrocytes. Reactions which have been omitted are indicated by interrupted lines. (1) DPNH (NADH)-methemoglobin reductase system. (2) TPNH (NADPH)-methemoglobin reductase system with methylene blue (Meth. Blue) or unknown natural cofactor (x). (3) nonenzymatic reduction of methemoglobin by reduced glutathione (GSH). (4) nonenzymatic reduction of methemoglobin by ascorbic acid. (Reprinted from the American Journal of Medicine, 41: 786, 1966).

globin. Ross, McDonald and Zipursky* have all shown that these erythrocytes have decreased DPNH methemoglobin reductase activity of the order that is observed in the erythrocytes of heterozygotes for hereditary methemoglobinemia.

Dr. Scott has made the point that the DPNH methemoglobin reductase system accounts for most of the capacity of normal human erythrocytes to reduce methemoglobin, that ascorbic acid and reduced glutathione can serve to reduce methemoglobin, and that the TPNH methemoglobin reductase system probably plays a very minor role, if any role at all (Figure 4). This conclusion is supported by the observation recently reported by Sass† from the Brooklyn Veterans Administration Hospital. A real example of serendipity was provided by a patient whose erythrocytes appeared to be deficient in the TPNH methemoglobin reductase system when this activity was assayed in hemolysates in the presence of methylene blue and TPNH by measuring either methemoglobin reduction or TPNH oxidation. The interesting point, of course, is that this patient does not have methemoglobinemia. The observations that Dr. Kaplan mentioned are of interest, too, in that Dr. Bastien Gompertz, in Dr. Huehn's laboratory in London, also has been able recently to demonstrate at least two bands of DPNH methemoglobin reductase activity on starch gel electrophoresis. Gompertz has suggested that there may well be polymorphism in this reductase. Although the patients may appear to have the same disorder with cyanosis and methemoglobinemia, there are certain observations which make it difficult to invoke just one simple mutation of one gene as the responsible mechanism. We may well find here, as has been the case in G-6-PD deficiency and in some other disorders, that there are a number of different mutations by which a hereditary disorder can be produced.

SIMON, ALBUQUERQUE: I wish to add a word of caution in regard to the interpretation of diaphorase activity when iron deficiency coexists. Apparently hypochromic iron-deficient cells have elevated diaphorase activities, when measured by the Scott assay. This is partly spurious since diaphorase activity is expressed per unit of hemoglobin, but even taking this into account and reporting activity on a per cell basis, elevated levels are still found. About two years ago, we saw an infant with presumed diaphorase deficiency and severe iron deficiency, with an MCHC of about 23. Methemoglobin comprised 17 per cent of the total hemoglobin. The diaphorase activity was in the lower limit of normal, when expressed per unit hemoglobin. Correction on a per cell basis put it squarely into the heterozygote range rather than in the expected homozygous-deficient range. A year later when the MCHC was 29, the methemoglobin was still 17 per cent, but now the diaphorase activity, expressed per unit hemoglobin, was in the lower limit of the heterozygote range or upper homozygous-deficient range. Thus, in populations such as the Navajo and Apache, in which both iron deficiency and diaphorase deficiency are prevalent, grouping into normal individuals, heterozygotes, and homozygous-deficients on the basis of the Scott diaphorase assay does not appear possible without some modification of the assay.

I would also question Doctor Waller's statement that red cells older than 80-90 days have a mean methemoglobin concentration of 7 per cent. Since such a cohort comprises ⅓ to ¼ of the normal cell population, normal methemoglobin levels of unfractionated blood should be 2 to 2.5 per cent instead of less than 1 per cent as is observed.

LÖHR, MARBURGH: Another reason for

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the lower activity of methemoglobin reductase in newborn blood is the presence of α-glycerophosphate dehydrogenase in the fetal erythrocytes.

JAFFÉ: The point that Dr. Löhr made was that, in cord blood cells, the increased α-glycerophosphate dehydrogenase activity would serve to oxidize some of the DPNH. This would remove it from the reaction and would either diminish the rate of methemoglobin reduction in the intact cell or would diminish the rate of dye reduction in the assay of the DPNH methemoglobin reductase system in the lysate. I would expect, however, if this were the case, that pyruvate would accumulate in these erythrocytes because DPNH would not be available for the reduction of pyruvate to lactate. We have had an opportunity to study erythrocytes from several cord bloods, including cord blood from a patient with hereditary methemoglobinemia associated with DPNH methemoglobin reductase deficiency. As Dr. Löhr and Dr. Waller mentioned before, the lactate-pyruvate ratio was very high. In these cells, there was no significant accumulation of pyruvate, even though DPNH methemoglobin reductase activity was low and the intact cells showed a decreased capacity to reduce methemoglobin. I am, therefore, not sure about the significance of the presence of α-glycerophosphate activity in cord blood cells.

MORRISON, DUARTE: I am somewhat puzzled by the discrepancies between Dr. Scott's report on the isolated diaphorase and Dr. Huennekens'. I wonder, if in Dr. Huennekens' absence, Dr. Scott might comment.

SCOTT: The only explanation that I have is that apparently he is separating out the major TPNH diaphorase that we purified earlier. This enzyme is very difficult to separate from the second DPNH diaphorase. For quite a while we felt that they were the same enzyme. I think the thing that really convinced us that they were different was that we let a sample of this enzyme sit in the refrigerator, frozen, for two or three months and we found that TPNH activity was retained and the DPNH activity was completely gone. I don't feel that there is any real discrepancy. Although Dr. Huennekens' enzyme had much more DPNH than TPNH activity, he was using a different assay system and I have no idea what this would do. He was using methylene blue plus methemoglobin while we were using 2,6-dichlorophenolendophenol as a receptor.

The Genetics of Glucose-6-Phosphate Dehydrogenase Deficiency

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LONG BEFORE the nature of the underlying red cell defect had been elucidated, clinicians noted a familial factor in the occurrence of favism and drug-induced hemolysis.[34] Since drug-induced hemolysis in G-6-PD-deficient subjects depends upon the coincidence of two factors, the basic red cell defect and exposure to drug, it was not possible to define the mode of genetic transmission. In the case of favism, matters were even more complex, since here only occasional individuals with the red cell defect developed hemolytic anemia when exposed to the offending bean. For these reasons, it was not until in vitro tests for the detection of the red cell abnormalities became available that the mode of transmission could be studied adequately.

Soon after the introduction of the glutathione stability test, Browne et al.[12,13] examined a large number of Negro families of propositi with abnormal glutathione stability tests. A difference was noted between males and females, both in the prevalence of the abnormality and in its severity. Males always appeared to express the defect fully. Females, although occasionally expressing the defect fully, more often manifested intermediate abnormality. These findings suggested that glucose-6-phosphate dehydrogenase deficiency might be sex-linked, the females with intermediate expression representing the heterozygotes. Indeed, examination of the pedigrees revealed that ordinarily transmission was from mother to son. Occasional exceptions were observed, however. Father-to-son transmission appeared to have occurred in several pedigrees. Furthermore, in subsequent studies, females with full expression sometimes bore normal sons.[16,32] Since apparent father-to-son transmission was unusual, it seemed likely that these represented instances where the father merely happened to be carrying a gene common in the population, and the mother, although heterozygous for this defect actually had normal enzyme activity. Similarly, fully expressed females with normal sons must have been heterozygous. Thus, it appeared that G-6-PD deficiency was a sex-linked trait, but one with marked variability in the phenotypic expression of female heterozygotes (Fig. 1).

It has been possible also to demonstrate that glucose-6-phosphate dehydrogenase deficiency is sex-linked in Equidae.[21,22,33] Horse and donkey G-6-PD proved to be readily distinguishable on starch gel electrophoresis (Fig. 2). Examination of the red cell enzyme from male mules and male hinneys revealed that the enzyme always resembled the maternal type: male mules always had horse G-6-PD; male hinneys always had donkey G-6-PD (Fig. 3). Subsequently,

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it has also been shown that rabbit G-6-PD is sex-linked.[25] It seems likely that G-6-PD is sex-linked in all mammals.

In 1960 we were stimulated by the coincidence of two events to examine the question of the marked variability of G-6-DP deficiency. First, Professor Harry Harris, in an informal discussion, raised the question of why females had no more G-6-PD than did males, even though females had two X-chromosomes and males only one. At this time we also became aware of the work of Ohno,[24] who had demonstrated that one of the two female X-chromosomes was heterochromatic while the other was euchromatic. It is well known that, in insects, heterochromatin is often genetically inactive. This raised the question of whether the heterochromatic-X of the female might also be genetically inactive. Since the pattern of X-linked inheritance precludes the possibility that an inactive X-chromosome was derived either from the paternal-X or from the maternal-X exclusively, it would be necessary to assume that in some cells the paternally-derived X was inactive; in other cells, the maternally-derived X was inactive.[1,10] Other investigators, particularly Lyon[20] and Russell[27] independly reached the same conclusion from study of X-linked coat color genes in mice.

Because of the interfering effect of hemoglobin, we were unable to demonstrate by histochemical means the G-6-PD activity of individual erythrocytes.[9] It was possible to show, however, by the use of methemoglobin reduction curves, and by the use of glutathione stability curves that the red cells of female heterozygotes for G-6-PD deficiency did, indeed, behave just as did artificial mixtures. Although these results were initially questioned,[11] on the basis of in vivo survival curves they have now been amply confirmed by a variety of

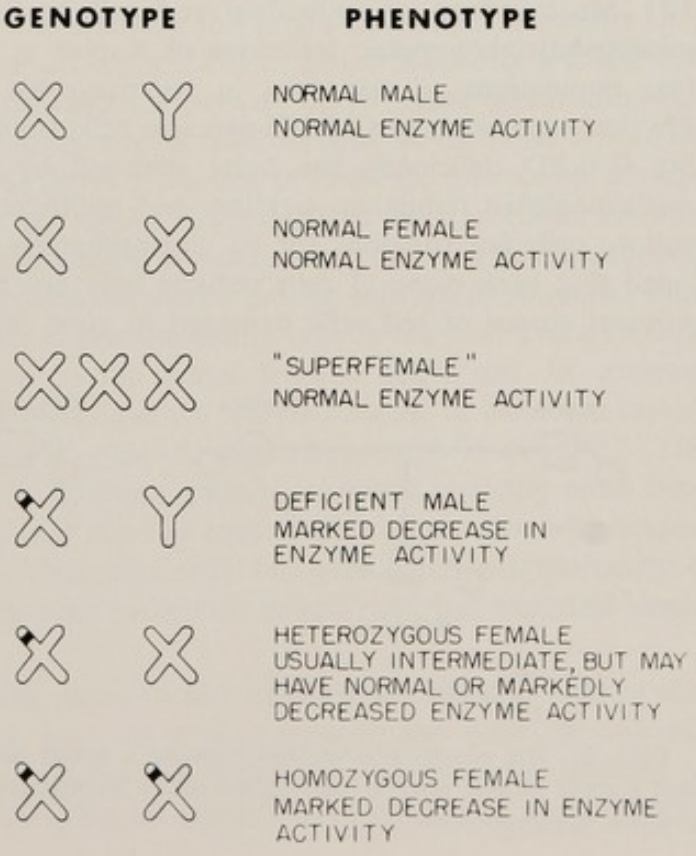


FIG. 1. Schematic representation of the relationship between G-6-PD genotype and phenotypic expression. A darkened area on one of the arms of the X-chromosome represents the gene for G-6-PD deficiency.

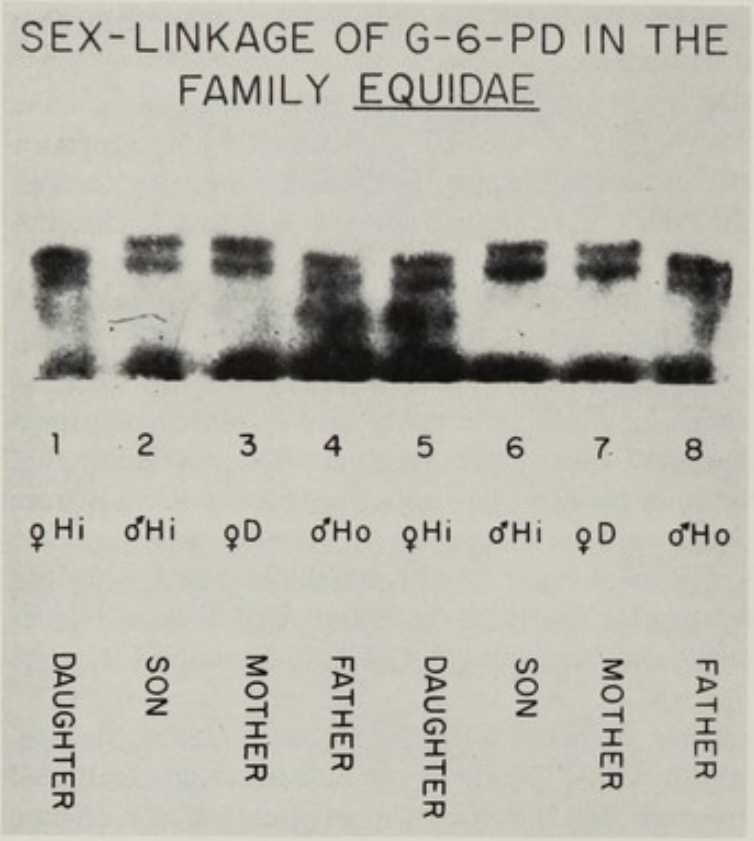


FIG. 2. Electrophoretic patterns obtained on starch gel electrophoresis of G-6-PD in a family group of *Equidae*. Hi represents hinney, D, donkey, and Ho, horse. Electrophoresis and staining for G-6-PD was carried out as described by Mathai et al.[21]

techniques. Evidence for the presence of two red cell populations in heterozygotes for G-6-PD deficiency includes the following: (1) Curves of the rate of reduction of methemoglobin and stability of glutathione have two components in heterozygotes for G-6-PD deficiency, just as found in artificial mixtures.[2,9,10] (2) Measurement of individual red cell hemoglobin levels by the elegant microspectrophotometric technique of Kaplan et al.[17] indicates the presence of two populations of red cells in heterozygotes for G-6-PD deficiency. (3) Physical separation of G-6-PD-deficient cells from the red cells of heterozygotes for G-6-PD deficiency has been achieved by a complex method involving methemoglobin reduction, sickling, and millipore filtration.[5] (4) Cloned tissue culture cells heterozygous for an electrophoretic variant of G-6-PD have indicated that each clone of cells reduces only one type of enzyme.[14] (5) In vivo survival curves of red cells damaged in vitro suggest the presence of two red

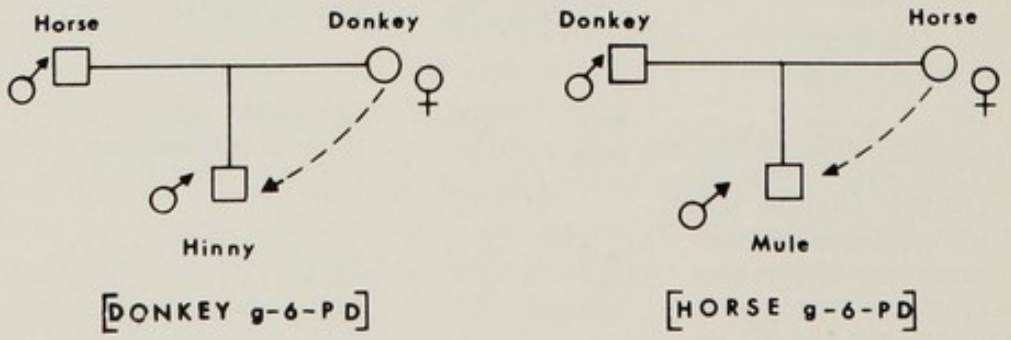


FIG. 3. The genetic relationship between a horse, donkey, hinney, and mule. It is seen that a male hinney always receives a donkey X-chromosome; a male mule always receives a horse X-chromosome.

cell populations.[29] (6) Examination of uterine myomas in heterozygotes for a common electrophoretic variant of G-6-PD reveals the presence of only one type of enzyme in each tumor.[19] We regard as unconvincing, evidence of X-inactivation based on histochemical examination of smears of red cells subjected to nitrite treatment and methemoglobin reduction in the presence of dye.[28,30,31] As Kleihauer and Betke[18] originally pointed out, the methemoglobin elution technique is not quantitative, and it is apparent that the threshhold effect is present. Thus, all cells which contain more than a certain amount of methemoglobin have their pigment eluted; cells which contain less than this amount of methemoglobin remain filled with pigment.[3] In view of this it is not surprising that "pseudomosaic" effects can be produced both in males with mild G-6-PD deficiency, and indeed, in normal subjects.[6,26]

Recognition of the fact that two red cell populations are present in heterozygotes for G-6-PD deficiency has been useful in devising tests for the detection of heterozygotes. We have developed a technique for detection of heterozygotes for G-6-PD deficiency consisting of nitriting red cells, washing the cells, and then incubating them in a system comprised of glucose, buffer, and Nile blue sulfate.[8] The latter dye is much preferable to methylene blue, since it does not permit interaction between red cell populations.[4] This method is capable of detecting heterozygotes for G-6-PD deficiency which are missed by other techniques, including enzyme assay. Tönz[30] has devised a similar procedure based on the histochemical demonstration of red cells containing residual methemoglobin. Gall et al.[15] have recently combined these biochemical and histochemical methods in a modified procedure which they report to be reliable in detection of heterozygotes.

One of the most interesting aspects of G-6-PD deficiency in X-inactivation concerns its use as a marker for studying the origin of malignant tumors. If a malignant tumor arises from a chance somatic mutation involving a single cell, all tumor metastases should demonstrate activity of the same X-chromosome. If this event occurs in an individual who is heterozygous for an easily detectable X-chromosome marker such as the A+ variant of G-6-PD, it becomes possible to determine whether, indeed, the tumor did arise from the transformation of a single cell. We have had the opportunity to study two patients with disseminated tumors using this approach.[7] In one instance, a patient with lymphosarcoma, each of the tumors examined contained predominantly type A enzyme. These findings are compatible with the origin of the tumor from a single cell. In contrast, examination of the metastases from a patient with carcinoma of the colon revealed that some metastases were of type A and others type B (Fig. 4). This indicates clearly that the tumor, although grossly unicentric, actually arose from more than a single cell. Obviously, this has considerable application with respect to the origins of tumors. Dr. Paul McCurdy has also had the opportunity of studying a number of patients with malignant tumors and has reported results similar to ours.[23]

It is beyond the scope of this paper to discuss the large body of linkage data which has accumulated in the past ten years regarding the gene for G-6-PD. Neither will we have time to discuss the malaria hypothesis, put forward by Dr. Motulsky. This hypothesis seeks to explain the fact that in some popula-

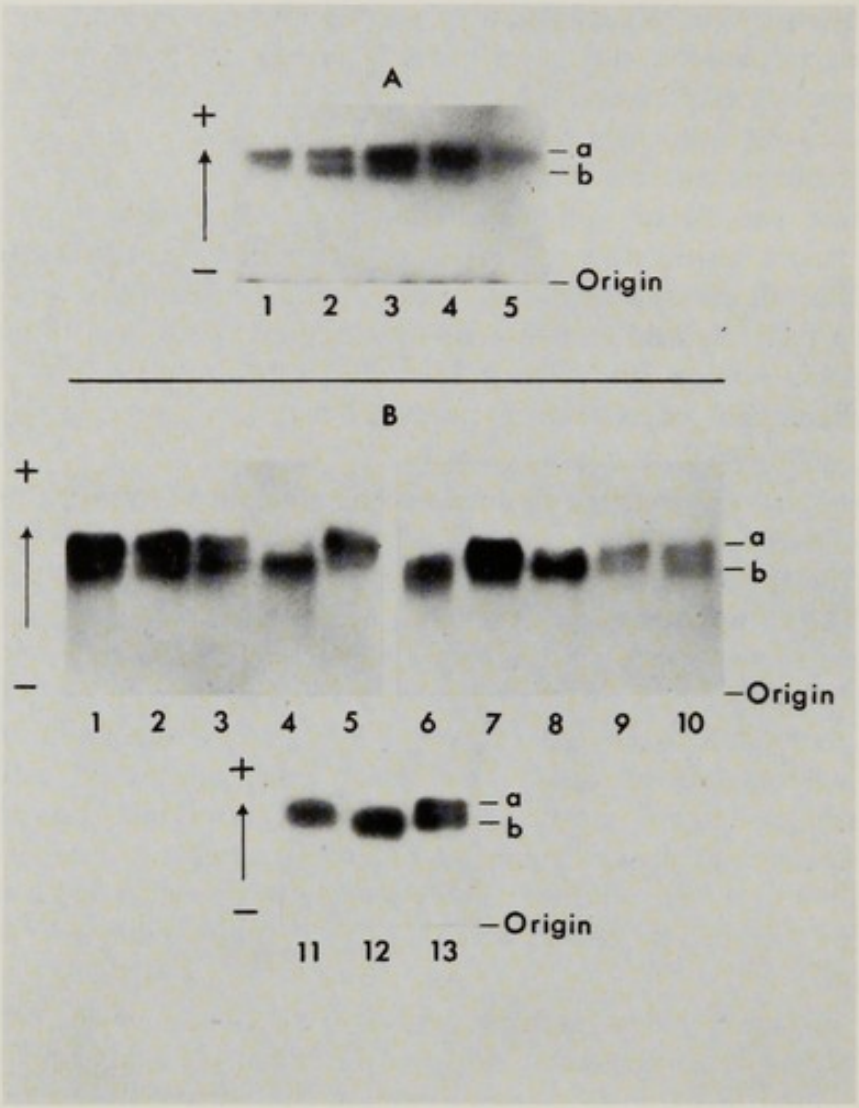


FIG. 4. The electrophoretic patterns of the red cells, tumors and normal tissues of a patient with lymphosarcoma (A) and a patient with metastatic carcinoma of the colon (B). A-1 mediastinal tumor, A-2 red blood cells, A-3, right inguinal tumor, A-4 left axillary tumor, A-5 left axillary tumor at greater dilution than A-4; B-1 liver nodule, B-2 liver nodule, B-3 red blood cells, B-4 normal liver, B-5 liver nodule, B-6 liver nodule, B-7 liver nodule, B-8 normal liver, B-9 red blood cells, and B-10 primary tumor (colon), B-11 liver nodule, B-12 liver nodule, B-13 red blood cells. J. Med. (From Beutler et al.,[7] courtesy New England J. Med.)

tions the gene for G-6-PD deficiency has become very prevalent in spite of the fact that it may have deleterious clinical effects under some circumstances. This problem will be discussed in greater detail by Dr. Motulsky.

It is apparent that the gene for G-6-PD deficiency and for electrophoretic variants of G-6-PD has proven to be an extremely useful one in human genetics. It has made possible studies of the activity of X-chromosomes in females, population movements, genetic linkage, and even of the origin of tumors. It promises to continue to be a useful tool for studying fundamental genetic problems in man.

Summary

The gene governing production of glucose-6-phosphate dehydrogenase (G-6-

PD) appears to be sex-linked in man and in all other mammals studied. An effective dosage-compensating mechanism maintains approximately the same level of enzyme activity in normal males, normal females, and subjects with more than two X-chromosomes. Evidence is reviewed that this dosage-compensating mechanism represents inactivation of all but one of the X-chromosomes in each cell. It has been possible to make use of this phenomenon in tracing the origin of both benign and malignant tumors.

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Discussion

SIMON, ALBUQUERQUE, CHAIRMAN: The discussion is open.

KOSOWER, NEW YORK: Could the two bands shown for malignant tumors be due not to multiple cellular origin but to derepression of previously inactive X-chromosome?

BEUTLER, DUARTE: The possibility that the inactive-X in tumor tissues has become derepressed cannot be ruled out entirely, but seems unlikely in view of some of our observations. As you recall, a number of tumor nodules showed the presence predominantly of one band or the other. If the inactive X became derepressed in the course of oncogenesis, we should anticipate that both bands would be present in all tumor tissues. Dr. Comings has recently carried out extensive studies that show how exceedingly difficult it appears to be to derepress the inactive X. I should like Dr. Comings to comment on these studies, if he will.

COMINGS, DUARTE: I have been ininterested in the inactive X chromosome, especially in relation to the mechanism of

it inactivation,* for quite some time. If you could find some agent to derepress the inactive X chromosome, this might offer some important clues as to the mechanism of its repression. To investigate this we grew fibroblasts from uterine fibromas of a Negro AB heterozygote. The fibromas were either A or B. This is an easy way to do cloning, a natural cloning technique. We then exposed these growing fibroblasts to a number of agents. These included polyethylene sulfonate, which Frenster has shown stimulates the condensed chromatin of calf thymus nuclei to increase its rate of RNA synthesis; canavanine, an inhibitor of histone synthesis; heat; cold; ultraviolet light; a number of hormones, including estrogen; and several other agents. In no case was a second band produced indicative of derepression of the inactive X chromosome. This doesn't mean that heterochromatin cannot be derepressed. Nur (personal communication) has shown that the heterochromatic chromosomes in the mealy bug may, in some cases, undergo, as he puts it, euchromatinization. An additional observation that I think is of some importance is that even when up to 30 per cent of these fibroblasts were sex chromatin negative, there was still only one G-6-PD band. To me this means that the sex chromatin negative state is not one of derepression of the inactive X chromosome, but rather an uncoiling of the X chromosome which still remains inactivated by histones and other nuclear proteins. Now to get back to the question Dr. Beutler asked—my reply would be that it is very difficult to derepress the inactive X chromosome, and other explanations should be sought.

TEPLITZ, DUARTE: If the inactive X were derepressed in tumors, one ought to lose X chromatin in malignant tumors, and many studies, including ours, have shown that this is not so, although an X chromatin body, differentiated from other chromatin, is hard to find in some cells. In most cells of malignant tumors the sex chromosome body is still present, so it is not derepressed.

MCCURDY, WASHINGTON: We also have studied with starch gel electrophoresis the G-6-PD enzyme of tumors from

*Comings, D. E.: The inactive X-chromosome. Lancet (Letter to the editor), Nov. 19, 1966, 1137-38.

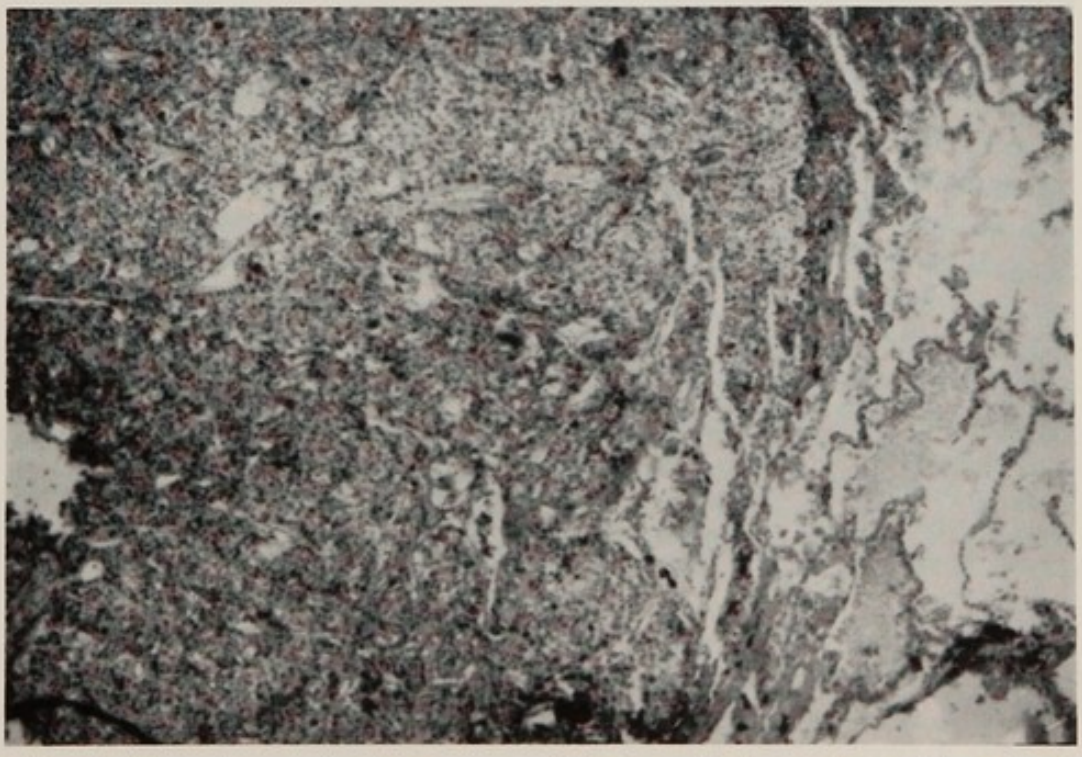


FIG. 1. Photomicrograph of a tumor nodule from the lung of a patient with disseminated hepatoma.

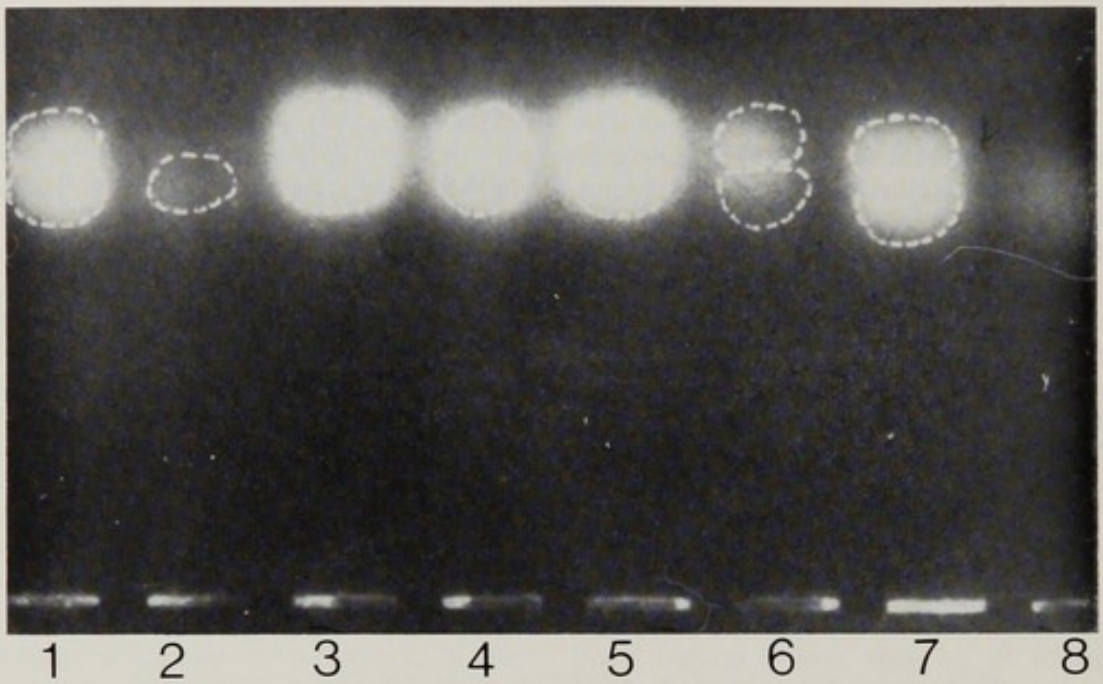


FIG. 2. Electrophoretic pattern of muscle and metastatic tumor tissue extracts from a patient with disseminated hepatoma.

Channel 1. Standard dilution, lung tumor nodule #1. Channel 2. 1:2 dilution of above. Channel 3. Standard dilution, lung tumor nodule #2. Channel 4. 1:2 dilution of above. Channel 5. Standard dilution, lung tumor nodule #3. Channel 6. 1:2 dilution of above. Channel 7. Normal muscle, standard dilution. Channel 8. Normal muscle, 1:2.

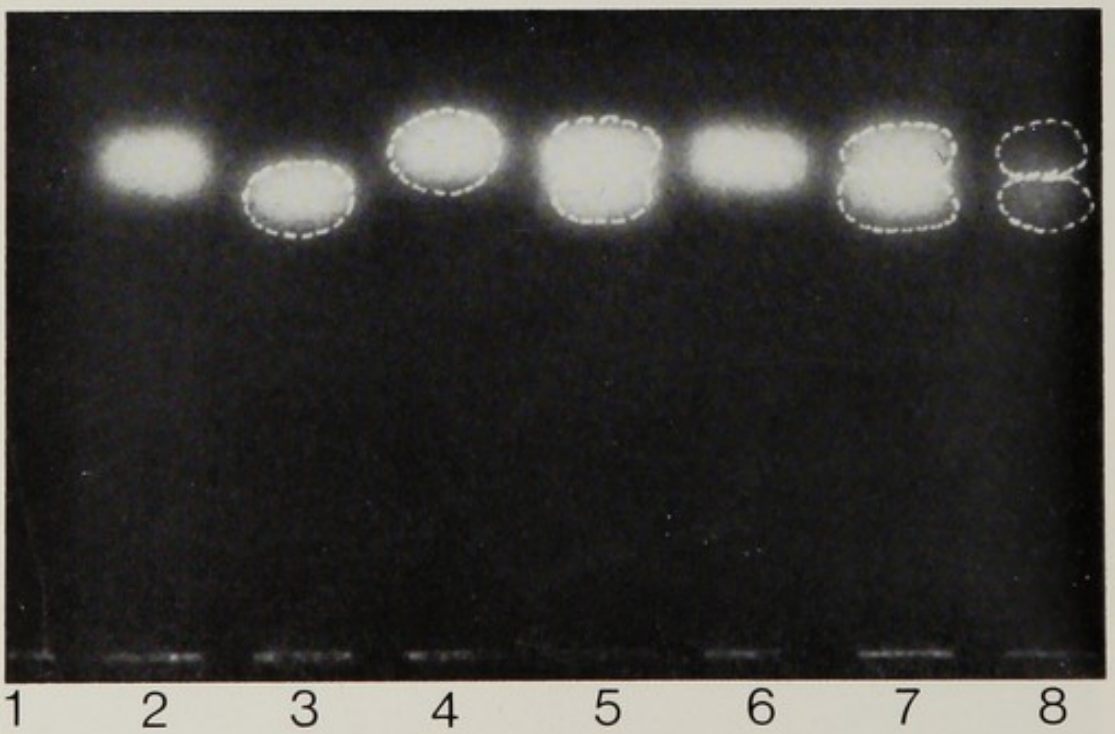


FIG. 3. Electrophoretic pattern of an extract of a tumor nodule from the liver of the same patient, along with blood samples of other patients.

Channel 1. G-6-PD AB (erythrocytes). Channel 2. G-6-PD A (erythrocytes). Channel 3. G-6-PD B (erythrocytes). Channel 4. G-6-PD A (erythrocytes). Channel 5. G-6-PD AB (WBC, same patient as Channel 1). Channel 6. G-6-PD A (Same patient as Channel 2). Channel 7. Standard dilution, hepatic tumor nodule. Channel 8. 1:2 dilution of above.

Negro females who were heterozygous for G-6-PD A and G-6-PD B. Tissue extracts were diluted to contain 0.16 international units of G-6-PD/ml, prior to electrophoresis in the starch gel system described by Kirkman. In some instances, further dilutions of this standard concentration were made and electrophoresed too. The first patient had disseminated hepatoma, and Figure 1 shows a photomicrograph of a tumor nodule in the lung. On the right is lung tissue which was easily removed from the nodule before the extracts were made for electrophoresis. There was relatively little stroma in the tumor of this nodule or a number of others. Unfortunately we did not obtain sections of the nodules studied for G-6-PD activity. On Figure 2, on the far right (channels 7 and 8), is shown an extract of muscle tissue from this patient. Double bands were found in the standard dilution and also in a further 1:2 dilution. In the left six channels are extracts of three pulmonary nodules of her tumor, each in the standard dilution and 1:2 of that. In the two nodules represented by channels 3 to 6, both G-6-PD bands were quite visible, even on dilution. For the nodule in slots 1 and 2, perhaps the A variant was diluted out before the B. In Figure 3, on the far right, is an extract of a large tumor nodule from the liver, showing both G-6-PD bands in standard and one-half dilutions. Channels 1–6 contain control type A, B, and AB hemolysates and leukolysates.

We also studied a patient with chronic lymphocytic leukemia. In the left 3 slots of Figure 4 is shown an extract of granulocytes which was separated by differential sedimentation and centrifugation. Slot 3 contains the standard dilution; slots 2 and 1 contain 1:2 and 1:4 further dilutions, respectively. This preparation contained 61 per cent granulocytes, 13 per cent red cells, and 26 per cent lymphocytes. As you can see, the

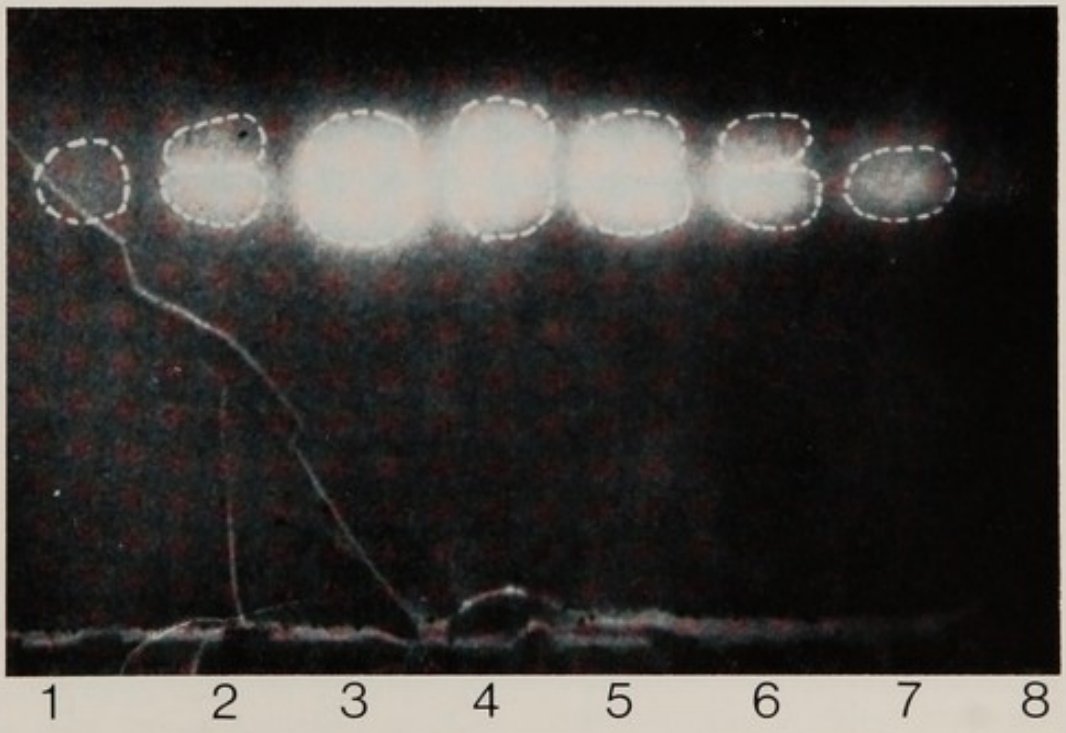


FIG. 4. Electrophoretic pattern of extracts of granulocytes and of lymphocytes from a patient with chronic lymphocytic leukemia.

Channel 1. Granulocytes,* dilution, 1:4. Channel 2. Granulocytes, dilution 1:2. Channel 3. Granulocytes, standard dilution. Channel 4. Red blood cell lysate. Channel 5. Lymphocytes,† standard dilution. Channel 6. Lymphocytes, 1:2 dilution. Channel 7. Lymphocytes, 1:4 dilution. Channel 8. Lymphocytes, 1:8 dilution.

(*61 per cent granulocytes, 13 per cent red blood cells, and 26 per cent lymphocytes. †95 per cent lymphocytes, 2 per cent red blood cells, and 3 per cent granulocytes.)

A variant is diluted out slightly before the B variant. The center slot (4) contains a red cell lysate. On the far right, in slots 5-8, are extracts of lymphocytes in standard dilution, 1:2, 1:4, and 1:8. The last is not very visible. This lymphocyte preparation contained 95 per cent lymphocytes, 2 per cent red cells, and 3 per cent granulocytes. Unfortunately, a fair number of platelets contaminated this lymphocyte preparation. An extract from the bone marrow of a patient with myeloma is shown in Figure 5. In the 3 slots on the far right are aliquots of this extract, all in the same dilution. The red cells were largely removed by shock lysis with hypotonic saline. The resultant preparation contained 81 per cent plasma cells. The rest were predominantly granulocytes with a few red cells. There is predominantly an A band visible with a possible faint trailing amount of B band, perhaps due to other contaminating cells.

We have also studied the primary tumor from a carcinoma of the colon and a carcinoma of the breast. As far as we could tell there was relatively little stromal contamination in these tumors. Both primary tumors had double bands electrophoretically.

From these studies we conclude that myeloma probably arises from a single cell and that chronic lymphocytic leukemia and carcinoma of the breast, colon, and liver probably arise from more than one cell.

PRINS, AMSTERDAM: I would like to ask Dr. Beutler whether his very fine demonstration of the mode of inheritance of G-6-PD deficiency necessarily mean that always G-6-PD deficiency is inherited via the X chromosome? For instance, in Holland we know of three families each having one male infant with G-6-PD deficiency associated with chronic nonspherocytic hemolytic anemia, and elaborate family studies did not reveal other G-6-PD-deficient siblings. One explanation might be that in those families there is a recessive autosomal

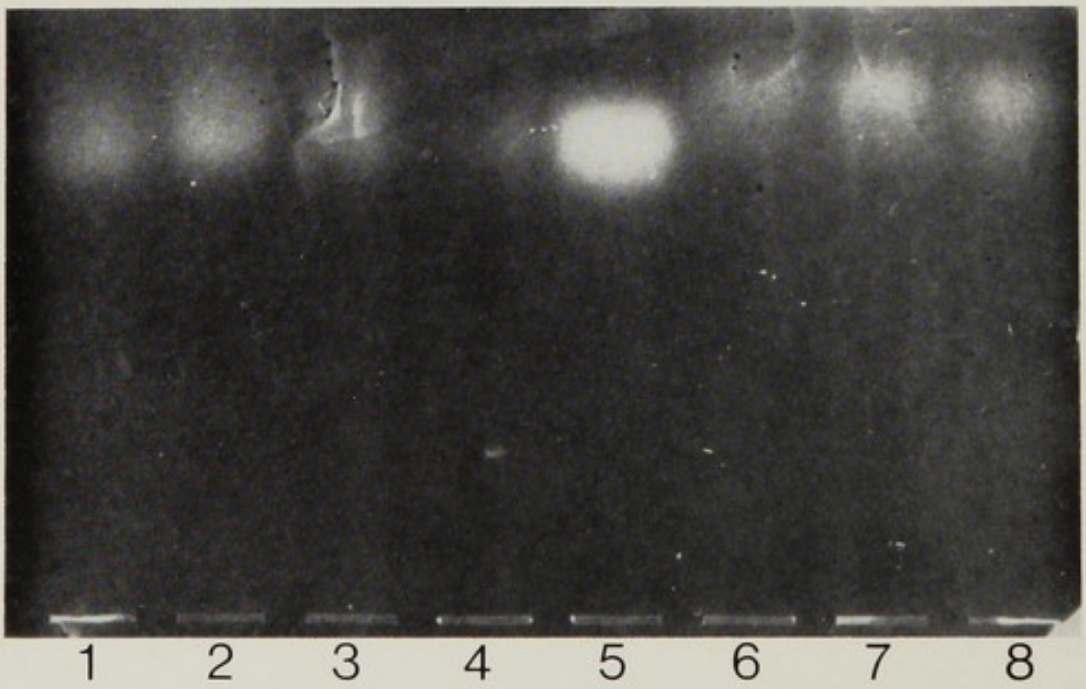


FIG. 5. Electrophoretic pattern of an extract of bone marrow from a patient with myeloma. All are same dilution of G-6-PD activity.

Channel 1. G-6-PD B—one-half standard activity (post-mortem tissue, another patient). Channel 2. G-6-PD B, Standard dilution of Channel 1. Channel 3. G-6-PD B, Standard dilution (post-morten tissue, another patient). Channel 4. G-6-PD B, one-half dilution of Channel 3. Channel 5. G-6-PD B, WBC from a normal patient. Channel 6. Bone marrow extract.* Channel 7. Bone marrow extract. Channel 8. Bone marrow extract.

(*81 per cent plasma cells, 8 per cent red blood cells, and 11 per cent "other" cells.)

mode of inheritance. Are there other explanations?

BEUTLER: The locus for G-6-PD-deficiency is, as far as is known, always sex-linked in man and probably sex-linked in all mammals. It is also sex-linked in *Drosophila*, although I think it is too much to expect homology there. The type of family that you have observed represents an example of the situation that is often observed in nonspherocytic congenital hemolytic anemia, because the abnormal red cell population is short-lived. Thus, in a female carrier who produces 50 per cent normal and 50 per cent deficient red cells, the cells sampled in the peripheral blood are almost entirely normal. For this reason heterozygotes usually or very frequently appear normal on enzyme assay. The other possibility to be considered is that this defect may have arisen as a new mutation. We have studied a number of patients with nonspherocytic congenital hemolytic anemia with G-6-PD-deficiency, and there is a great deal of heterogeneity in this group, suggesting that these traits are not broadly propagated in the population. Hence, a high proportion of cases detected may actually represent new mutations.

There was a report by Dr. Shaw about a year and one-half ago of an autosomal G-6-PD in *Peromyscus*.* Drs. Ohno, Morrison, and I have found that this enzyme is actually a galactose-6-phosphate dehydrogenase that has approximately equal glucose-6-phosphate dehydrogenase activity and is autosomally inherited.† It is not found in red cells, and recent studies carried out by us in collaboration with Dr. Morrison show that it is microsomal in localization, and probably identical to glucose dehydrogenase.

SMITH, STILLWATER: I wonder if Dr. Beutler would comment about the fact that there was predominance of B enzyme in normal liver. Why has this occurred and has he investigated any other livers from AB persons?

BEUTLER: When we wrote initially about X-inactivation,‡ we did not take into proper account the factor of selection within red cell populations after activation. It now appears as if this is a very important factor, not by virtue of the G-6-PD locus itself, except in the isolated instance of the peripheral blood of individuals with nonspherocytic hemolytic anemia, but in relationship to other genes on the X-chromosome. It is quite conceivable that there may have been, in this particular patient, a gene on the X-chromosome that gave some type of selective advantage to the cells in the anlage of the liver. Although the liver may have started out with 50 G-6-PD A and 50 G-6-PD B cells, G-6-PD B cells finally overgrew the others. We have not had very much experience with human liver tissue from other patients. In all of the other tissues of this particular patient, both A and B bands were present in equal concentrations, but the liver was a striking exception in this case. Perhaps Dr. McCurdy has more data on expression of G-6-PD in livers.

MCCURDY: Double bands appeared electrophoretically in liver samples from the patient with hepatoma. We have also done a few other liver samples with similar results. We have selected that tissue to use in trying to find our heterozygotes among those subjected to post-mortem examinations. We also have diluted out the G-6-PD in white cells from a patient who was a B+/A+ heterozygote to see what normal white cells would show. It looked as though the B band was diluted out somewhat before the A band, suggesting again that there might be variability either in the X inactivation or possibly later selection for cells with one or the other X.

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*Shaw, C. R. and Barto, E.: Autosomally determined polymorphism of glucose-6-phosphate dehydrogenase in Peromyscus. Science 148: 1099, 1965.

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‡Beutler, E., Yeh, M., and Fairbanks, V. F.: The normal human female as a mosaic of X-chromosome activity studies using the gene for g-6-pd deficiency as a marker. Proc. Natl. Acad. Sci. 48: 9-16, 1962.

Variants of Human Glucose-6-Phosphate Dehydrogenase. Studies of Samples from New Guinea

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OVER THE PAST DECADE the studies of investigators from many different disciplines and countries have revealed a high degree of prevalence and genetic heterogeneity of abnormalities of human glucose-6-phosphate dehydrogenase (G-6-PD). Dr. Beutler[2] has reviewed the genetic uses which some of these markers have served. Dr. Yoshida[29] will discuss recent advances in understanding of the structure of human red cell G-6-PD and the nature of certain defects of this enzyme. This report deals with the peculiar thermostabilities, electrophoretic patterns, and functional properties of the abnormal G-6-PD's, as exemplified by variants of the enzyme recently isolated from indigenes of New Guinea.

In 1956 Carson et al.[4] showed that a deficiency in activity of G-6-PD exists in the red cells of certain persons who are susceptible to a hemolytic reaction induced by the antimalarial, primaquine. Others demonstrated a deficiency in activity also in the red cell G-6-PD of persons prone to a hemolytic reaction following ingestion of fava beans (*Vicia faba*), and the studies of Newton and Frajola[20] and subsequent workers revealed that a deficiency in activity of red cell G-6-PD also underlies certain types of congenital nonspherocytic hemolytic disease (CNHD). Although the deficiency in each of these conditions is severe, it is seldom complete. Frequently, in point of fact, the enzyme can be extracted and at least partially purified in sufficient quantity to allow characterization of the enzyme if some of the same precautions are taken as have been found necessary to stabilize the normal enzyme.

Such techniques made possible in 1960 the demonstration that the G-6-PD of two male cousins with CNHD had thermostabilities and functional properties different from those of G-6-PD-deficient Negroes, which, in turn, were similar to those of G-6-PD from normal persons.[9,10] Marks, Banks, and Gross[16] reported an electrophoretically fast variant of G-6-PD, with an altered Michaelis constant (K_m) in a family of Italian subjects with mild G-6-PD deficiency. Other variants of G-6-PD have been reported not only in the rare group of CNHD due to G-6-PD deficiency but in other isolated instances as well (Table

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1). For the population geneticists, however, particular significance is ascribed to abnormal forms of G-6-PD that may be found in over one per cent of people in a large population. Especially when obvious disadvantages of such genes can be recognized, counterbalancing selective advantages may exist. In 1962, Boyer, Porter, and Weilbacher[3] demonstrated the first abnormal qualitative characteristics of a relatively prevalent G-6-PD abnormality. G-6-PD-deficient Negro males, representing about 12 per cent of American Negro males, were found to have a G-6-PD that migrated faster than normal enzyme during starch gel electrophoresis. A similar abnormal electrophoretic migration was found in another 22 per cent of Negro males who were not substantially deficient in activity of the enzyme. Kirkman, Schettini, and Pickard[12] and Ramot, Bauminger, et al.[23] demonstrated entirely different qualitative characteristics of the enzyme from G-6-PD-deficient Caucasian males of the Mediterranean area (Table 1). Earlier observations of levels of activity in white blood cells and platelets had also suggested that the mutations in G-6-PD-deficient Negroes and Mediterranean Caucasians were dissimilar.[17],[25] Recently, still another set of characteristics was found for the enzyme of five, unrelated Cantonese Chinese males with deficiency in activity of red cell G-6-PD.[18] In all instances, the distribution of these qualitative abnormalities in the population, like the distribution of the deficiency itself, has suggested an X-linked pattern of inheritance. This suggestion has been supported in those instances in which family studies have been possible.

Table 1 summarizes the characteristics of the more prevalent variants of G-6-PD, as well as those of less common variants for which more than a few



FIG. 1. Known location of the more common variants of human red cell G-6-PD. 1 and 2: G-6-PD A and G-6-PD A-. These variants may exist among Negroes in other areas of Africa. They are common among American Negroes. 3: G-6-PD's very similar, if not identical, to G-6-PD Mediterranean. 4: G-6-PD Canton. 5: Group III G-6-PD of New Guinea (possibly common). Also probably prevalent is G-6-PD Athens.

TABLE 1. *Variants of human red cell G-6-PD*

Variant	Population	RBC activity (% of normal)	Electrophoretic mobility (% of normal)	K_m G-6-P (μM)	K_m NADP (μM)	2d G-6-P utilization (%)	Thermostability	pH optima	References
				Common variants, in order of diminishing activity					
Normal B	All	100	100	50-78	2.9-4.4	< 4	normal	truncate	
A	Negro	88	110	normal	normal	< 4	normal	truncate	Boyer et al.[3]
Greece	Greek	12-45	98-100	20-30	1.5-2.0	3-12	reduced	mildly biphasic	Stamatoyannopoulos et al.[28] Kirkman, Doxiadis, et al.[7]
A-	Negro	8-20	110	normal	normal	< 4	normal	truncate	Boyer et al.[3] Kirkman[5]
Canton	Cantonese Chinese	4-24	104-108	20-36	2.0-2.4	4-15	slightly reduced	mildly biphasic	McCurdy et al.[18]
Mediterranean	Greek, Sardinians, Sephardic Jews, Asiatic NW Indians	0-7	100	19-26	1.2-1.6	23-37	reduced	biphasic	Kirkman, Schettini, and Pickard[12] Ramot, Bauminger et al.[25] Azevedo et al.[1]
				Uncommon variants, without apparent congenital nonspherocytic hemolytic disease					
"Madison"	Norwegian	100	70(TEB) 90(Tris)	?	?	?	?	?	Nance and Uchida[19]
Baltimore-Austin	Negro	75	90	68	3.1	< 4	normal	truncate	Boyer et al.[3] Long et al.[14]
Ibaden-Austin	Negro	72	80	62-72	3.3	< 4	normal	truncate	Boyer et al.[14] Long et al.[14]

TABLE 1. (*Cont.*) *Variants of human red cell G-6-PD*

Variant	Population	RBC activity (% of normal)	Electrophoretic mobility (% of normal)	K_m G-6-P (μM)	K_m NADP (μM)	2d G-6-P utilization (%)	Thermostability	pH optima	References
			Uncommon variants, without apparent congenital nonspherocytic hemolytic disease						
Barbieri	Italian	40-50	135	Increased	Increased	?	normal	?	Marks et al.[16]
Kerala	Asiatic SE Indian	50	75(TEB) 90(Tris)	23	1.5	7.4	normal	mildly biphasic	Azevedo et al.[3]
Tel Hashomer	Tunisian Jew	25-40	60-70	30-40	?	normal	normal	mildly biphasic	Ramot and Brok[24]
Columbus	Negro	35	100	normal	normal	normal	?	?	Pinto et al.[22]
Seattle*	Welsh-Scottish ?Greek	8-21	90	15-25	2.4-2.8	7-11	normal	mildly biphasic	Kirkman, Simon, and Pickard[12]
West Bengal	Asiatic Indian	9	90-94	31	6.6	4	normal	truncate	Azevedo et al.[3]
			Uncommon variants, with congenital nonspherocytic hemolytic disease						
Chicago	Western Europe	9-26	100	58-76	3.1-3.7	< 4	greatly reduced	truncate	Kirkman, Rosenthal et al.[13]
Oklahoma	Western Europe	4-10	100	127-200	20	< 4	reduced	narrow peak	Kirkman et al.[10]
Ohio	Italian	2-16	110	slightly increased	slightly increased	normal	greatly reduced	?	Pinto et al.[22]

*See also electrophoretically similar variant of Shows *et al.*[27]

characteristics have been reported. The variety of expressions which these alterations of a human enzyme can take is evident from this table. A rough estimate of the geographic distribution of the more prevalent variants may be gained from Figure 1.

The discovery of apparently distinct variants among African Negroes, Caucasians from the Mediterranean area, and Cantonese Chinese, prompted this investigation of the enzyme from G-6-PD-deficient indigenes of New Guinea. Anthropological tradition holds that these people are located near the end of one of the longer paths of the migration of man. It seemed desirable to learn whether or not these subjects had acquired a variant similar to one of those found far to the west or north. The alternate possibility also seemed to warrant examination: that people in this remote area might have evolved a variant of G-6-PD that is unique. The present report will reveal that both possibilities were encountered.

Experimental Methods

Samples of blood were taken from 35 males of Butibum village, which is near the town of Lae at the mouth of the Markham River, on the mainland of New Guinea. These males were of the Bukawa linguistic group. One or two fathers and brothers of the subjects were included in the study. The blood from each subject was injected into Vacutainers® containing acid-citrate-dextrose solution. These were placed in ice for shipment to an intermediate station, where activities of G-6-PD were determined. Three Vacutainers (24 to 30 ml.) of blood from each of the 35 subjects were flown to Chapel Hill, North Carolina, for further characterization. Within two weeks of the time of venipuncture, all samples were again assayed for activity of G-6-PD, and the enzyme from 27 samples was partially purified. All samples were essentially free of visible hemoglobin in the supernatant plasma.

The method of assay has been described previously.[6,9] Red cells were lysed by exposure to a solution consisting of 10 μM NADP and 7 mM β-mercaptoethanol. One unit of activity of G-6-PD is defined as that amount of the enzyme that converts one micromole of NADP to NADPH in one minute at 25° C. G-6-PD was partially purified by absorption on diethylaminoethyl-cellulose (DEAE-cellulose), elution, and ammonium sulfate fractionation.[10] These preparations were essentially free of hemoglobin and 6-phosphogluconic dehydrogenase. Except where otherwise indicated, all procedures were performed at 0 to 2° C. The enzyme, suspended in 2.7 M ammonium sulfate, was dissolved in dialysis solution for studies of thermostability. All other characteristics were determined with dialyzed enzyme. Dialysis solution consisted of tris (chloride), 0.05 M, pH 8.0; ethylenediaminetetraacetic acid (EDTA), 0.27 mM; NADP, 10 μM; and β-mercaptoethanol, 7 mM. The samples were dialyzed in groups, several samples of mutant enzyme and at least one sample of normal enzyme being present in the same beaker during dialysis. At the end of each period of dialysis, used dialysis solution was saved for dilution of the samples to the desired concentration. Vertical starch gel electrophoresis was performed with a continuous buffer system, as described by Kirkman and Hendrickson.[8]

Hemolysates were assayed for activity of G-6-PD and were treated with

DEAE-cellulose within a few hours of the time that they were prepared. Characteristics of the G-6-PD were determined as soon as possible after dialysis of the enzymic preparations. These precautions, coupled with the large number of samples, necessitated omission of several measurements used in previous studies: the G-6-PD reaction of hemolysates was not followed for the lengthy periods necessary to determine very low levels of activity. Michaelis constants (K_m's) for NADP were not determined. Thermostabilities were examined only on selected samples.

Results

Of the 35 samples of blood, 14 had normal or above-normal levels of G-6-PD activity. Six of these were selected for detailed characterization. These exhibited activities from 5.3 to 8.6 units per g of hemoglobin, with an average activity of 6.6 units per g of hemoglobin. The enzyme from this group (Group I) was obtained in 18 to 36 per cent yield, with specific activities of 0.53 to 0.70 units per mg. of protein, representing 80-fold to 127-fold purification. The 21 samples with deficient activity were separable into two distinct groups (II and III) on the basis of utilization of 2 deoxyglucose-6-phosphate (2dG-6-P)

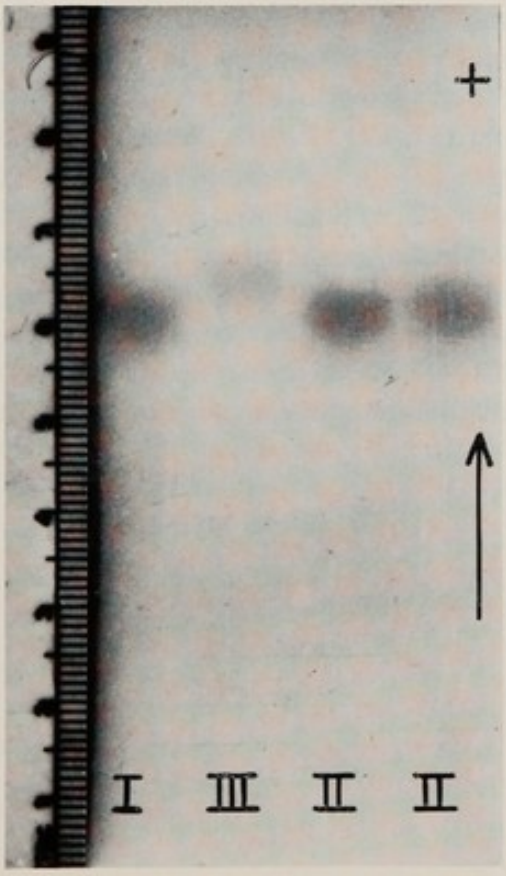


FIG. 2a. Vertical starch gel electrophoretic patterns of G-6-PD. Each channel received 1.6 x 10^{-4} to 3.9 x 10^{-4} units of G-6-PD. From left, enzymes from: Group I (normal), Group III (sample 618), Group II (sample 619), and Group II (sample 620).

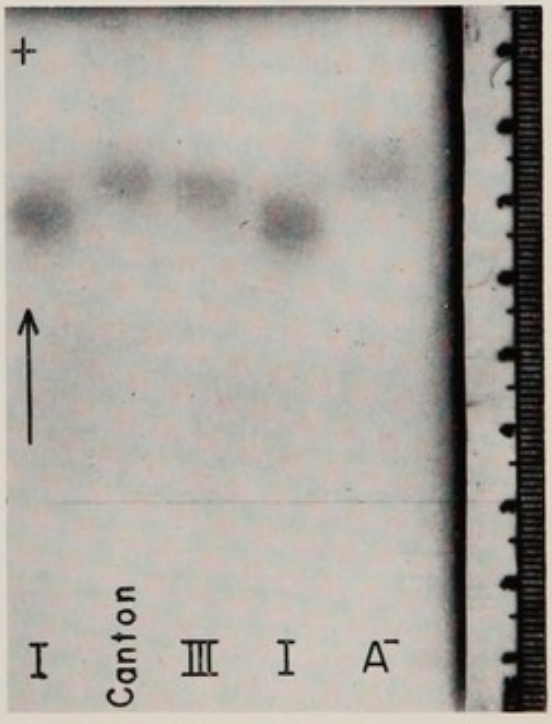


FIG. 2b. Comparative electrophoretic pattern of G-6-PD variants. Each channel received 4 x 10^{-4} to 5 x 10^{-4} units of activity. From left: Group I (normal) enzyme, G-6-PD Canton, Group III enzyme, Group I enzyme, and G-6-PD A-.

as will be shown later. Of the 18 samples of Group II, 12 had activities between 0.10 and 0.44 units per gram of hemoglobin, or between 1.4 and 6.5 per cent of the average activity of Group I. The enzyme from this group was obtained in 10 to 23 per cent yield with specific activities of 3.6×10^{-3} to 18×10^{-3} units per mg, or 51-fold to 129-fold purification. Activities of the remaining 6 samples of Group II were too low to measure on brief assay, but the total activity recovered after partial purification indicated that they had more than 0.5 per cent normal activity. Specific activities were 1.7×10^{-3} to 18×10^{-3} units per mg. One sample exhibited 2dG-6-P utilizations in the range of other samples of Group II but had too little total activity to allow further characterization. The three samples of Group III exhibited 0.65, 0.17, and 0.10 units of activity per g of hemoglobin or 10, 2.6, and 1.5 per cent of the average activity of Group I. Specific activities were 3.52×10^{-3}, 2.24×10^{-3}, and 1.87×10^{-3} units per mg, but the yields of 2 of these were 4 to 5 per cent. This loss in activity occurred principally during the DEAE-cellulose step. Losses of this magnitude during the DEAE-cellulose step were not observed with several hundred previous samples of normal and variant G-6-PD. The three males of Group III were not closely related (brothers or father-sons).

The G-6-PD's of Group I had a normal electrophoretic migration in starch gel, as did also the G-6-PD of hemolysates of 4 other New Guinea samples with normal activity. All enzymic preparations from Group II also had normal electrophoretic patterns, but the G-6-PD of each of the 3 samples of Group III moved 5 to 8 per cent faster than normal G-6-PD (Figures 2a, 2b).

Portions of the enzyme, stored as a suspension in 2.7 M ammonium sulfate, were mixed with at least 7 volumes of dialysis solution for determination of thermostability. A sham solution was obtained by centrifugation of unused portions of the enzyme suspension and comparable dilution of the G-6-PD-free supernatant fluid (Figure 3). Three incubation mixtures, containing mutant enzyme, control enzyme, and a mixture of both, were prepared as outlined in Table 2. Activities were followed during incubation of these mixtures at 41° C.

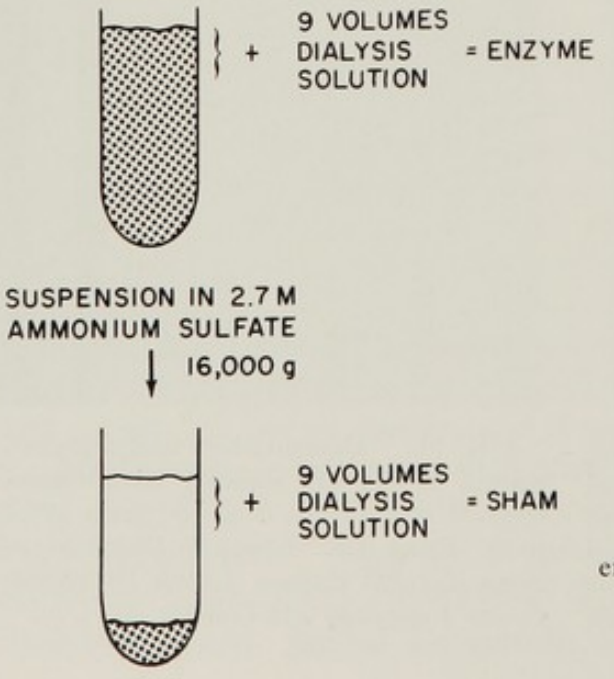


FIG. 3. Scheme for preparation of sham enzyme.

TABLE 2. *Studies of thermostability of a Group II enzyme (sample 602) at 41° C.*

	Mutant	Normal	Both
	(volumes, μl)		
Mutant enzyme (602)	200	—	200
Mutant sham solution	—	200	—
Normal enzyme (614)	—	20	20
Normal sham solution	20	—	—
Bovine albumin, 20 mg/ml	10	10	10
Total	230	230	230
Time (min.)	Activity (units/ml) x 10^3		
0	9.65	15.4	27.2
20	2.27	10.3	13.6
40	1.61	9.87	12.1
60	1.44	7.65	10.4

The moderately reduced thermostability of a sample from Group II may be seen in Table 2. Comparably diminished thermostability of two other samples from Group II was observed when these were paired with another control preparation. The total activity of samples of Group III was insufficient to allow studies of thermostability.

Table 3 lists the K_m's for G-6-P and the relative rate of utilization of 2dG-6-P. The latter is here defined as the rate at which the enzyme will utilize 0.6 mM 2dG-6-P, expressed as a percentage of the rate at which the same amount of enzyme will utilize 0.6 mM G-6-P. Noteworthy is the reversed specificity of Group III samples, as indicated by the relative rate of utilization of 2dG-6-P. A similar alteration in specificity for other substrate analogs is noticeable in Table 4. As determined by absorbance at 340 mμ wavelength of light, no reaction occurred when samples of Group III enzyme were added to standard assay mixtures from which either G-6-P or NADP had been omitted, nor did a reaction occur with a mixture of buffer, Group III enzyme, and NAD. Measurement of rates

TABLE 3. *Michaelis constants (K_m) for G-6-P and substrate analog utilization*

Group	Number of Subjects	K_m G-6-P μM			Relative 2dG-6-P Utilization (%)		
		Avg.	Max.	Min.	Avg.	Max.	Min.
I	6	45.4	49.9	41.5	3.5	4.0	2.7
II	17	11.8	15.2	7.8	39.4	46	31
III	3	5.5	6.3	4.4	186	222	162

K_m values were obtained from the regression (least-square fit) of V against V/s. V velocity; substrate concentration. Relative 2dG-6-P utilization is the rate of utilization of 2dG-6-P, expressed as a percentage of the rate of utilization of G-6-P at the same substrate concentration (0.6 mM). Substrate utilizations are the average of duplicate determinations.

TABLE 4. *Utilization of other substrate analogs*

Group	Relative rate of utilization (%)		
	Gal-6-P 0.6 mM	NAD 2 mM	Glucose 0.2 M
I	1.4-1.6 (2)	1.7 (1)	0.14 (1)
II	14-20 (2)	—	—
III	66 (1)	111-141 (2)	10-20 (2)

Figures within parentheses refer to number of samples tested.

of a Group III enzyme at 6 different concentrations of 2dG-6-P revealed that the maximal velocity for this substrate was 375 per cent of that for G-6-P. The corresponding rate for a Group I preparation was 8 per cent of that for G-6-P. Specificity of Group III enzyme for natural substrate was retained in other respects, however; the K_m for 2dG-6-P (6.7×10^{-4}M) was almost as high as that observed for a sample from Group I (9.1×10^{-4}M) and for previously studied controls.[11] Although Group III enzyme generated NADPH faster with 2dG-6-P than with G-6-P as a substrate, the rate with both substrates was the same as the rate with G-6-P alone. The experiment described in Table 5 and Figure 4 indicated that the Group III enzyme utilizes principally G-6-P when both G-6-P and 2dG-6-P are present. The relative affinity of the enzyme for G-6-P, therefore, remains high. Indeed, the K_m for G-6-P is much lower for Group III enzyme than for enzymes of the other two groups (Table 3).

TABLE 5. *Study of competitive effect of G-6-P on 2dG-6-P reaction*

Incubation mixture #	1	2	3
	(volumes, μl)		
Stock	425	425	425
Group III enzyme	50	50	—
G-6-P, 10 mM	10	10	10
2d G-6-P, 20 mM	15*	15	15
Dialysis solution	—	—	50
Total	500	500	500
	(μmoles/ml)		
NADPH generated	0.13	0.13	0.00
G-6-P consumed	0.14	0.14	0.00

*Delayed addition.

2dG-6-P was withheld from mixture #1 until NADPH had been generated to a concentration of 0.13 μmoles per milliliter, at which time 2dG-6-P was added to mixture #1 and the three mixtures were inactivated by placement in a 100° C bath for 2 minutes. The mixtures were clarified by centrifugation, and the supernatant fluids were used for estimation of residual G-6-P (Fig. 4). Stock solution consisted of Tris (chloride), 0.106 M, ph 8; $MgCl_2$, 10.6 mM; and NADP, 0.21 mM.

White blood cells were isolated from some of the samples. Bovine fibrinogen was added in the proportion of 12 mg per ml of whole blood; the samples were mixed, then allowed to stand at room temperature for 20 to 30 min. The supernatant phase was centrifuged at 2° C, and the resulting sediment was washed with cold 0.15 M sodium chloride. These packed white blood cells were lysed by freezing and thawing twice, then mixed with 9 volumes of the solution used for hemolysis. Unlike the white cell preparations from fresh blood samples, the 16,000 g supernatant fluid was contaminated with hemoglobin. The very high ratios of 6-PGD to hemoglobin nevertheless suggested that the activities were predominately from white blood cells. The ratio of G-6-PD activity to 6-PGD activity was: 1.35 to 1.75, for 3 samples of Group I; 0.46, for a sample of Group II; and 0.05 and 0.08 for 2 samples of Group III. The corresponding relative rates of utilization of 2dG-6-P were 5.6 to 9, 42, and 107 to 120 per cent.

As previously noted, partial purification of G-6-PD from Group III males provided the enzyme in relatively poor yield. The high 2dG-6-P utilization seemed representative of the red cell enzyme, however; an hemolysate from a Group III male had a 2dG-6-P utilization of 136 per cent, whereas that of a Group II male was 35 per cent.

The reaction rates of the partially purified G-6-PD's at various pH's were

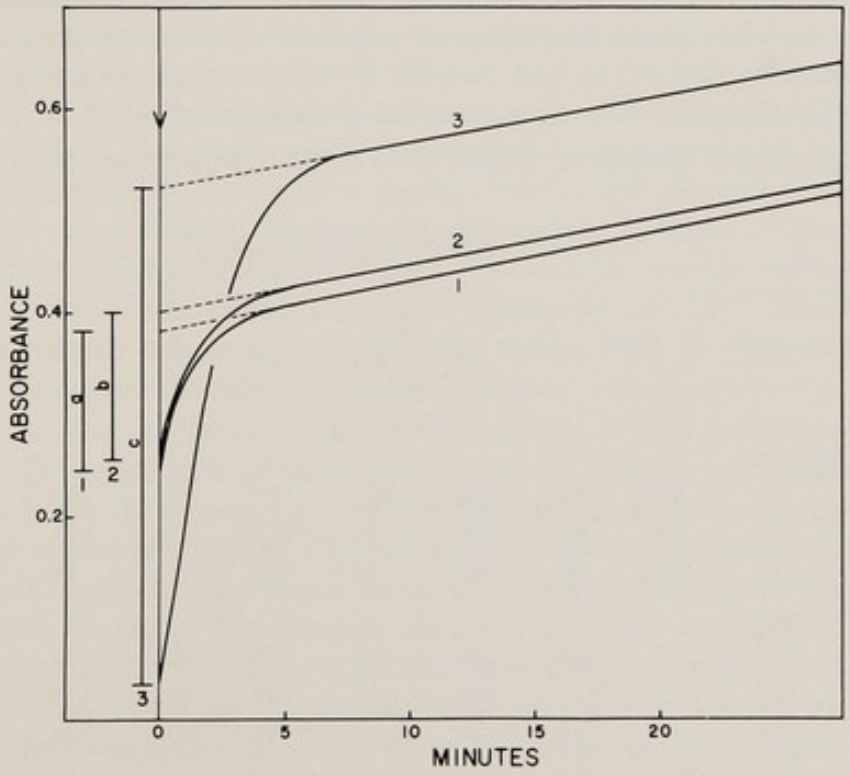


FIG. 4. Determination of residual G-6-P after incubation under conditions of Table 5. Each cuvette (1 cm. light path) received 200 μl of supernatant fluid from the corresponding incubation mixtures of Table 5, 0.1 micromole NADP, tris buffer, and sufficient water to bring to 0.495 ml. Reactions were started by the addition of 5 μl of normal G-6-PD. Lines a, b, and c represent the increment in absorbance due to residual G-6-P. The gradual slope after 5 minutes was indentical to that obtained with a comparable concentration of 2dG-6-P and normal enzyme.

determined in duplicate in a triple buffer consisting of tris, glycine, phosphate, and sufficient sodium hydroxide or hydrochloric acid to bring to the specified pH.[10] The results appear in Table 6. For Group I samples the pH-activity relationship is similar to that observed previously for normal males. Group II samples had a moderately bimodal pH optimum curve; Group III, a markedly bimodal curve.

Discussion

Comparison of the characteristics of Group II G-6-PD with those of Table 1 suggests that it is similar, if not identical, to G-6-PD Mediterranean. K_m's for both the normal group (I) and the Mediterranean-like group (II) are slightly lower than those listed for the corresponding groups in Table 1. Despite enzymatic standardization of substrate concentrations, K_m's differ slightly from one batch of reagent to the next,[11] and the present batch of reagents gave K_m's for the enzyme of three Mediterranean G-6-PD- deficient males and several American male controls that were slightly below the previously-reported ranges. The levels of activity in hemolysates and in white cell lysates are also consistent with the possibility that Group II enzyme is the Mediterranean variant. Yoshida (personal communication) has found that G-6-PD Mediterranean moves differently from normal G-6-PD on columns of calcium phosphate gel. This technique may also prove effective in establishing similarity or dissimilarity between Group II and the G-6-PD Mediterranean, but proof of structural identity between these two enzymes must await peptide analysis of the pure protein, such as that reported for G-6-PD A and G-6-PD B.[29] Techniques are not yet available for accomplishing this with severely deficient samples of blood.

Although similar, prevalent variants of G-6-PD have not been noted previously

TABLE 6. *Effects of pH on activity*

	pH									
Group	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	9.75	10.0
	Rates									
I (6 subjects)										
Avg.	30	65	92	100	108	113	117	103	79	52
Max.	32	69	96	100	109	118	128	111	86	59
Min.	27	61	90	100	104	111	112	98	75	49
II (17 subjects)										
Avg.	96	107	105	100	101	110	129	154	160	140
Max.	115	122	111	100	117	119	141	174	172	160
Min.	71	97	99	100	92	98	116	149	146	129
III (3 subjects)										
Avg.	148	139	112	100	103	108	128	187	204	203
Max.	151	150	124	100	105	115	131	198	208	215
Min.	141	132	102	100	101	100	126	178	196	190

All rates are the average of duplicate determinations. The rates are expressed as the percentage of rates at pH 7.5.

in genetically distinct groups, the great distances between New Guinea and the Mediterranean area could mean that these similar variants arose independently. Caution must be taken in dismissing the possibility, however, that these variants came from a common source. Genetic and cultural contacts have occurred since prehistoric times between Mediterranean people and those of the Indian peninsula, and contact has occurred over a long period between India and Indonesia. Recently, Azevedo et al.[1] reported the presence of a Mediterranean-like variant of G-6-PD among men from India. The studies described in this paper, it should be noted, were confined to material from one village in a single linguistic group. G-6-PD deficiency is widespread among many linguistic groups in this ethnically highly heterogeneous population, so it is possible that other variants exist in New Guinea.

The enzyme of Group III is electrophoretically similar to G-6-PD Canton. Both migrate faster than normal enzyme but not quite so fast as G-6-PDA- (Figure 2B). Among the G-6-PD variants, however, electrophoretic phenocopies occur. Several variants migrate about 10 per cent slower than normal G-6-PD but may be readily distinguished from one another by other criteria (Table 1). Two electrophoretically fast variants, G-6-PD A and G-6-PD A-, remained qualitatively indistinguishable until recently, when Luzzatto and Allan[15] succeeded in separating the two enzymes on DEAE-Sephadex columns, and Yoshida et al.[30] separated them on carboxymethyl-Sephadex. Similarly, the enzyme of Group III differs from G-6-PD Canton in the extreme alterations of substrate specificity which the former exhibits (Tables 1, 3, 4). These findings raise the question of whether or not Group III enzyme is G-6-PD at all. Ohno et al[21] and Shaw[126] have reported a minor electrophoretic component of hepatic G-6-PD that utilizes Gal-6-P even more readily than G-6-P. Several variants of red cell G-6-PD from G-6-PD-deficient subjects exhibit a slight to moderate loss of substrate specificity (Table 1). Might this residual activity represent simply an enzyme that is determined at another genetic locus? The characteristics of such a genetically-independent component would tend to predominate when severe deficiency of the major component exists.

Several findings serve to refute this possibility. The amount of 2dG-6-PD activity in the hemolysate of one of the Group III samples was determined and was found to be 0.76 units per g of hemoglobin, a value over three times that for normal hemolysates. After electrophoresis of the red cell enzyme, moreover, the 2dG-6-PD activity was found to be at the same position on the starch gel as the G-6-PD activity. The samples used in Figure 2B were diluted to similar concentrations of 2dG-6-PD activity and were added to other channels on the same gel. When developed for activity with 2dG-6-P as a substrate, the same migration distances and relative positions were observed as in Figure 2C. Electrophoretic coincidence of 2dG-6-PD and G-6-PD activity has also been observed with G-6-PD's Seattle and Mediterranean.[18] Such electrophoretic coincidence of the two activities and concomitant electrophoretic alteration in several electrophoretically-different variants would be very unlikely if the residual activity arose from a genetically-independent enzyme.

Several general concepts and questions become apparent during comparison of the New Guinea variants with other variants of human red cell G-6-PD.

We are accustomed to thinking of a deficiency in activity whenever a metabolic abnormality arises. If severe alterations in substrate specificity of an enzyme can occur, however, might not this also have pathological consequences at times? The product of such an enzyme could be an abnormal and toxic metabolite.

The phenotypic differences among G-6-PD variants (Table 1) also have bearing on the caution which must be taken in assuming that the severity of each defect is reflected by the level of activity exhibited by hemolysates in an assay in vitro. Clinical problems are associated principally with G-6-PD defects in which the red cell activity is between 0 and 30 per cent of normal activity; but within this range, little correlation exists between clinical severity and enzyme level. At least two characteristics would seem capable of causing a serious misestimation of the severity of the defect. Concentrations of substrates within a cell are often considerably lower than those used in assays in vitro. At these lower substrate concentrations, variants with unusually high K_m's should be impaired more than would be reflected by a standard assay. Of interest is the greater clinical severity of the Oklahoma variant than of the Mediterranean variant, despite the higher level of red cell activity of the former. The K_m's for the Oklahoma variant are unusually high; those of the Mediterranean variant are, in point of fact, unusually low. Stability is another characteristic that is relevant to function in vivo. Although stability of the enzyme in the cell may not necessarily parallel thermostabilities in vitro, the existence of decided differences in thermostabilities of the variants suggests that differences can also occur in the rate of decay of the G-6-PD during the life span of the red cell. It should be recalled that early in the life span of the red cell the ability to synthesize protein is lost. The activities obtained during a standard assay would not reflect the severity of deficiency in the older cells of the red cell population.

Summary

Blood samples were taken from 35 males of Butibum village in New Guinea. Twenty-one of these samples exhibited deficiency in activity of red cell G-6-PD. The qualitative characteristics of the partially purified G-6-PD of 18 of these samples resembled those of G-6-PD-deficient males from the Mediterranean area and India. Three of the G-6-PD-deficient males had a unique enzyme with severe alterations in substrate specificity. These characteristics are compared to those observed with other variants of human red cell G-6-PD.

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DISCUSSION

SIMON, ALBUQUERQUE, CHAIRMAN: Dr. Kirkman's paper is now open for discussion.

BEUTLER, DUARTE: Dr. Kirkman, you have discussed the in vitro stability of red cell G-6-PD and you have established this as an important criterion of the differentiation of different variants of G-6-PD from one another. Your closing remarks, however, were directed to a discussion of the significance of the rate of intracellular destruction of G-6-PD in vivo. What method have you used to determine the in vivo stability of various types of G-6-PD? What are your findings?

KIRKMAN, CHAPEL HILL: It should be remembered that early in its life, a red cell loses the ability to synthesize proteins. In a certain sense, it is coasting downhill with respect to the proteins that it contains. If the rate of destruction of G-6-PD is high, the older red cells of a sample of blood would be far more deficient in activity of G-6-PD than would be apparent from a standard assay of the whole population of red cells in the sample. Various techniques are now available for obtaining hemolysates of red cells of different ages. Using a technique of differential osmotic hemolysis, P. A. Marks offered evidence that G-6-PD A- is destroyed intracellularly more rapidly than normal G-6-PD.*

Yoshida et al.† have used phthalate esters to isolate red cells by density and hence, by age. Their studies indicate that the activity of G-6-PD A- is nearly normal in very young red cells. With many of these methods, white cells or white cell lysates tend to contaminate the younger red cell preparations. For G-6-PD-deficient variants that have nearly normal activity of white cell G-6-PD this can lead to a serious over-estimation of G-6-PD levels in young red blood cells. Mrs. Kennedy and I wish to report the preliminary results of special studies of G-6-PD levels in young and old red cells. These results offer both agreement and differences with the findings from previous studies.

Samples of blood from normal and G-6-PD-deficient subjects were collected in tubes containing acid-citrate-dextrose solution. These samples were subjected to fractionation between 12 and 24 hours after venipuncture. The method of fractionation is a modification of that of Danon and Marikovsky,‡ which is based on the general tendency of older red cells to be more dense than young red cells. Mixtures of various proportions of methyl phthalate and di-*n*-butyl phthalate provided a series of water immiscible liquids of different specific gravities. Whole blood was added to a series of microhematocrit capillary tubes, followed by several microliters of phthalate ester. Each capillary tube received phthalate ester mixture of different density. The capillary tubes were centrifuged in a microhematrocrit centrifuge at 14,000 g for 3 minutes at 2-4° C. To the top of each capillary tube were added several microliters of a phthalate ester mixture of slightly lower density than that of the first. The capillary tubes were then centrifuged at 12,000 g for 30 minutes at 5-6° C in a swinging cup ultracentrifuge rotor. This was made possible by the shaping of wooden adapters

*Marks, P. A.: *In:* Molecular Genetics and Human Disease, L. I. Gardner, ed. Springfield, Ill., Charles C. Thomas, Publishers, 1961, p. 126.

†Yoshida, A., Stamatoyannapoulos, G., and Motulsky, A.: Negro variant of glucose-6-phosphate dehydrogenase deficiency (A-) in man. Science 155: 97-99, 1967.

‡Danon, D., and Marikovsky, Y.: Determination of density distribution of red cell population. J. Lab. Clin. Med. 64: 668-674, 1964.

to fit an SW 39L Spinco rotor. Close control of rotor speed was possible, and a radiation thermometer and viewing port made possible close control of rotor temperature, which was found necessary for reproducibility. These procedures resulted in two zones of phthalate ester in each capillary tube. After repeating the addition of phthalate ester several times, the red cells were divided into 4-5 fractions, the upper fraction being separated from white cells by the lightest mixture of phthalate ester. The G-6-PD, 6-PGD, and glutamate-oxaloacetate transaminase levels of each zone of red cells were determined. Reticuloycyte counts and transaminase levels were much higher for red cells in the upper fractions than for those in lower fractions, indicating, as did the earlier studies with Fe^{59}-labelled red cells by Danon and Marikovsky (see previous footnote), that the lighter red cells were the younger cells.

Decided differences in the apparent rate of decay of G-6-PD activity were noticed among the samples. These differences were obvious whether G-6-PD activity was plotted against phthalate ester density or against vertical position of the red cell zone in the column of packed red cells. The most reproducible plot seemed to be that of log G-6-PD or log 6-PGD activity against log transaminase activity, a plot suggested by the probability that the activities of any two enzymes should be exponentially related through time or through a function of time. Unlike the unusually rapid decay in activity of G-6-PD A- and G-6-PD Seattle (Figure 1), no difference was noted for 6-PGD activity (Figure 2). Smears of the upper zone of red blood cells revealed that the level of contamination of these zones by white blood cells was too low to account for the difference between G-6-PD A- and normal G-6-PD or between G-6-PD Seattle and normal G-6-PD. Of greater significance was the fact that the differences in decay were apparent even if the upper zone (point farthest to the left in Figure 1) of each sample were omitted. Dr. Motulsky will comment later on the possibility that isoalleles of G-6-PD may exist. Pertinent to this are the differences in decay slopes for G-6-PD A- (Figure 1). It will be of interest to see whether or not moderate and steep decay slopes serve to separate G-6-PD A into two genetically-distinct classes of G-6-PD A-.

KOLER, PORTLAND: Have you made any attempts to see if there is *in vitro* complementation on mixing two different variants of G-6-PD?

KIRKMAN: Yes, as the number of variants increases, the number of possibilities becomes almost astronomical, but I think we have tried some seven to nine different combinations and have, in no instances, seen anything suggestive of complementation on simple mixing. One could argue that if the enzyme con-

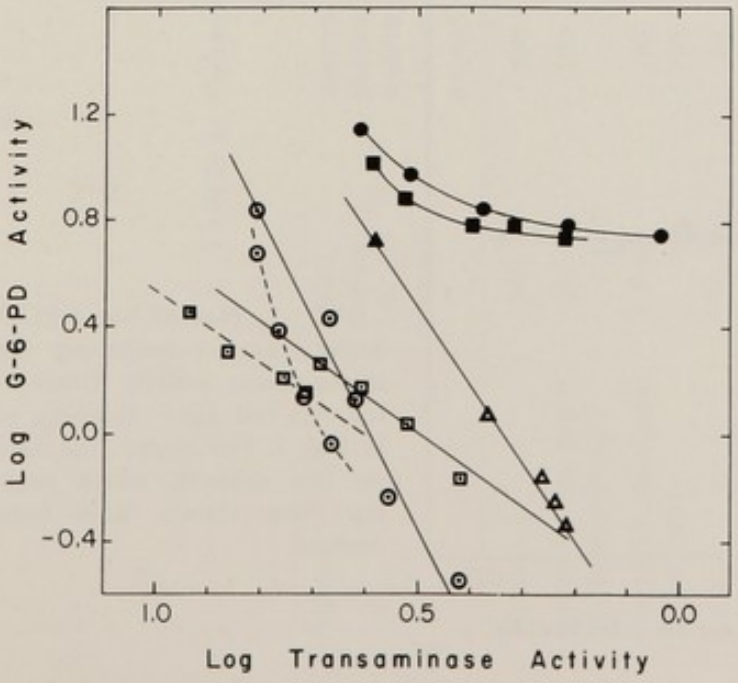


FIG. 1. Plot of log G-6-PD activity against decreasing log transaminase activity (increasing red cell age). ● and ■, normal Caucasian men. △, Caucasian man (propositus of J. Lab. Clin. Med. 66: 834, 1965) with G-6-PD Seattle. ○ and □, solid lines, Negro men with G-6-PD A-, ⊙ broken line, Negro boy with G-6-PD A- and elliptocytosis, and ⊡ broken line, Negro boy with G-6-PD A- and thalassemia minor.

sisted of two or more dissimilar tightly-bound polypeptide chains, perhaps more than simple mixing would be required. You might need to dissociate the chains and try to reassemble them after mixing them. We have done this only in a few instances. Perhaps Dr. Beutler can comment on his more extensive attempts at this.

BEUTLER: We have tried to do this with the Mediterranean variant, the A-variant, and a nonspherocytic congenital hemolytic variant (Chicago). We tried to complement them with each other and to complement them with TPNH oxidase.* TPNH oxidase activity, for some poorly understood reason, is always diminished in G-6-PD deficiency, and we thought that there might be a common sub-unit. In no case were we able to obtain complementation. Using electrophoretic methods of detection, we have been able to form hybrid enzymes between rat and human enzyme† and between cow and rat enzyme,‡ but never between two human enzymes.

HARRIS, LONDON: I think, Dr. Kirkman, that what you were referring to as Group III is not listed in Table 1 of your paper.

KIRKMAN: That is correct.

HARRIS: And the name of this variant?

KIRKMAN: At a recent meeting in Geneva, geographic terms were cited as being preferable to racial, alphabetical, or family names. Butibum is the name of the village from which the samples came, but perhaps it should be called G-6-PD Lae, after a nearby town. That would be shorter.

GIBLETT, SEATTLE: That is not appropriate. There is a transferase variant by that name.

KIRKMAN: That leaves us only Butibum, or Markham, the name of the nearby river. Markham is phonetically better. Which do you think, Dr. Motulsky?

MOTULSKY, SEATTLE: Markham is best.

HARRIS: I was wondering whether it would be helpful if you could just run over the headings of Table 1 in your paper, Dr. Kirkman, and give us the characteristics of type III (Markham) in that way.

Editor's note: These have been incorporated in Table 1 of this discussion.

*Beutler, E., in discussion of Kirkman, H. N., McCurdy, P. R., and Naiman, J. L.: Functionally abnormal glucose-6-phosphate dehydrogenases. Cold Spring Harbor Symp. 19: 391-398, 1964.

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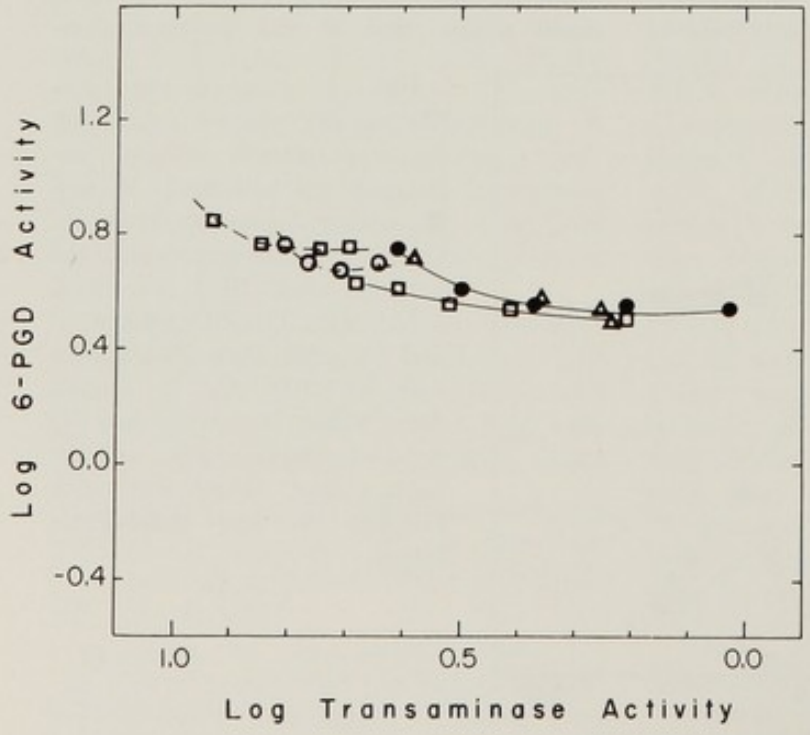


FIG. 2. Plot of log 6-PGD activity against decreasing log transaminase activity (increasing red cell age). Symbols as in Fig. 1. For clarity, the data on two subjects, which overlap those shown, have been omitted.

TABLE 1. *Comparative characteristics of normal G-6-PD, New Guinea variants, and other new variants brought out in discussion*

Variant	RBC activity (% of normal)	Electrophoretic mobility (% of normal)	K_m G-6-P (μM)	K_m TPN (μM)	2dG-6-P utilization (%)	Thermostability	pH optima
Normal B	100	100	50-78	3-6	<4	normal	truncate
Group II, this paper (? Mediterranean)	1.4-6.5	100	7.8-15.2		31-39	reduced	biphasic
Group III, this paper (Markham)	1.5-10	105-108	4.4-6.3		162-222	? reduced	very biphasic
Athens	20	98	19	3	15	sl. reduced	sl. biphasic
Albuquerque	1	100	115	11	0	markedly reduced	peaked with optimum at 8.5
Duarte	8.5	100	58	5	5.4	markedly reduced	7.0

SMITH, STILLWATER: Have you considered, in the discrepancy between the G-6-PD activity and the clinical syndrome, the possibility that the TPN-TPNH pool may be altered in different variants? If the amount of available TPN increases, it would be possible to accelerate the enzyme activity in vivo.

KIRKMAN: Yes, I think this is possible but I would need to refer you to the work of others for information about the ratio and the absolute amounts of TPN and TPNH under these various conditions. I am not aware that these ratios are greatly different from normal. Of the two substrates, TPN and G-6-P, the concentration of G-6-P may be more likely to affect the rate. The K_m for TPN is quite low. Some inverse correlation between K_mG-6-P and clinical severity is seen.

SMITH: One reason that this may be of some importance is brought out by the problem of sheep. Sheep have low G-6-P activity, and the K_m of their G-6-P is about half that found in human beings or in most other species. Yet, they are able to resist massive doses of primaquine as Dr. Carson showed first and which Dr. Beutler and I confirmed, using intravenous primaquine and Cr^{51} survival studies.

STAMATOYANNOPOULOS, SEATTLE: You could increase the list of G-6-PD mutants by one which was recently studied by Dr. Yoshida, Dr. Bacopoulos, and myself. The new mutant, G-6-PD Athens (see Discussion Table 1), was found in a 25-year-old male without signs or history of hemolytic anemia. G-6-PD Athens is associated with erythrocyte G-6-PD activity 20% of normal. On starch gel electrophoresis (TEB buffer, pH 8.6), the new mutant migrates slightly slower than the normal B+ enzyme, but faster than G-6-PD Seattle (relative mobilities: G-6-PD Seattle 90 per cent, G-6-PD Athens 98 per cent). Better separation from the B+ enzyme was achieved by high voltage electrophoresis applied on thin starch gels (Figure 3). Additional characteristics of the new variant are: K_m for G-6-P, 19 μM; for TPN, 3.0 μM; 2-deoxy-G-6-P utilization, 15 per cent of G-6-P. The optimum pH is 8.5 and slightly biphasic; it is slightly more labile than normal. Diamino TPN utilization was 126 per cent of the utilization of TPN compared with 58 per cent for the B+ enzyme, 58 per cent for the Mediterranean enzyme, and 56 per cent for the Seattle variant. Quantitative neutralization by anti-normal B+ serum: same as the normal B+ and the mutant A+ and A− enzymes. Chromatographically the enzyme was slower than B+ on CM-sephadex.

BEUTLER: I would like to contribute two more variants to the list. These have been characterized in collaboration with

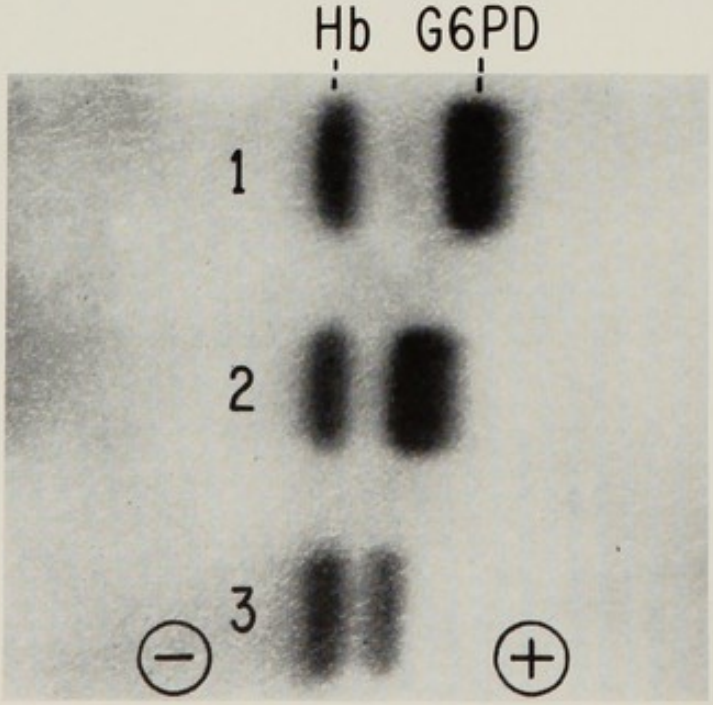


FIG. 3. High voltage electrophoresis of G-6-PD variants. 1: Variant A. 2: Variant B. 3: Athens variant.

Dr. Smith and Dr. Mathai. Both specimens are from patients with nonspherocytic congenital hemolytic disease. We have designated the first of these Albuquerque. The activity of the enzyme in the red cells of the affected hemizygous subjects was only approximately 1 per cent of normal. The K_m for G-6-P is 115 μM and the K_m for TPN is 11 μM. 2-deoxyglucose-6-phosphate utilization was 0 per cent of that with G-6-P. Electrophoretic mobility of the enzyme was normal and it showed marked thermal instability. Its pH optimum curve was sharply peaked with an optimum of 8.5. We have named the other variant Duarte. The enzyme activity in the red cells of the CNHD patient with this variant was 8.5 per cent of normal. The K_m for G-6-P and TPN were normal, but the pH optimum curve was markedly skewed to the acid side with a pH optimum of only 7.0. There was also a borderline abnormality of 2 deoxyglucose-6-phosphate utilization, and the enzyme had marked thermal lability (Discussion Table 1).

The Structure of Normal and Variant Human Glucose-6-Phosphate Dehydrogenase

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SINCE PAULING ET AL.[21] described sickle cell anemia as a "molecular disease" caused by sickle cell hemoglobin, and Ingram[6] elucidated the molecular difference—amino acid substitution—between the normal and sickle cell hemoglobins, it is generally believed that other hereditary disorders are also related to genetic alteration of structure and activity of the enzymes that are involved in certain metabolic pathways. In view of the evidence obtained from studies of many hemoglobin variants in man and mutation studies of *E. coli* tryptophan synthetase,[29,30] most mutationally-altered enzymes seem to be produced by a single amino acid substitution. Proof of this hypothesis for mutant enzymes in mammals is not yet available because it is difficult to obtain the necessary quantities of pure enzyme proteins for the required biochemical analyses.

Recent developments in methodology of protein purification, using various ion exchangers and molecular sieving materials, together with the development of microscale analyses of amino acid and peptides, have made it possible to study the structural characterization of genetically-altered enzymes in greater detail. This work is an example of such an attempt on the normal and mutant human glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP oxidoreductase, E. C. 1. 1. 1. 49).

I. PURIFICATION[32]

The glucose-6-phosphate dehydrogenase content of normal human erythrocytes is less than one part in 50,000 of total red cell protein. Rather prolonged and complicated procedures are necessary for purification. Particular care must be taken to avoid hydrolysis of the enzyme by proteolytic enzyme contamination and denaturation on the surface of glassware and to minimize the physical loss of the enzyme.

The purification procedures used for isolation and crystallization of the normal (B+) enzyme consisted of (1) hemolysis, (2) elimination of hemoglobin by DEAE-cellulose column, (3) carboxymethyl cellulose column chromatography, (4) calcium phosphate gel column chromatography, (5) DEAE-Sephadex column chromatograph, (6) second calcium phosphate gel column chromatography, (7) fractionation with ammonium sulfate, (8) second DEAE-Sephadex column chromatography, and (9) crystallization and precipitation with ammonium sulfate. Thus, after more than 20 steps of purification, a

This investigation was supported by Research Grant HD-02497-01 and H-3901 from the National Institutes of Health.

TABLE 1. *Yield and activity of B+ enzyme in purification*

Steps of purification	Total volume (ml)	Total protein (mg)	Total activity		Specific activity of glucose-6-P dehydrogenase	Accumulative purification*
			Glucose 6-P dehydrogenase units	6-Phosphogluconate dehydrogenase		
Hemolysate	~15 x 10^3	2,030 x 10^3	5.75 x 10^3	2.1 x 10^3	2.83 x 10^{-3}	1
DEAE-cellulose column effluent	~1.3 x 10^3	24.6 x 10^3	5.5 x 10^3	<100	2.33 x 10^{-1}	82
Ppt with $(NH_4)_2SO_4$ and resuspended in buffer	300	13.7 x 10^{-3}	5.5 x 10^3	<10	4.02 x 10^{-1}	142
CM-cellulose column effluent		5.35 x 10^3	4 x 10^3		7.5 x 10^{-1}	265
Ppt with $(NH_4)_2SO_4$	65	2.8 x 10^3	3.9 x 10^3		14 x 10^{-1}	495
First calcium phosphate gel column effluent		5.5 x 10^2	3.7 x 10^3		6.72	2,380
First DEAE-Sephadex column effluent		1.85 x 10^2	3.5 x 10^3		19	6,700
CM-Sephadex column effluent		55	3.2 x 10^3		58	20,600
Ppt with $(NH_4)_2SO_4$ and dissolved in buffer	13	50	7.5 x 10^3 (3.7 x 10^3)†		150 (74)†	53,000 (26,100)†
Second calcium phosphate gel column effluent	280	37	3.4 x 10^3		92	32,300
$(NH_4)_2SO_4$ fractionation, dissolved in buffer	3.5	28	16 x 10^3 (3.5-4.1 x 10^3)†		570 (127-146)†	201,000 (45,000-51,500)†
Second DEAE-Sephadex column effluent	65	18	3 x 10^3		165	54,500
Crystallization and precipitation	1.7	15.6	11.5 x 10^3 (2.8 x 10^3)†	<0.1	730 (180)†	258,000 (63,500)†

*Ratio of specific activity of the preparations taking that of hemolysate as 1.00.

†Activity measured after dilution inactivation (see text). The concentrated enzyme solution was diluted (100- or 200-fold) in a buffer and kept at 0-4° for several hours before the activity measurement.

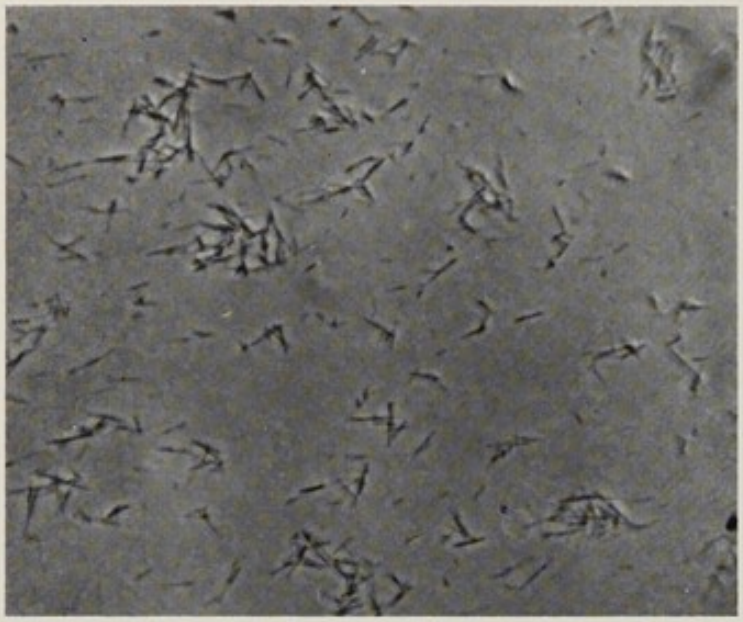


FIG. 1. Micrograph of glucose-6-phosphate dehydrogenase crystals. About 650 magnification.

homogeneous enzyme preparation was obtained in an overall yield of about 50 per cent. About 16 mg of purified enzyme were obtained from about 10 liters of blood. Yield and activity of the enzyme at various steps of purification are shown in Table 1. A microphotograph of crystalline normal enzyme is shown in Figure 1.

The common Negro variant with normal erythrocyte enzyme activity (A+) was also purified by procedures similar to those used for the purification of B+ enzyme.[33]

Both purified B+ and A+ enzymes are homogeneous by physicochemical

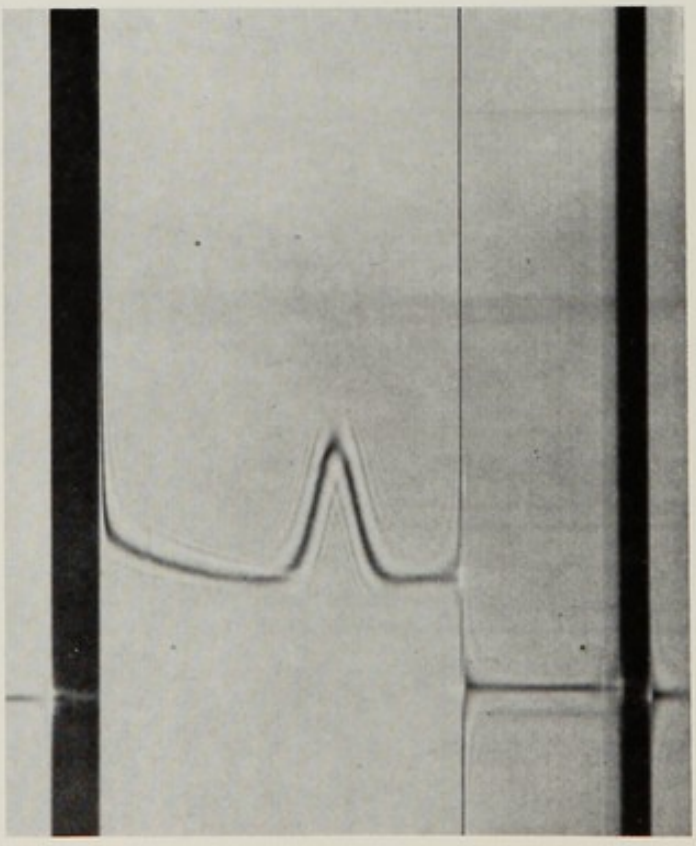


FIG. 2. Schlieren pattern of purified normal glucose-6-phosphate dehydrogenase in the centrifuge at 50,740 rpm 32 min after reaching final speed at 20°. The concentration of the protein was about 0.3 per cent in 0.05M acetate buffer (pH 6.0) containing 10^{-3} M EDTA, 10^{-3} M mercaptoethanol, and 2 x 10^{-5} M NADP.

criteria such as Schlieren pattern in analytical ultracentrifiguration (Fig. 2), interference patterns in sedimentation equilibrium (Fig. 3), and disc electrophoresis in polyacrylamide gel.

II. Structure of the Normal, Wild Type, Enzyme (B+)[32]

The sedimentation constant ($S_{20,w}$) was 10.0 S at a protein concentration of about 0.3 per cent in 0.05M acetate buffer (pH 6.0) containing 10^{-3}M EDTA, 10^{-3} M mercaptoethanol, and 2 x 10^{-5} M NADP. The molecular weight of the enzyme was estimated to be about 240,000 by the sedimentation equilibrium method[37] at protein concentrations of 0.015 and 0.008 per cent in the buffer described above.

When the enzyme was dissolved in the same buffer containing 4M guanidine hydrochloride and 0.01M mercaptoethanol (the enzyme was completely inactive in this solvent), the molecular weight was estimated to be approximately 43,000±3000. Since the interference pattern in this solvent showed monodispersion of the protein (Fig. 3B), these data indicate that the enzyme consists of six similar-sized subunits. This conclusion was further substantiated by the protein chemical method as follows.

As will be shown later, the digestion of the enzyme by trypsin and fingerprinting yields about 40 peptides, and amino acid analysis indicated the existence of 6.2 per cent lysine and 8 per cent arginine residues in the enzyme (Table 2). From these data, it could be concluded that the enzyme consists of six identical (or nearly identical) repeating units of molecular weight of

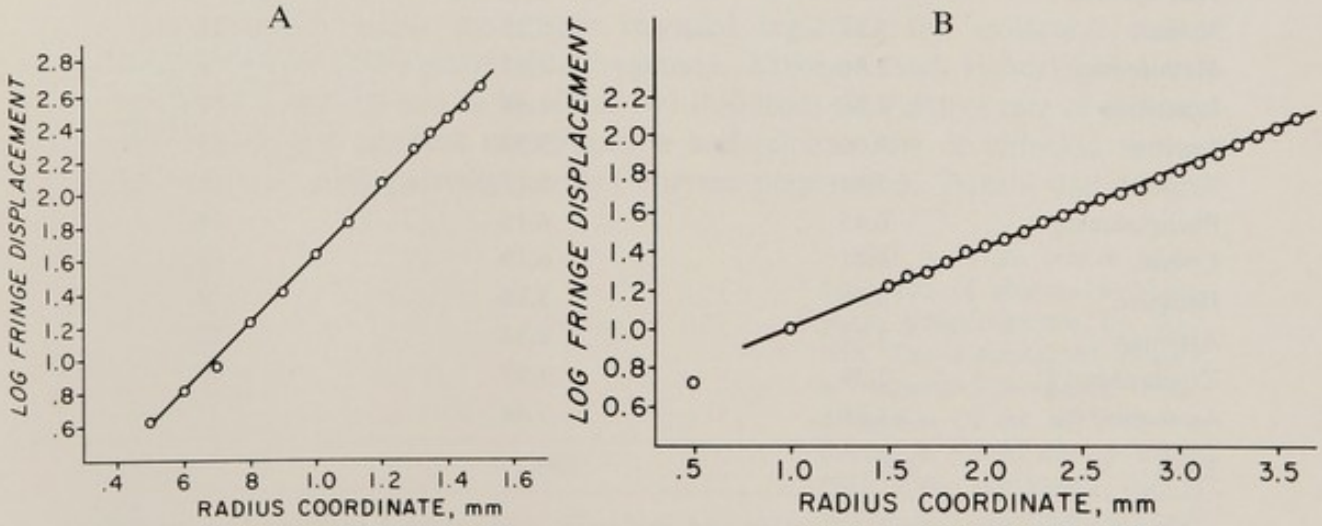


FIG. 3. The logarithm of the fringe displacement as a function of the distance from the center of the rotor. The data were obtained from the interference pattern of sedimentation equilibrium by comparator reading. Magnification factor = 2.229; vertical axis, log (millimeters of fringe displacement x 100); axis of abscissa, millimeters of comparator coordinate.

A: Initial concentration of the enzyme was 0.015 per cent in 0.05M acetate buffer (pH 6.0) containing 10^{-3} M EDTA, 10^{-3} M mercaptoethanol, and 2 x 10^{-5} M NADP. After 18 hours at 23.150 rpm at 10.1°.

B: Initial concentration of the enzyme was 0.02 per cent in 4M guanidine-HCl containing 0.05M acetate buffer (pH 6.0), 10^{-3} M EDTA, and 10^{-2} M mercaptoethanol. After 20 hours at 27,690 rpm at 14.3°.

about 40,000, since the expected number of tryptic peptides in the protein of a molecular weight of 40,000 is about 40 (about 19 lysine residues and about 22 arginine residues per subunit, see Table 2).

The sedimentation equilibrium experiment showed that removal of bound NADP from the enzyme by treatment with acidic ammonium sulfate caused dissociation into inactive (or weakly active) subunits (trimer) of a molecular weight of about one-half of the native enzyme (hexamer). Judging from the reports of previous investigators,[9,28] the enzyme (hexamer) may dissociate into partially active subunits (trimers) at very low concentrations (>0.01 mg per ml), even in the presence of NADP, although any accurate molecular weight determination is impossible at such low protein concentrations.

TABLE 2. *Amino acid composition of glucose-6-phosphate dehydrogenase*

Amino acid	Percentage of amino acid residues*		Assumed number of residues per subunit†
	Normal enzyme (B+)	Negro type variant (A+)	
Aspartic acid	10.83	10.88	38
Threonine‡	3.96	4.04	16
Serine**	4.07	4.01	19
Glutamic acid	13.77	13.78	43
Proline	5.26	4.75	21
Glycine	3.89	3.46	26
Alanine	3.87	4.11	22
Half-cystine††	1.67	1.91	7
Valine	5.21	5.25	21
Methionine	2.86	2.67	9
Isoleucine	4.36	4.68	17
Leucine	9.06	9.52	33
Tyrosine	5.44	5.12	13
Phenylalanine	6.45	6.15	18
Lysine	6.21	6.19	19
Histidine	3.06	3.16	9
Arginine	7.74	8.34	22
Tryptophan‡‡	2.29	1.97	5
Ammonia***	1.56	1.46	
Total	100	100	

*Mean value of analysis of native and performic acid-oxidized protein. The contents of methionine sulfone and tryrosine of performic acid-oxidized protein have not been included in the mean value because of uncertainty of their recovery. Total of individual amino acid residues is taken as 100.

†The nearest integrated number to the calculated number of amino acid residues per subunit, assuming that the enzyme consists of six identical subunits (see text).

‡The recovery after hydrolysis is assumed to be 95 per cent.[22]

**The recovery after hydrolysis is assumed to be 90 per cent[22]

††Calculated from the cysteic acid content in performic acid-oxidized protein. The recovery of cysteic acid is assumed to be 95 per cent.[23]

‡‡Estimated by p-dimethylaminobenzaldehyde method.[26,27]

***Not included in the total.

When the enzyme was diluted in buffer (pH 6.0 to 8.0, acetate or tris-HCl buffer) from a concentrated solution (more than 2 mg per ml) to give a concentration less than 0.1 mg per ml, the specific activity* (about 750-800 at concentrated state) decreased rapidly, reaching a stationary specific activity of about 180 (Fig. 4). The partial inactivation by dilution was reversible since precipitation of the diluted enzyme by ammonium sulfate, followed by redissolving of the enzyme at a higher concentration, restored the activity. It was rather surprising that no significant molecular weight change accompanying the dilution inactivation could be detected. From the sedimentation constant at higher concentrations and the molecular size estimated by the sedimentation equilibrium method at lower concentrations, it has to be concluded that the fully active enzyme, which exists at higher concentrations, and the partially active enzyme, which exists at lower concentrations, have the same molecular weight of approximately 240,000.

Since the concentration of the enzyme in red cells is about 0.005 mg per ml, assuming a homogeneous distribution, one may assume that the actual specific activity of the enzyme in red cells is about 180 (i.e., the maximum turnover rate is about 5×10^4 moles of substrate per min per mole of enzyme). On the other hand, since the enzyme was not inactivated by dilution in the reaction mixture, which contained both NADP and glucose-6-phosphate (or its analog), even at very low concentrations (>0.001 mg per ml), it is also plausible that the enzyme is fully active (specific activity about 800) in red cells. Such reversible activation-inactivation of the enzyme may play an important role in the regulation of glucose metabolism in cells.

Controversial results have been reported regarding the molecular size of human glucose-6-phosphate dehydrogenase. Kirkman and Hendrickson[9] estimated the molecular size to be about 105,000 from the relative rate of sedimentation in sucrose gradient centrifugation and relative rate of diffusion through sintered glass using partially purified enzyme preparation. Tsutsui and Marks[28]

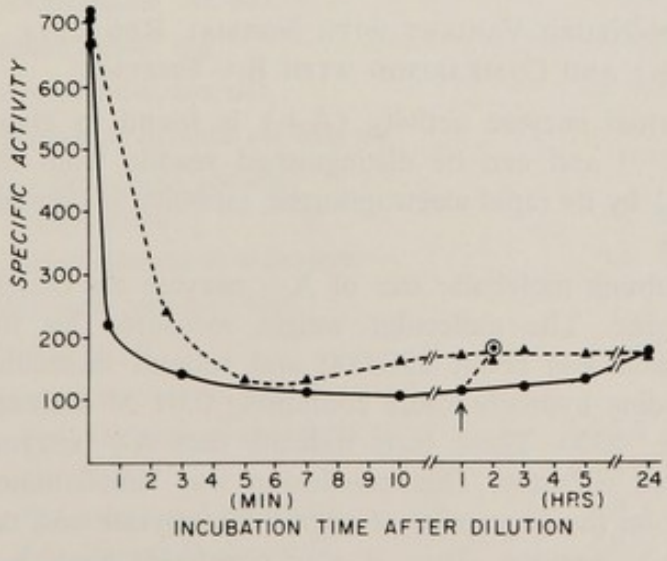


FIG. 4. Time course of inactivation of glucose-6-phosphate dehydrogenase by dilution. The concentrated solution of glucose-6-phosphate dehydrogenase (7 mg per ml) was diluted in 0.05M acetate buffer (pH 6.0) containing 10^{-3} M EDTA, 10^{-2} M mercaptoethanol, and 2×10^{-5} M NADP to give a final concentration of 0.07 mg per ml. ●——●, kept at 0°, ▲ - - - ▲, kept at 25°. ↑, part of the diluted enzyme solution kept at 0° was transferred to 25°, and the activity (⊙) was measured after keeping at 25° for one hour.

*Enzyme activity is defined as micromoles of NADP reduced per min at 25° C. Specific activity is the number of enzyme units per mg of protein.

also reported a similar molecular weight from the sucrose gradient centrifugation. On the other hand, Chung and Langdon,[3] using a more purified enzyme preparation (probably about 60 per cent pure), estimated the molecular weight to be 190,000 from a rough estimation of the sedimentation constant by ultracentrifugation and the diffusion constant by the porous diaphragm method. All these investigators observed that the removal of bound NADP resulted in dissociation of the enzyme into smaller subunits. The molecular weight of the enzyme is estimated to be 240,000 in the present work.

These discrepancies could be related to the following factors: (1) the enzyme may have various polymerization forms, depending upon the concentration of the enzyme and physicochemical environments (such as solvent, presence of molecular absorbing or molecular sieving materials, etc.); and (2) the assumptions that were required for calculation of molecular weight from the observed values might not be adequate or accurate.

The molecular weight of 240,000, found in the homogeneous enzyme preparation described here, is probably quite reliable since it was determined by the sedimentation equilibrium method. This is one of the most accurate methods for determining molecular weight, both practically and in principle, involving no arbitrary assumptions. The accuracy of this method is estimated to be $\pm$ 5 per cent. Removal of bound NADP causes dissociation of the enzyme into smaller subunits of a molecular weight about one-half of the native enzyme, and, judging from the results of previous investigators,[9,28] the enzyme may dissociate into subunits (MW about 120,000) at lower concentrations ($>$ 0.01 mg per ml) even in the presence of NADP.

The molecular size of glucose-6-phosphate dehydrogenase from other mammalian species seems to be similar to that of human enzyme. Nevuldine and Levy[19] estimated the molecular weight of rat mammary enzyme to be about 130,000 in the diluted state and about a quarter million at higher concentrations in the presence of NADP. Crystalline yeast glucose-6-phosphate dehydrogenase also seems to have the same molecular size as the human enzyme.[38]

III. Structure of Common Negro Variant with Normal Red Cell Enzyme Activity (A+) and Comparison with B+ Enzyme

The Negro variant with normal enzyme activity (A+) is found in about 18 per cent of Negro males[2,11,13] and can be distinguished readily from the normal wild type enzyme (B+) by its rapid electrophoretic mobility at alkaline pH.

The molecular weight and subunit molecular size of A+ enzyme are closely similar to those of B+ enzyme. The molecular weight measured by the sedimentation equilibrium method was about 230,000 and subunit molecular weight measured in 4 M guanidine hydrocholoride containing 0.01 M mercaptoethanol was about 45,000 $\pm$ 4000. These facts indicate that A+ enzyme also consists of six similar-sized subunits. This conclusion was substantiated by the number of peptide spots on the fingerprint of tryptic hydrolysate and the amino acid composition of A+ enzyme. Thus it was concluded that A+ enzyme consisted of six identical (or nearly identical) subunits of molecular weight of about 40,000.

No significant difference was found in amino acid composition between the B+ and A+ enzymes (Table 2). Since conventional amino acid analysis cannot reveal, in general, a single amino acid difference between two proteins, this does not necessarily mean that none exists.

The sedimentation constant of A+ enzyme ($S_{20,w}$ = 9.0 S) is slightly less than that of B+ enzyme ($S_{20,w}$ = 10.0 S). Since the molecular weights of the two enzymes are closely similar, the A+ enzyme molecule presumably is more asymmetric than the B+ enzyme molecule. From the molecular weight and the sedimentation constant, the Stokes radius was calculated as 5.6 $m\mu$ for the B+ enzyme and as 6.2 $m\mu$ for the A+ enzyme. It has been known that A+ and B+ enzymes are difficult to separate in disc electrophoresis, using standard 7.5 per cent polyacrylamide gel at alkaline pH, while A+ enzyme moves faster than B+ enzyme in starch gel electrophoresis. It is likely that the faster electrophoretic mobility of A+ enzyme is compensated for by the molecular sieving effect of polyacrylamide gel since the A+ enzyme has a larger Stokes radius than the B+ enzyme.

The A+ variant is similar to the B+ enzyme in enzymatic properties, there being no large differences in specific activities, turnover rates, or Michaelis constants for the major substrates (Table 3). The A+ enzyme was also partially inactivated by dilution without dissociating into subunits.

The optimum pH of the A+ enzyme was slightly higher (pH 8.8) than that of the B+ enzyme (pH 8.0). In contrast to the B+ enzyme, magnesium ion increased the activity of the A+ enzyme with NAD as substrate.

Serological similarity between the A+ and B+ enzymes was examined, using

TABLE 3. *Comparison of normal (B+) and Negro variant (A+)*

	Normal enzyme (B+)	Negro variant (A+)
Molecular weight	230,000-240,000	230,000-240,000
Sedimentation constant, $S_{20,w}$	10.0	9.0
Stokes radius, $m\mu$	5.5	6.2
Subunit molecular size	ca. 40,000	ca. 40,000
Number of subunits per enzyme	6	6
Specific enzyme activity		
Fully active	ca. 800	ca. 900
After dilution in activation	ca. 180	ca. 190
Maximum turnover rate	2.2×10^5	2.4×10^5
Optimum pH	8.0	8.8
Michaelis constants (M)		
For D-glucose-6-phosphate	$3.9\text{-}5.2 \times 10^{-5}$	4.8×10^{-5}
For NADP	$4.4\text{-}5.7 \times 10^{-6}$	7.9×10^{-6}
For galactose-6-phosphate	8.0×10^{-3}	6.2×10^{-3}
For 2-deoxy-D-glucose-6-phosphate	6.0×10^{-3}	4.4×10^{-3}
For NAD	4×10^{-3}	7.8×10^{-3}



FIG. 5. Precipitation pattern of double diffusion in an agar plate on a microslide (Ouchterlony plate). Enzyme samples were dissolved in 0.05M phosphate buffer (pH 6.8) containing 10^{-3} M EDTA, 10^{-3} M mercaptoethanol and 2 x 10^{-5} M NADP to give a concentration about 5 units per ml, and placed in a well No. 1 to 6.

1. Partially purified (0.2 unit/mg protein) B+ enzyme.
2. Partially purified (0.2 unit/mg protein) A+ enzyme.
3. and 6. Partially purified (0.03-0.04 unit/mg protein) A– enzyme.
4. Purified, homogeneous B+ enzyme.
5. Purified, homogeneous A+ enzyme.

rabbit anti-serum that was obtained by immunizing a rabbit with crystalline B+ enzyme. The precipitation pattern in an Ouchterlony plate indicated a single continuous line between the A+ and B+ enzymes and the anti-serum, and no spur was detected (Fig. 5). Neutralization of enzyme activity as a function of anti-serum concentration is shown in Figure 6. The results indicate that the purified B+, partially purified B+, purified A+, and partially purified A+ showed no differences in neutralization by the anti-B+ serum. The microcomplement fixation test done by Drs. V. Sarich and A. C. Wilson, University of California, using our enzyme preparations and anti-serum, showed no difference between the A+ and B+ enzymes.

Altogether, these findings indicate that the structural difference between the two enzyme forms must be very minor.

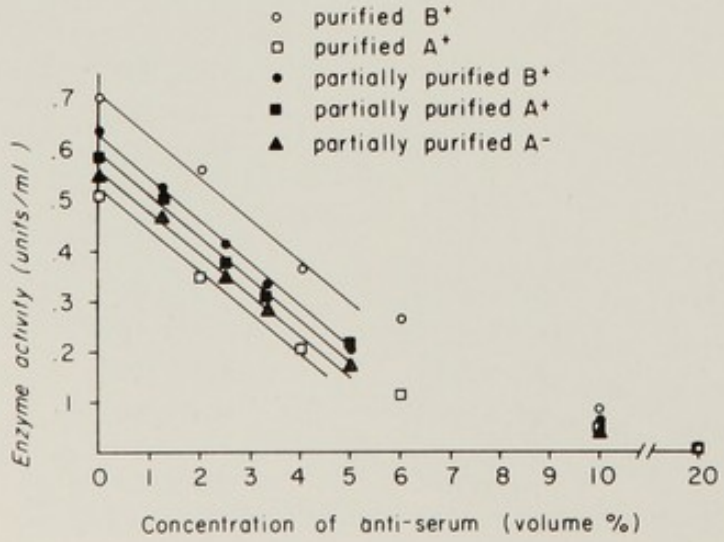


FIG. 6. Neutralization of glucose-6-phosphate dehydrogenase by anti-serum. Enzyme samples were dissolved in 0.05M phosphate buffer (pH 6.8) containing 10^{-3} M EDTA, 10^{-3} M mercaptoethanol, and 2 x 10^{-5} M NADP and mixed with anti-B+ serum of various dilution. Enzyme activity was assayed after keeping the mixture at 30° for one hour.

IV. Amino Acid Substitution between the Normal Enzyme (B+) and the Negro Variant (A+)[34]

Amino acid substitution between the A+ and B+ enzymes was studied by fingerprinting of tryptic hydrolysates of the enzymes. In order to insure sufficient digestion by trypsin, the proteins were denatured, reduced, and S-carboxymethylated in advance of the digestion. The tryptic hydrolysates were separated by paper chromatography (Whatman 3 mm; n-butanol: acetic acid: water = 4:1:5), and paper electrophoresis (pyridine: acetic acid: water = 1:10:289, pH 3.6-3.7).

The fingerprints of tryptic peptides obtained from the B+ and A+ enzymes are shown in Figures 7-I and 7-II. Among approximately 40 peptide spots appearing in each fingerprint, only one peptide spot (marked I) of the B+ enzyme was replaced by a peptide (marked II) in the variant enzyme; all other peptide spots corresponded in both enzymes. The results strongly suggest a single amino acid substitution between the normal and the variant enzymes. Since peptide I moved more rapidly than peptide II on electrophoresis at pH 3.6-3.7, it is likely that an amino acid residue of peptide I might be substituted by a more acidic amino acid residue of peptide II.

When the acid hydrolyzates of peptide I and peptide II were subjected to amino acid analyses, both peptides showed the same amino acid composition (Table 4). Considering the difference in electrophoretic mobility, asparagine or glutamine in peptide I should be replaced by aspartic acid or glutamic acid in peptide II, since asparagine and glutamine are converted to aspartic acid and glutamic acid by acid hydrolysis.

To distinguish between the two possibilities, peptides I and II were digested by carboxypeptidase A and B and leucine-amino peptidase, and the amino acids that appeared were identified by high voltage electrophoresis and paper chromatography. It was found that the hydrolyzates of peptide I contained very little aspartic acid (approximately in the same order of the blank) and

TABLE 4. *Amino acid composition of tryptic peptide of human glucose-6-phosphate dehydrogenase*

Amino acid	Normal enzyme, B+ peptide I μ mole*	Negro variant, A+ peptide II μ mole*	Ratio of amino acid residues†
Aspartic acid	0.043	0.040	2
Serine	0.021	0.020	1
Glutamic acid	0.019	0.016	1
Glycine	0.020	0.018	1
Alanine	0.032	0.032	2
Leucine	0.039	0.038	2
Histidine	0.036	0.033	2
Arginine	0.021	0.018	1

*Amount of amino acid found in peptide I and peptide II eluted from one fingerprint each.

†Closest integrated number of amino acid residues taking arginine as 1.

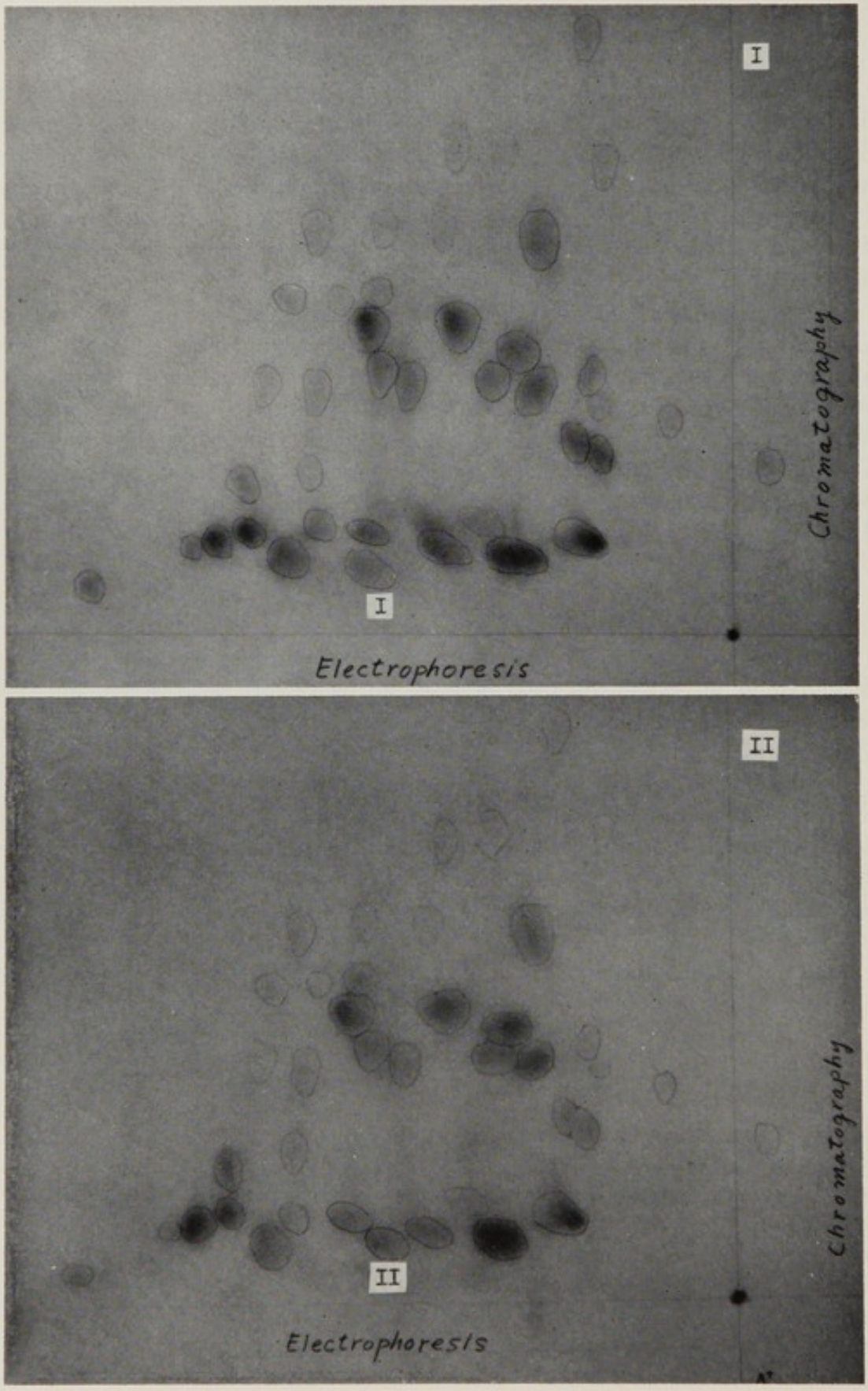


FIG. 7. Fingerprint of tryptic hydrolyzates of denatured, reduced and S-carboxymethylated human glucose-6-phosphate dehydrogenase. I: Normal enzyme (B+); II: Negro variant with normal enzyme activity (A+). First dimension, chromatography, n-butanol-acetic acid-water (4:1:5) for 18 hours. Second dimension, electrophoresis, pyridine-acetic acid-water (1:10: 289, pH 3.6-3.7), 2000 volts for 60 min.

a large quantity of asparagine. Peptide II contained both asparagine and aspartic acid in apparently the same quantity. Both peptides contained glutamine instead of glutamic acid. From these findings, together with the results of amino acid composition of acid hydrolyzates of the peptides (Table 4), the following amino acid compositions for the peptide I and II were established:

Peptide I (from the normal, B, enzyme): Asn_2, Ser_1, $Gln_{\sim}$, Gly_1, Ala_2, Leu_2 His_2, Arg_1.

Peptide II (from the A+ variant): Asn_1, Asp_1, Ser_1, Gln_1, Gly_1, Ala_2, Leu_2, His_2, Arg_1.

Accordingly, asparagine in the normal enzyme is replaced by aspartic acid in the A+ variant.

The substitution of aspartic acid for asparagine is frequently found in mammalian hemoglobin.[39] Since the mRNA codons for asparagine are AAU or AAC, and those for aspartic acid are GAU or GAC, this conversion is associated with a single step transition from AT to GC in the gene.

It has been reported that 15-20 per cent of chimpanzees also have an electrophoretic variant of glucose-6-phosphate dehydrogenase that corresponds in electrophoretic mobility to the human A+ variant.[4] Microcomplement fixation tests (done by Drs. V. Sarich and A. C. Wilson, using our enzyme preparation and anti-serum, indicated that the "normal" enzyme from chimpanzees can be distinguished from both the normal (B+) and the variant (A+) human enzymes that could not be distinguished from each other with this technique. It is, therefore, most likely that the human A+ and the chimpanzee "A+" mutations are not structurally identical but represent independent single mutations from their respective wild type enzymes.

V. The Negro Variant Associated with Red Cell Enzyme Deficiency (A–)[35]

Ten to 12 per cent of Negro males have glucose-6-phosphate dehydrogenase deficiency (10-15 per cent of normal) in red cells.[2,10] The enzyme levels of A– deficient Negro males in nucleated tissues such as leukocytes and liver are normal or only slightly decreased.[16] A– enzyme cannot be distinguished from A+ enzyme by electrophoresis, and no consistent differences have been found between A+ and A– enzymes in enzymatic properties such as Michaelis constants, pH optima, substrate specificities, and immunological properties.[12,17,18] Kirkman and Crowell[8] suggested that enzyme deficiency in red cells may be caused by a quantitative reduction in the number of the enzyme molecules.[7,8] Luzzato and Allan[15] observed differences in heat stability and elution patterns on DEAE-Sephadex column chromatography between the A+ and A– enzymes.

Sufficient starting material to prepare and study the A– enzyme in a manner similar to that described for the normal and A+ enzymes has not yet been obtained. Thus far, we can discuss whether the low level of activity in the

glucose-6-phosphate dehydrogenase-deficient red cells from Negro males resulted from a structural abnormality producing a less active enzyme molecule, from a decreased rate of production, from an increased degradation rate, or from a combination of these effects.

A+ and A– enzymes could be separated by DEAE-Sephadex column chromatography, but the recovery of the activity of A– enzyme from the column was low as first shown by Luzzato and Allan.[15] Since the A– enzyme is inactivated in the absence of NADP, the low recovery of A– enzyme from the DEAE-Sephadex column may have been partly due to loss of NADP by the anion exchanger during chromatography. Another chromatographic system—carboxymethyl Sephadex column—gave a more effective separation of the A+ and A– enzymes, and the recovery of enzyme activity was better than 90 per cent (Fig. 8). These findings indicate a structural difference between the A+ and A– enzymes.

A– enzyme has serologic characteristics similar to the A+ and B+ enzymes, since the precipitation pattern in an Ouchterlony plate showed a single continuous line between the A+, B+, and A– enzymes and the anti-serum, and no spur was detected (Fig. 5). Neutralization of enzyme activity by anti-serum (Fig. 6) showed no difference in the neutralization behavior of A+, B+, and A– enzymes. From the slope of enzyme activity in the enzyme anti-serum mixture versus anti-serum concentration, it was estimated that one ml of this undiluted anti-serum neutralized 8-9 units of each enzyme. This means that one ml of anti-serum absorbed about 50 μg of B+ (or A+) enzyme protein (calculated from the specific activity of the purified B+ enzyme preparation). The B+ enzyme is the homologous antigen for the anti-serum used for this experiment; the anti-serum, therefore, should have maximal capacity to absorb B+ enzyme protein. Thus, the amount of A– enzyme protein absorbed by one ml of the anti-serum should not exceed 50 μg. This fact, together with the finding that one ml of the anti-serum neutralized 8 to 9 units of A– enzyme, indicates that the specific activity of A– enzyme should be at least as high as that of the B+ and A+ enzymes.

If the serological activity of A– enzyme was much lower than that of the normal B+ or A+ enzyme (i.e., a lesser amount of A– enzyme protein was absorbed by the anti-serum), the specific activity of A– enzyme would necessarily be much higher than that of B+ and A+ enzyme. This seems highly unlikely. It may be concluded, therefore, that A– enzyme has about the same serological activity and, at the same time, is enzymatically as active as the normal (B+) and A+ enzymes. Consequently, the low level of enzyme activity of deficient A– subjects results from reduction in the number of enzymatically active A– enzyme molecules in red cells.

When red cells from deficient Negro males with the A– mutation were fractionated by the two-phase centrifugation techniques of Danon et al.,[5] the younger A– cells contained more than 10 times as much enzyme activity as the total cell population (Table 5). Thus, the enzyme activity in the younger A– cells was close to that of the normal (B+) younger cells even though the enzyme activity in unfractionated A– red cells was only 10-15 per cent as compared with normal cells.

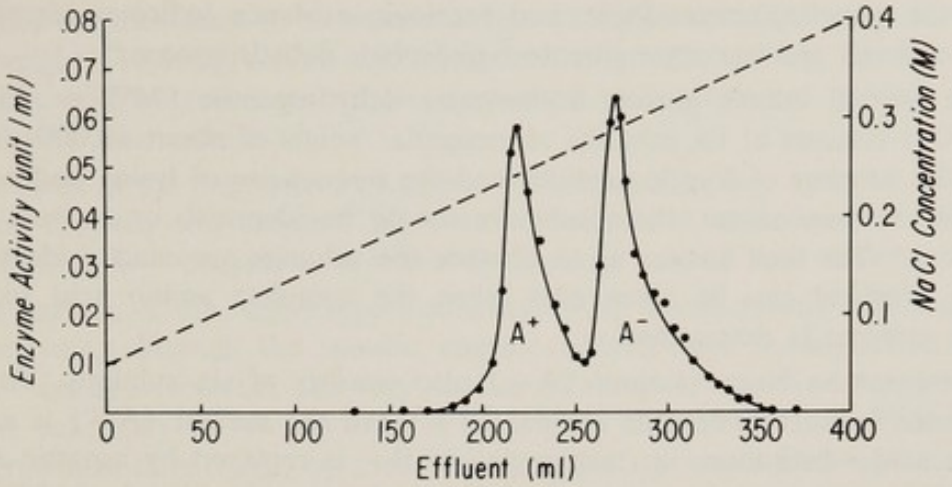


FIG. 8. Separation of A+ and A– enzymes by carboxymethyl-Sephadex column chromatography. A mixture of A+ enzyme (1.65 units) and A– enzyme (1.9 units) was placed on a carboxymethyl-Sephadex column (1 x 30 cm) buffered with 0.01M acetate buffer, pH 5.7 and eluted with linear gradient of NaCl from 0.1 to 0.4M.

Considering all these findings, it appears that the A– variant represents a structural mutation different from the A+ variant. The mutation does not affect the rate of synthesis and the enzymatic activity of the A– enzyme. The deficiency of red cell enzyme activity is principally caused by more rapid loss of enzyme activity upon red cell aging. The mechanism of accelerated inactivation of A– enzyme is yet unknown, but may relate to the more rapid loss of enzyme activity in vitro in the absence (or at very low concentration) of NADP. The A– enzyme inactivated in vitro had no serologic cross reactivity with the anti-B+ serum.

Discussion

The X-linked glucose-6-phosphate dehydrogenase in red cells and other tissues seems to be structurally identical.* Recently, it has been claimed that significant structural differences exist between red cell and leucocyte enzymes.[1] Such differences would imply separate genetic control of red cell and leucocyte enzymes.

TABLE 5. *Glucose-6-phosphate dehydrogenase activities in red cells of different age groups*

	Normal (B+)†	Deficient (A–)†
Younger cells	17 (16.1–18.2)	13 (12–15)
Total cells	6.6 (4.4–7.8)	0.93 (0.76–1.1)
Older cells	4.1 (3.4–4.9)	0.60 (0.4–0.7)

†The values are mean values and range (parenthesis) of the number of units of glucose-6-phosphate dehydrogenase activity per gram of hemoglobin in red cells obtained from three A– and three normal (B+) individuals.

*The liver enzyme, which is more active for galactose-6-phosphate than glucose-6-phosphate as a substrate and is linked to an autosomal locus,[20,24,25] is obviously structurally different from the X-linked glucose-6-phosphate dehydrogenase discussed in this paper.

However, genetic, enzymologic, and serologic evidence indicates identity between red cell and leucocyte glucose-6-phosphate dehydrogenase.[36]

The normal human glucose-6-phosphate dehydrogenase (MW = 230,000-240,000) consists of six subunits of molecular weight of about 40,000. Judging from the number of tryptic peptides and the frequencies of lysine and arginine residues in the enzyme, these subunits should be identical, or at least nearly identical.* The final answer as to whether the subunits are exactly identical or nearly identical can be given only when the complete amino acid sequence of the enzyme is determined.

The common Negro variant (A+) also consists of six subunits. The only difference found between the normal (B+) and the variant (A+) is a single amino acid substitution, i.e., asparagine in B+ is replaced by aspartic acid in A+. This conversion is associated with a single step transition from AT to GC in the structural gene. Considering the similarity of peptide spots on the fingerprints (Figs. 7-I and 7-II), this substitution is probably the only structural difference between the normal (B+) and variant (A+) enzymes. Although another substitution that could not be detected by the fingerprinting system used might exist, this possibility is highly unlikely on genetic grounds.

The finding of a single amino acid substitution in the A+ variant suggests that other genetic variants of human glucose-6-phosphate dehydrogenase are probably caused by single amino acid substitutions. For example, in the case of the Negro variant with reduced red cell enzyme activity (A–), the substitution causes no significant change of enzymatic activity, serologic properties, or rate of production of the enzyme, but does cause more rapid degradation of the enzyme during red cell aging. Another example, the common variant found in many Mediterranean populations (B–), has very weak red cell ezyme activity for glucose-6-phosphate as a substrate (0-5 per cent of normal) but has increased relative activity for galactose-6-phosphate (or 2 deoxyglucose-6-phosphate) as a substrate.[14] A single amino acid substitution, therefore, presumably causes alteration of specificity of the enzyme. Such mutations might play an important role in the differentiation and evolution of enzymes.[31]†

Summary

The normal human glucose-6-phosphate dehydrogenase (B+) and the common Negro-type variant (A+), which is distinguishable from the normal enzyme by its rapid electrophoretic mobility, were purified by column chromatographic techniques. The preparations obtained were homogeneous, judging from analytical ultracentrifugation and interference patterns of sedimentation equilibrium. B+ and A+ enzymes are very similar, there being no significant

*It has been suggested that the enzyme (B+) has at least two peptide chains, one with tyrosine and the other wtih alanine as amino-terminals.[3] However, Chung and Langdon's sample was not homogeneous and contained impurities of smaller molecular weights. The observed amino-terminals, therefore, may not be attributed to glucose-6-phosphate dehydrogenase itself.

†Preliminary study showed that the B– enzyme had reduced turnover rate (about 20-30 per cent of normal B+) for glucose-6-phosphate as a substrate and increased turnover rate (about 3 times as normal B+) for galactose-6-phosphate as a substrate (Yoshida, unpublished observation).

difference between them in molecular weight (230,000-240,000), subunit molecular weight (40,000), amino acid composition, specific activity, turnover rate, Michaelis constants for the major substrates, and serologic activity as determined by anti-serum B+.

The study of the fingerprints of tryptic hydrolysates of B+ and A+ enzymes revealed that the two enzymes differ structurally in a single amino acid, i.e., asparagine in B+ enzyme is replaced by aspartic acid in A+ enzyme.

The enzyme of the Negro type deficiency (A–) is structurally different from A+ enzyme, although the specific enzyme activity and serologic activity of the A– enzyme molecule are very similar to those of the A+ enzyme. The enzyme deficiency in A– red cells is principally caused by an increased rate of degradation rather than by a decreased rate of production of the enzyme.

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DISCUSSION

SIMON, ALBUQUERQUE, CHAIRMAN: Dr. Yoshida's paper is open for discussion.

MORRISON, DUARTE: With regard to the molecular weight of G-6-P-dehydrogenase, I wonder if you have attempted to evaluate the effect of dilution. If you dilute out your enzyme, what happens to your sedimentation equilibrium patterns?

YOSHIDA, SEATTLE: The lowest initial concentration used for the sedimentation equilibrium measurement was about 0.08 mg per ml. The enzyme had reduced specific activity at this concentration, but the interference pattern showed monodispersion of the protein solution, suggesting no dissociation of the enzyme into subunits. Molecular weight was estimated as 230,000-240,000.

There was no evidence of dissociation at the concentration used for the sedimentation equilibrium measurements. However, at very low concentrations (lower than 0.01 mg/ml), the enzyme probably dissociates into smaller subunits as was shown by sucrose gradient centrifugation and Sephadex gel filtration by other investigators.

MORRISON: Sedimentation equilibrium studies should provide a good criterion for dissociation, and I just wondered why you have not observed any.

YOSHIDA: The partial inactivation by simple dilution without dissociating into subunits is really rather astonishing. However, a similar phenomenon was observed also in some other dehydrogenases such as bacterial alanine dehydrogenase and lactate dehydrogenase as I have reported some time ago. We have to admit that the enzymes have two molecular forms with the same molecular size, but with different enzymatic activity, depending upon protein concentration. In the case of human glucose-6-phosphate dehydrogenase, it may be shown schematically as: 000
N N
000
(fully active, stable at high concentration) and N
000
000
N
(partially active, stable at low concentration).

MORRISON: As a last question, may I ask why gel filtration does not distinguish between the B+ and A+ enzymes? As you know, separation of proteins by gel filtration is really based on the differences in their Stokes radius.

YOSHIDA: Resolving power of gel filtration is not sufficient to separate A+ and B+ enzymes. The electrophoresis in polyacrylamide gel (disc electrophoresis), which usually gives better separation of proteins than starch gel electrophoresis, could not separate well B+ and A+ enzymes. The plausible explanation for this is that faster electrophoretic mobility of A+ enzyme at alkaline pH is compensated by the molecular sieving effect of polyacrylamide gel, since the A+ enzyme has a larger Stokes radius than the B+ enzyme.

KIRKMAN, CHAPEL HILL: Dr. Yoshida, I think you do get, as did earlier workers, dissociation when you remove the NADP and dilute the enzyme. If you remove NADP and dilute the enzyme it dissociates into the trimer [Yoshida: Yes.], and in the presence of guanidine it goes down to a monomer, so that disassociation does occur but requires both dilution and removal of NADP or the addition of guanidine. I should like to point out that at low concentrations of 0.08 mg per ml at a specific activity of 180, you then have 14 units per ml, which is approximately 10 times the enzyme concentration in red blood cells. [Yoshida: Exactly.] Recently reported differences in molecular weights may result not from estimations carried out by different people using different techniques, but from differences in enzymic concentration and other experimental conditions and probably reflect the fact that the enzyme can exist in a number of different molecular sizes. I should like to ask the following question relevant to the dissociation of the enzyme into dissimilar subunits. Have you had occasion to subject the pure enzyme to electrophoresis in a gel in the presence of guanidine to see if these subunits move as a single band?

YOSHIDA: No, I have not tried that.

BEUTLER, DUARTE: I think that the question of whether the units are identi-

cal or not or whether the subunits are identical or not is particularly interesting from a genetic point of view. Evidence from some other mammalian species suggests that there may be dissimilar polypeptides produced in some species such as in horses. Two bands are present on starch gel electrophoresis. In hybridizing rat and cow enzyme, one obtains four bands which could also be explained on the basis of reassociation-disassociation of subunits. In the evolution of proteins, presumably polypeptide changes often arise by duplication of genes. I suppose that the subunits may be very similar to one another. Are the methods that you have used, the counting of tryptic fragments and comparing them with the number of lysine and argenine residue, sufficiently sensitive to pick up small differences between subunits affecting, let's say, only 3 or 4 amino acids in the entire polypeptide?

YOSHIDA: It would be impossible to determine whether the subunits are identical or closely identical from the number of tryptic peptides and the frequencies of arginine and lysine residues. Since (1) the digestion of trypsin is not absolutely specific, some particular arginine and lysine bonds are resistant against tryptic digestion; (2) the resolution of fingerprinting may or may not be high enough to separate all tryptic peptides; and (3) the amino acid analysis has limited accuracy (about 3 per cent). At this stage, we can say that human glucose-6-phosphate dehydrogenase consists of closely similar subunits of molecular weight of about 40,000. Whether the subunits are identical or closely similar can be answered only when the complete amino acid sequence of the enzyme is determined.

KIRKMAN: Before the discussion closes on Dr. Yoshida's work, I should like to point out something that we may lose sight of in asking these questions. Dr. Yoshida has carried over 50,000-fold purification of the enzyme with a 50 per cent overall yield. This is a fantastic accomplishment and may be without parallel in enzymology. Dr. Yoshida should really be congratulated.

Glutathione Deficiency

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THE CONDITION IN WHICH the total glutathione content of the circulating red cells is at most 10 per cent of the normal value is termed "glutathione deficiency." In a Dutch family, in which 5 members possess this red cell abnormality,[14] the clinical and hematologic findings can be summarized as follows: The defect is associated with a clinical picture of congenital nonspherocytic hemolytic anemia, which is fairly well compensated. According to a red cell survival study, using the Ashby method, 50 per cent of the cells survived at 15 days, which indicated that the life span of the glutathione-deficient red cell is 3 to 4 times shorter than that of the normal cell. Red cell destruction could be increased by the administration of primaquine, and the history of some patients strongly suggests that fava beans can give rise to hemolytic crises. Furthermore, incubation with radioactive chromate in vitro results in the rapid disappearance of red cells in vivo. No Heinz bodies are present under normal circumstances. We did not have the opportunity to observe the red cells during a hemolytic crisis. Incubation of the red cells in vitro with acetylphenylhydrazine results in increased Heinz body formation.

From their studies on two unrelated patients with glutathione deficiency, Boivin and Galand[1] obtained evidence that the abnormal red cells are not able to attach glycine to glutamylcysteine. In this symposium we discuss glutathione deficiency from the point of view of the investigator who wishes to learn more about the function of glutathione in the normal red cell. Glutathione is involved in a number of reactions, and the importance of each reaction in maintaining the viability of the red cell has been considered by many investigators. The first statement we can make with respect to this is that obviously a red cell can exist in vivo without glutathione.

Let us consider the different reactions in which glutathione is known or supposed to be involved.

THE GLYOXALASE REACTION

Glutathione is a cofactor in the glyoxalase reaction:

$$CH_3\text{-}CO\text{-}CHO + GSH \rightarrow \underset{OH}{\overset{H}{CH_3\text{-}C\text{-}CO\text{-}SG}} \xrightarrow{H_2O} CH_3\text{-}CHOH\text{-}COOH + GSH$$

Racker[15] demonstrated that methylglyoxal inhibits hexokinase and glyceraldehyde phosphate dehydrogenase. Methylglyoxal can be formed spontaneously from triosephosphate; therefore, the glyoxalase reaction might be important in the protection of these two glycolytic enzymes.

In glutathione-deficient erythrocytes, the activity of the glyoxalase system

is very low (about 10 per cent of the normal activity) as a result of the glutathione deficiency. Although there is normal glycolytic activity in erythrocytes, we cannot exclude the contribution of glyoxalase-deficiency to the decreased life span of erythrocytes.

The Glutathione Peroxidase Reaction

$$2\ GSH + H_2O_2 \rightarrow GSSG + 2\ H_2O$$

Several investigators[4,8] have indicated that this reaction may be very important in the red cell, not only during the administration of certain drugs, but also under normal physiological conditions. Vitamin C, for instance, is always present and is known to generate hydrogen peroxide in combination with oxyhemoglobin.

There are indications that the glutathione peroxidase activity of glutathione-deficient erythrocytes is abnormally low. During incubation at 37° C with Na-ascorbate and KCN, according to the method of Jacob and Jandl,[9] fresh EDTA blood from a GSH-deficient patient turned brown within 30 minutes (Fig. 1). In the absence of KCN there was hardly any change of color. In another experiment, it was shown that oxygen consumption, during incubation with glucose, adenosine, and methylene blue was equally high in glutathione-deficient and normal red cells, whereas there was hardly any consumption in G-6-PD deficient red cells (Fig. 2). Oxygen consumption was attributed to the hexose-monophosphate pathway, and the combined results of both experiments were



FIG. 1. Glutathione-deficient and normal blood after incubation with ascorbate, glucose and cyanide, according to Jacob and Jandl.[9] The suspensions were prepared from fresh EDTA blood of a glutathione-deficient patient and from a healthy normal subject. They were incubated for 30 minutes at 30° C in a Dubinoff shaker. Photographed through a red B+W filter 070.

interpreted as evidence of a lack of glutathione peroxidase activity in glutathione-deficient red cells, due to the lack of GSH.

In addition to these experiments, we performed incubations with ascorbate and KCN, using normal red cells rendered glutathione-reductase-deficient by treatment with chromate.[11] However, no change of color was observed, not even a decrease in GSH during a 4½-hour period of incubation. Although there was some reactivation of glutathione reductase during the incubation, it is possible that glutathione reduction by NADPH is not restricted to the enzyme glutathione reductase under all circumstances.

Protection of Thiol Groups

$$GSH + RSSR \rightleftharpoons GSSR + RSH$$
$$GSSR + GSH \rightleftharpoons GSSG + RSH$$
$$GSSG + NADPH \rightleftharpoons 2\ GSH + NADP$$

In the above reactions, RSSR may stand for disulphide bridges in hemoglobin, glycolytic enzymes, etc., inside the red cell, disulphides on the cell membrane, or even disulphide bridges in the plasma proteins. In the last two cases there is no direct contact with GSH inside the cell. Cystamine and derivatives, however, can pass through the red cell membrane, and Eldjarn and associates[7] demonstrated that cystamine (1 mM) is reduced when incubated with red cells

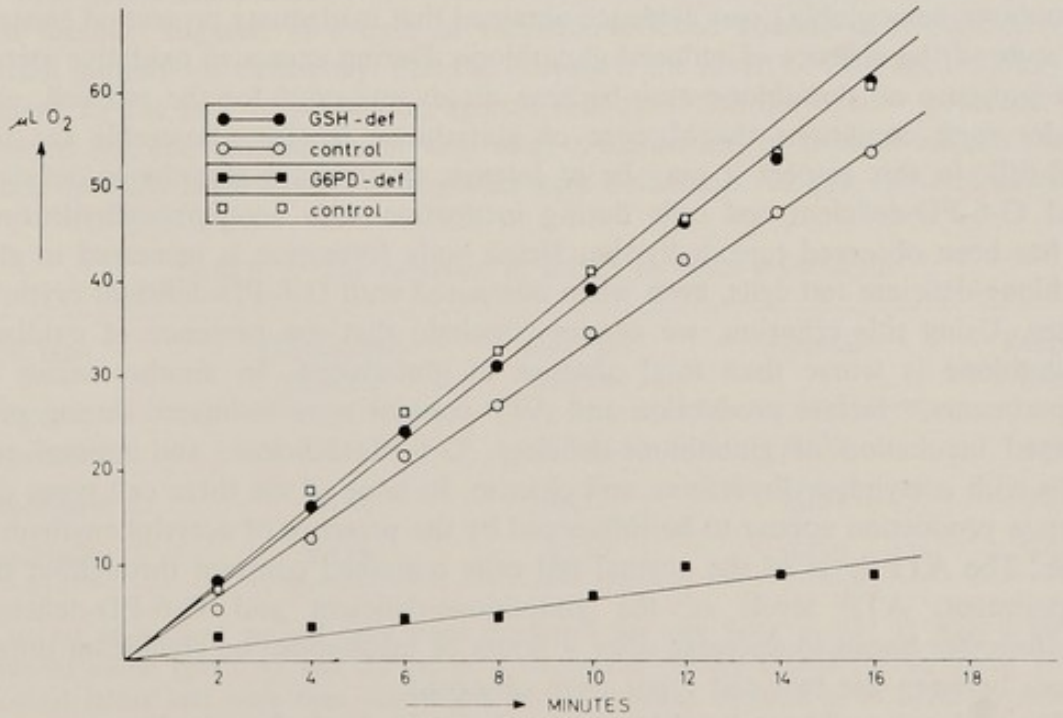


FIG. 2. Oxygen consumption of normal, G-6-PD-deficient, and glutathione-deficient red cells during incubation with methylene blue. 0.8-ml portions of washed packed cells, derived from ACD-blood, were suspended in 1.6 ml of a solution containing 0.12 M NaCl, 0.0048 M KCl, 0.0026 M $CaCl_2$ 0.0012 M $MgSO_4$, 0.016 M phosphate, 0.0035 M adenosine, 0.014 M glucose, and 0.003 M methylene blue (pH 7.4). Incubation was started in Warburg flasks with 0.15 ml of a 10 per cent KOH solution in the center well and with open valves. After 20 minutes of incubation at 37° C, the valves were closed and oxygen consumption was measured. Oxygen consumption (μL/Warburg flask) is represented as a function of time.

and glucose. Therefore, there may be two other reactions in which cystamine is a carrier:

$$NH_2\text{-}CH_2\text{-}CH_2\text{-}SH + RSSR \rightleftharpoons NH_2\text{-}CH_2\text{-}CH_2\text{-}S\text{-}SR + RSH$$

$$NH_2CH_2CH_2SH + NH_2CH_2CH_2\text{-}S\text{-}S\text{-}R \rightleftharpoons NH_2\text{-}CH_2CH_2SSCH_2CH_2NH_2 + RSH$$

These reactions may proceed when the NADPH generation capacity of the red cell is intact, and in this way GSH may act as a detoxifying agent. On the other hand, the presence of GSH may become a disadvantage when the oxidative stress becomes too high. When this happens, the increasing GSSG level may result in the formation of mixed disulphides with hemoglobin, enzymes, etc. Eldjarn and Bremer[6] demonstrated that high concentrations of cystamine (17 mM) inhibit the utilization of glucose in human red cells, whereas utilization of adenosine and inosine is unaffected.

We estimated a number of enzyme activities, performed chromatography of the hemoglobin on columns of Amberlite IRC-50, and carried out analyses of glycolytic intermediates to try to answer the question of whether there is a lack of protection of thiol groups in glutathione deficiency.

Neither from the activities of the enzymes glucose-6-phosphate dehydrogenase, glutathione reductase, 6-phosphogluconate dehydrogenase, glyceraldehyde phosphate dehydrogenase, and triosephosphate isomerase, nor the lactate production from glucose, nor the amount of Hb-A_3 (which may be regarded as partially denatured hemoglobin) was evidence obtained that inadequate protection existed, in spite of the absence of reduced glutathione. During excessive oxidative stress, the presence of glutathione may become disadvantageous for the red cell, and under such conditions the absence of glutathione is more favorable for the red cell. In this respect it may be of interest to compare glutathione-deficient and G-6-PD-deficient red cells during incubation with acetylphenylhydrazine. It has been observed repeatedly that Heinz body formation is increased in glutathione-deficient red cells, even when compared with G-6-PD-deficient erythrocytes. Using this criterion, we cannot conclude that the presence of oxidized glutathione is worse than total absence of glutathione. In another series of experiments,[13] lactate production and ATP content were estimated during prolonged incubation of glutathione-deficient, G-6-PD-deficient, and normal red cells with acetylphenylhydrazine and glucose. In none of the three cell types did lactate production appear to be influenced by the presence of acetylphenylhydrazine. The ATP level of the normal red cells remained constant throughout the experiment; ATP levels of the glutathione-deficient and G-6-PD-deficient erythrocytes began to decrease after 4 hours of incubation; no significant differences between the two cell types were observed.

From this experiment it could not be determined whether the hexose monophosphate pathway or GSH is required for the protection of the Embden-Meyerhof pathway during oxidative stress. Although the cause of ATP loss is not known, it seems that the absence of GSH is more important in ATP loss than the presence of GSSG.

Intact red cells were incubated with dithio-bis (nitrobenzoic acid) (DTNB) in order to detect possible differences between the number of SH- groups on the membrane of normal, glutathione-deficient and G-6-PD-deficient red cells

(Table 1). No significant differences among the three cell types were detected. Obviously no direct relationship exists between the DTNB-reactive groups on the membrane and the intracellular GSH-content. It should be noted that in this respect N-ethylmaleimide (NEM)-treated normal red cells are not representative of GSH-deficient erythrocytes (Fig. 3); during incubation of normal red cells with NEM, the number of DTNB-reactive groups on the intact erythrocyte decreases.

Regulation of Shunt Activity

Jacob and Jandl[10] conclude that the hexose monophosphate pathway of red cells, assayed by $C^{14}O_2$ production from glucose-1-C^{14}, is regulated primarily by glutathione (the GSSG/GSH ratio) and that methemoglobin has little effect on the activity of this pathway.

In Jacob and Jandl's demonstration of a sudden decrease in activity of the HMP Pathway when GSH is almost completely blocked by NEM treatment, it should be kept in mind that by the same NEM treatment there is a considerable inactivation of glutathione reductase and, to a lesser extent, G-6-PD. In these studies real GSH-deficient red cells are especially indispensable.

Protection against Radiation Injury

It is well-known that sulfhydryl compounds, possibly by reaction with radicals or with peroxides, protect other cell constitutents from radiation damage. Waller and Gerok[17] suggest, in a case of radiation-induced anemia in a patient with partial glutathione deficiency, that the anemia is the result of increased hemolysis caused by the lack of protection against radiation. Schiffer et al.[16] observed increased red cell destruction in vivo after x-irradiation of normal human ACD blood in vitro with 35,000 r. Together with decreased red cell survival in vivo,

TABLE 1. *The reduction of DTNB by intact erythrocytes*

Cell type	Glutathione-deficient	G-6-PD-deficient	Normal	Reagent blank
Experiment 1	0.110	0.140	0.132	0.041
Experiment 2	0.158	0.151	0.157	0.053
Experiment 3	0.113	0.130	0.110	0.052

10 ml of washed packed cells were incubated for one hour at 37° C with 2 ml of buffered saline (pH 7.4) and 0.2 ml of a solution of 40 mg DTNB in 100 ml of physiological saline and were then centrifuged for 10 minutes at 2000 g. In order to remove hemoglobin, 2 ml of the supernatant was filtered through an 0.6 x 1-cm column of Amberlite IRC-50 XE 64 (equilibrated with buffered saline, pH 6.0). The column was washed with 1.5 ml of physiological saline, and to the combined filtrates (3.5 ml) 1 ml of 0.15 M phosphate buffer pH 8.4 was added, giving a final pH of 7.3-7.4. The amount of 5-recapto-2-nitrobenzoate was estimated photometrically at 410 mμ. In a control experiment it was found that if the supernatant was replaced by a mixture of 2 ml of an 0.050 mM GSH solution and 0.2 ml of DTNB reagent, the final Ext 410_{410}^{1cm} was 0.231, when compared with the reagent blank.

All data in the table are expressed as Ext 410_{410}^{1cm} of the final reaction mixture.

an increase in red cell osmotic resistance at 37° C was observed after 24 hours of incubation.

Normal and glutathione-deficient red cells were irradiated with 36,000 r. The red cells were inspected for change of color and for Heinz bodies; furthermore, glutathione reductase and glyceraldehyde phosphate dehydrogenase activities were estimated, and osmotic fragility curves were made after 24 hours at 4° C. The only change observed was the decrease in osmotic fragility of glutathione-deficient red cells after irradiation. This may be an indication, that radiation-induced changes, also occurring in normal red cells, occur in glutathione-deficient red cells subjected to smaller doses.

Regulation of the Ratio of Membrane Saturated to Unsaturated Fatty Acids

The possible regulation of the ratio of saturated to unsaturated fatty acids by GSH is highly speculative. Boyland and Williams[3] demonstrated that rat liver supernatant preparations catalyse the conjugation of some epoxides with glutathione:

$$\mathrm{GSH} + \mathrm{R'\text{-}CH\text{-}CHR} \rightarrow \mathrm{GS\text{-}CHR'\text{-}CHR''\text{-}OH}$$

(epoxide: R′-CH(O)-CHR)

This reaction may be the first step in a reduction process in which the originally present double bond is reformed.

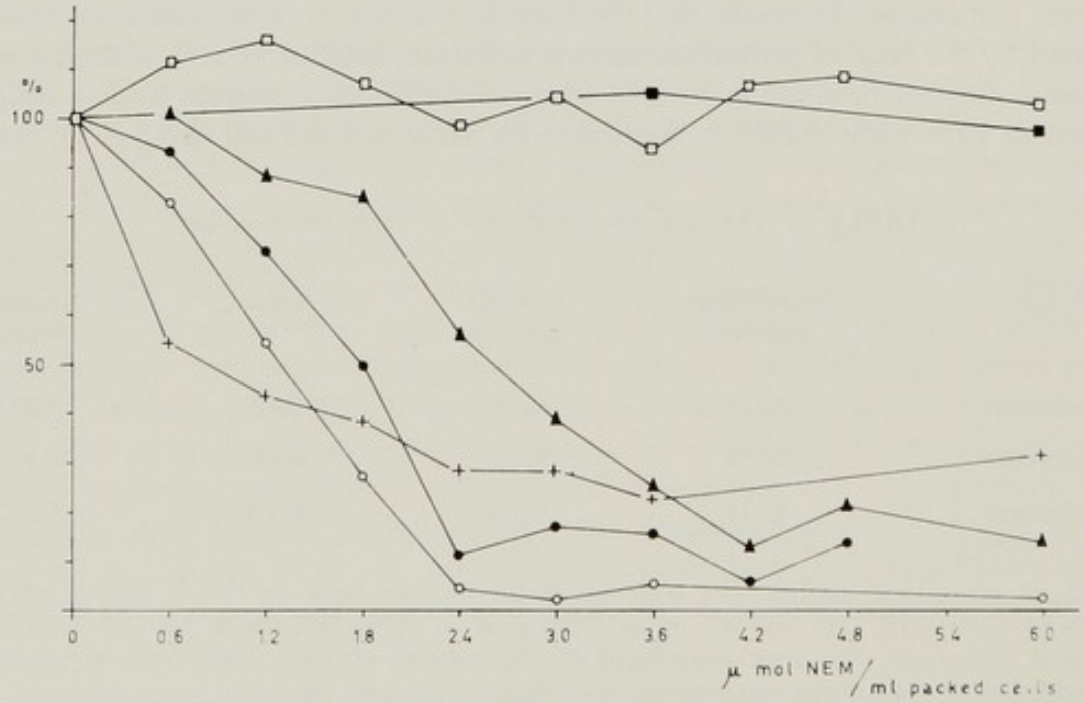


FIG. 3. The effect of NEM-treatment on normal erythrocytes. 1 volume of washed packed cells was incubated for 15 minutes at room temperature with 2 volumes of Ringer-phosphate-glucose solution containing increasing amounts of N-ethyl maleimide (0.3-3.0 mM). After incubation, the cells were washed with saline-containing glucose and once with glucose-free saline at 4° C. The data, obtained on incubation without NEM, are arbitrarily taken as 100 per cent. +——+ Reduction of DTNB by intact erythrocytes. O——O GSH. △——△ G-6-PD activity. ●——● Glutathione-reductase. □——□ ATP. ■——■ Lactate production in Ringer-phosphate-glucose solution.

Boyland and Chassaud[2] also described an enzyme in rat liver supernatant that catalyzed the addition of glutathione to double bonds. This again is an indication that glutathione may be involved in the regulation of the ratio of saturated to unsaturated fatty acids. However, the enzymatic activities described here have so far not been demonstrated in red cells; furthermore, we have not yet carried out experiments in order to obtain information on the composition of lipids in the glutathione-deficient red cell.

Acceleration of Chromate-Conversion

The general interpretation of the binding of chromium by the red cell is that chromate enters the red cell by diffusion and that after, or in the course of reduction to trivalent chromium, it is attached chiefly to the beta chain of hemoglobin. In 1955 Donohue and associates[5] observed that red cell survival is not affected when the red cells are labelled with less than 1 millimole of chromate per liter of red cells, but that above this concentration there was a definite shortening of red cell survival.

In a series of experiments, we incubated blood for one hour at 37° C with labelled chromate; the chromate concentration varied from 10^{-5} mole/l packed cells to 10^{-2} mole/l packed cells. Up to 1 millimole of chromate per liter of packed cells, the binding of chromate by normal cells after 1 hour of incubation is almost 100 per cent. At levels of chromate above 1 mM the binding

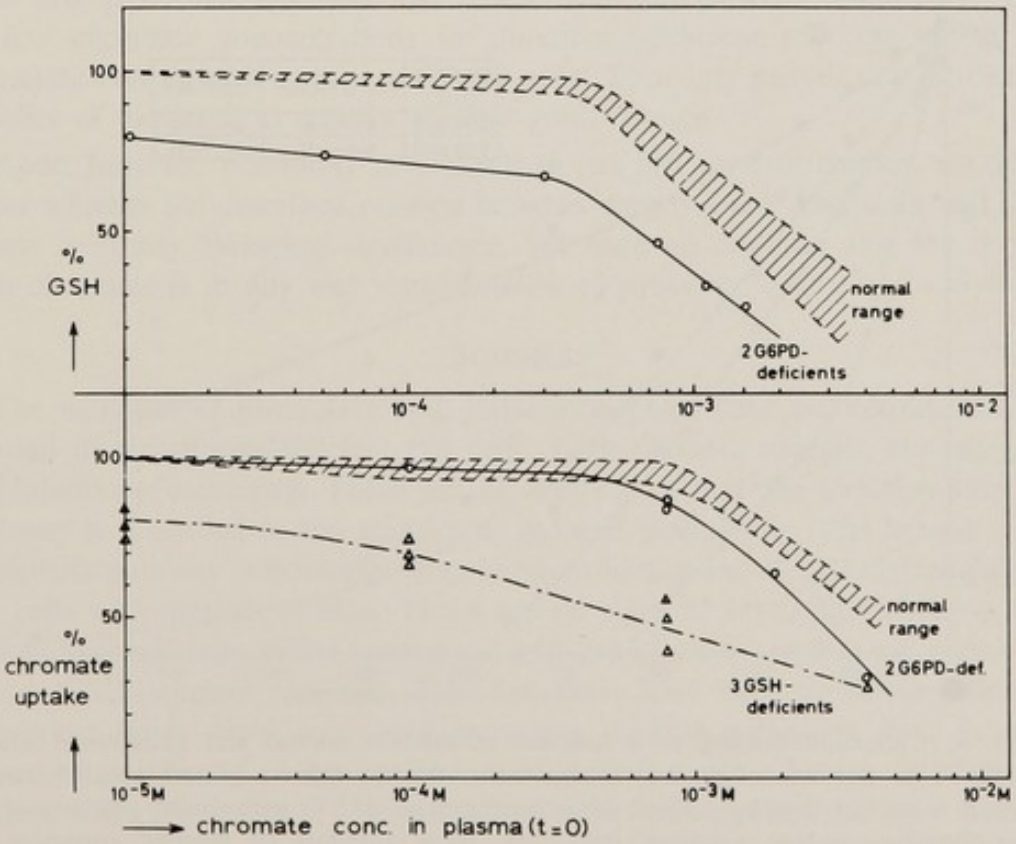


FIG. 4. The binding of chromium by glutathione-deficient, G-6-PD-deficient, and normal erythrocytes. Both the amount of GSH and the chromate uptake in normal red cells, on incubation with a trace amount of chromate, have been arbitrarily taken as 100 per cent. (Reprinted from Blood 27: 156, 1966.)

percentages decrease, indicating that after that one hour unconverted chromate is present and that the red cell constituents are still exposed to oxidative damage by chromate (Fig. 4). Also in the same range of chromate concentrations, the red cells are no longer able to maintain the original level of reduced glutathione. We made two observations with respect to glutathione-deficient blood: (1) Chromate binding is lower than normal after one hour of incubation, even with the lowest chromate concentrations. (2) In vivo survival experiments indicated that there is a marked decrease in red cell viability, even after labelling with these low amounts of chromate. These two findings suggest a correlation between glutathione and the chromate-binding capacity on one hand, and between lack of chromate binding capacity and cell damage on the other.

When glycolysis was blocked in normal red cells and ATP levels were reduced to zero by incubation with fluoride, or when catalase was inhibited by cyanide or azide, or when almost all hemoglobin was converted to ferrihemoglobin cyanide, no or hardly any effect was observed on chromate binding. A minor but significant decrease in chromate-binding capacity was found in G-6-PD-deficient red cells, probably as a reflection of the inability of those cells to maintain a sufficient level of GSH during oxidative stress. It may be that in the absence of GSH the red cells are more easily "saturated" with chromate, or that in the absence of reduced glutathione, the red cells react more slowly with chromate.

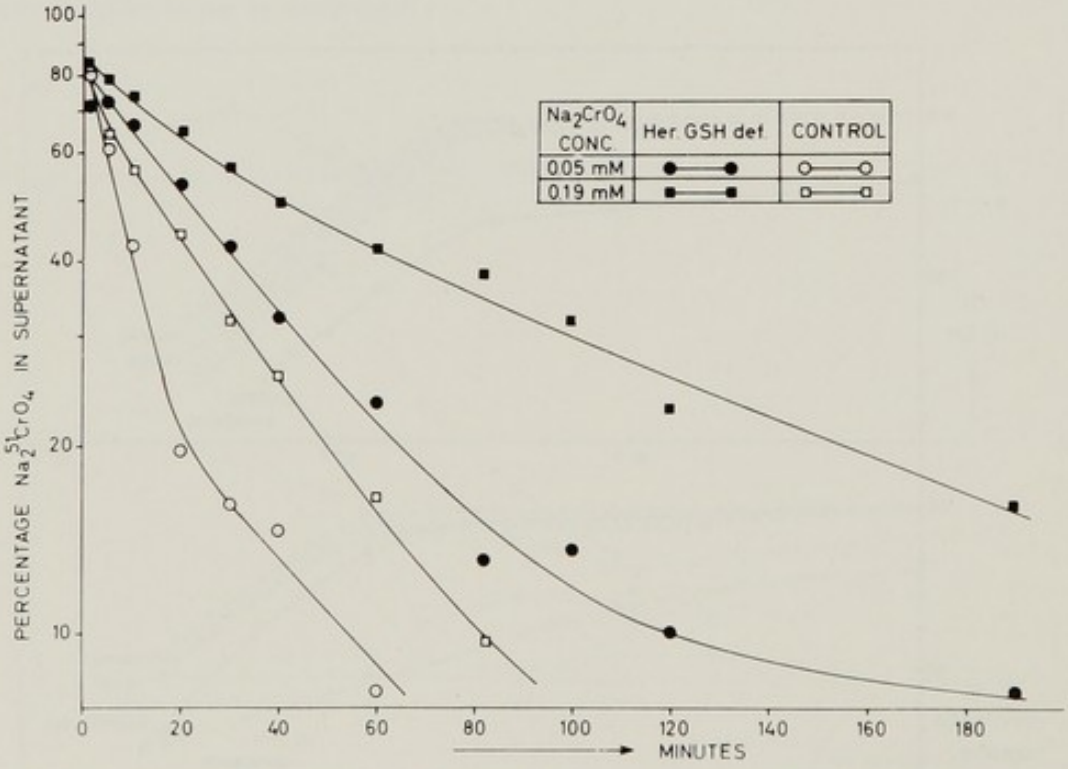


FIG. 5. Chromium-binding as a function of time in normal and glutathione-deficient red cells. 8-ml portions of washed packed cells, derived from a glutathione-deficient patient and a normal healthy subject, were incubated at 37° C with 16-ml portions of two Ringer-phosphate-glucose solutions containing trace amounts of labelled chromate and concentrations of 0.05 or 0.19 mM carrier Na_2CrO_4. After different time intervals, 2-ml aliquots of the incubation mixtures were mixed with 0.10 ml of a 10 per cent vitamin C solution, and chromium-binding percentages were determined. Log (100-binding percentage) is plotted against time.

We performed a number of experiments in order to answer this question. Normal and glutathione-deficient red cells were incubated with chromate at two different concentrations, and chromium binding was measured for 3 hours. We observed comparatively rapid disappearance of chromate within normal cells and a slow disappearance in glutathione-deficient cells. As shown in Figure 5, incomplete chromium binding after one hour appears to be the result of a low reaction velocity; there is no evidence of the existence of a "saturation point." In order to confirm the conclusion that glutathione is a rate-limiting factor, we demonstrated that the addition of reduced glutathione to hemolysates of glutathione-deficient red cells results in acceleration of the reaction up to the normal rate. We had to perform these experiments with hemolysates because the red cell membrane is impermeable to glutathione. These results are compatible with the view that GSH accelerates a reaction in which chromate is reduced and attached to hemoglobin, and in this way GSH contributes to the protection of the red cell.

I am afraid we cannot define the damaging side reactions. It is known from the work of Koutras and associates[12] that chromate causes inactivation of glutathione reductase in the presence of NADPH, and we found that at higher chromate concentrations there is also a loss of ATP and glycolytic activity. Furthermore, there is methemoglobin formation. Each of these effects may contribute to a loss of viability of red cells, but each effect occurred in glutathione-deficient red cells at almost exactly the same chromate concentrations as it did in normal red cells. No biochemical effects were observed to occur at low chromate concentrations in glutathione-deficient red cells or at high chromate concentrations in normal red cells. This only proves that, so far, the number of biochemical criteria was not great enough.

Apart from the possibility of artifacts in red cell survival studies, we do not know whether the described relation between conversion of chromate and glutathione is of any biological significance. We have no evidence that the reaction with chromate is in any way representative of processes occurring in vivo.

Summary

The activities of glyoxalase and glutathione peroxidase are considerably decreased in glutathione-deficient red cells. Both of these changes are secondary to glutathione deficiency. There are no indications that the Embden-Meyerhof pathway is disturbed in the absence of reduced glutathione. The hexose monophosphate pathway is normally stimulated on incubation of glutathione-deficient red cells with methylene blue. There are no signs of instability of hemoglobin in the abnormal cells. NEM-treated red cells are in many respects not representative of GSH-deficient red cells. Thus far, there have been no investigations on membrane lipids, lipid loss, lipid peroxides, sodium-potassium flux, or metabolism of glucose-1-C.[14] An adequate biochemical explanation for the chromate-induced loss of viability of glutathione-deficient red cells has not been found.

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DISCUSSION

ROSS, LOS ANGELES, CHAIRMAN: Let us begin the discussion.

VALENTINE, LOS ANGELES: We were particularly interested in Dr. Prins's demonstration of this peculiar effect of chromate in his glutathione-deficient subjects; showing that not only was it not bound well, but that it was uniquely toxic to the cell under these circumstances. We had been studying the chromate effect for some time, and it is a curious thing, but the maximal inhibition of glutathione reductase by chromate requires the cycling of TPNH to TPN and back again. Chromate will not inhibit the glutathione reductase of hemolysates that have been dialyzed; the action of chromate on glutathione reductase in hemolysates can be restored by adding physiological levels of TPNH. If you try to inhibit glutathione reductase in patients who are G-6-PD-deficient, you observe an inhibition that, just as an example, instead of going to 80 per cent goes only to 20 per cent and then levels off. So we wondered whether, in some way, the

chromate toxicity for red cells in the cases of Dr. Prins paradoxically would be associated with a situation in which you didn't inhibit glutathione reductase, and that perhaps the inability to cycle TPNH to TPN adequately in GSH-deficient cells is concerned with the chromate toxicity. Interestingly, chromate itself does not seem to result in Heinz body production, in the test tube anyway, but it predisposes to Heinz body production if you carry out the acetylphenylhydrazine test. For example, as little as 1.25 mg of elemental chromium per milliliter of blood (this would be about .03 μM per ml), produces no effect on glutathione reductase. If you then wash these cells and expose them to acetylphenylhydrazine in the Beutler test, you find that Heinz body formation is greatly exaggerated by preincubation with that relatively small amount of chromium.

PRINS, AMSTERDAM: We did not do these experiments, first incubating with chromate and afterwards incubating with acetylphenylhydrazine, but I wonder what you think about it. Is it possible that chromate creates a series of derivatives, possibly of hemoglobin, in this way creating a pathway for oxygen transport?

VALENTINE: Of course, at pH 7.0, even in the absence of hemolysates, glutathione does reduce chromate to the chromic state, though quite slowly. If you put in a micromole of chromate, you could convert it to the chromic state over a period of three to five hours at pH 7.0 in the presence of glutathione. I have no idea what goes on within the cells.

WALLER, TUEBINGEN: I think that the foregoing experiments demonstrate the importance of GSH for cell integrity also in radiation. We carried out the Heinz body test on cells irradiated in vitro. In the erythrocytes of the patient with GSH deficiency mentioned by Dr. Prins, there is increased Heinz body formation in the Heinz body formation test with increased radiation doses. In normal cells, there is no increase in Heinz body formation, and in cells from a patient with G-6-PD-deficiency, there is again an increase of Heinz body formation with increasing radiation. Independently of this observation, it is also very interesting that Heinz body formation test is almost always higher in radiologists than in the rest of the population.

ROSS: Would you repeat your last statement again? Did I understand what you said—that in radiologists, the Heinz body numbers are higher than normal? Is that what you said?

WALLER: Dr. Gerhard in Tuebingen has found that after long exposure to radiation, Heinz body formation, as revealed by the Beutler test, shows that about 20 to 25 per cent of red cells form more than four Heinz bodies. The normal range is between 10 to 15 per cent of red cells. Interpretation of this observation is very difficult. Perhaps it is possible that during radiation, a substance is produced that catalyses formation of Heinz bodies by APH. You can observe the same thing in the Heinz body formation test in rabbits after total-body irradiation.

BEUTLER, DUARTE: These very interesting findings on Heinz body formation in radiologists have, I believe, created a certain amount of consternation among health physicists. Radiologists are usually regarded as being rather well-protected against ionizing radiation. Furthermore, erythrocytes are usually considered particularly resistant to the effects of irradiation.

When we first developed this test in about 1953, we thought that we would use it for some family studies, and I got together some bottles and anticoagulant and went out to the home of a Negro family in Lockport, Ill. There were about 7 or 8 children in a small room, and I must have become somewhat nervous. In any case, I had a considerable amount of trouble getting the blood samples that I wanted, especially from the smaller children, and had to resort to multiple finger puncture, after multiple antecubital puncture had failed. I transported these samples back to my laboratory and found that all of them had positive Heinz body tests. It was only at that point that I began to realize that this test is very sensitive to extraneous factors, and the extraneous factor that I later found to be responsible for these results was that the test is exquisitely sensitive to oxygen tension. When I carried these samples back in my 1951 Plymouth, I had very thoroughly oxygenated all of the samples. In addition, the finger puncture samples

were very well oxygenated to begin with. I know that Dr. Waller is very much aware of the different things that may alter this test. I think that one must reemphasize that it is quite susceptible to influence by a variety of external effects.

KOSOWER, NEW YORK: Not all of us who are interested in the role of GSH in the red blood cell are so lucky as to have patients with no GSH in their erythrocytes. Reagents like APH have been used to induce loss of GSH in the erythrocyte, but have the disadvantage of being slow and inefficient. Many important questions such as the rate of regeneration of GSH in the intact erythrocyte, the role of NADH in the reduction of GSSG, etc., could not be studied.

There are a few physical organic chemists who want to apply their knowledge of chemical mechanisms to problems in medicine. One of these happens to be my husband. When we discussed how a reducing agent like APH could induce the oxidation of GSH, especially in light of Beutler's original finding that oxyhemoglobin was required for APH to be effective, we concluded that the active agent might have been an intermediate called phenyldiimide. We, therefore, substituted for the APH a compound that had been shown to generate phenyldiimide.* The source of phenyldiimide is the ester, methyl phenylazoformate, the synthesis for which is shown in Figure 1. The preparation involves the formation of methyl phenylhydrazoformate from phenylhydrazine and methyl chloroformate, followed by oxidation with lead

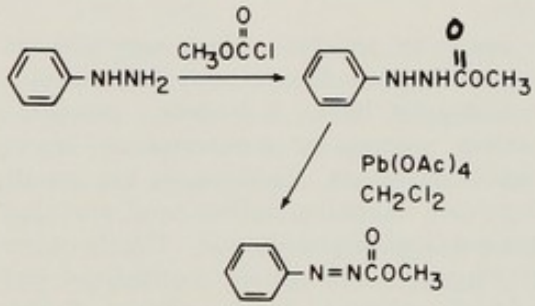


FIG. 1. Synthesis of methylphenylazoformate (azoester).

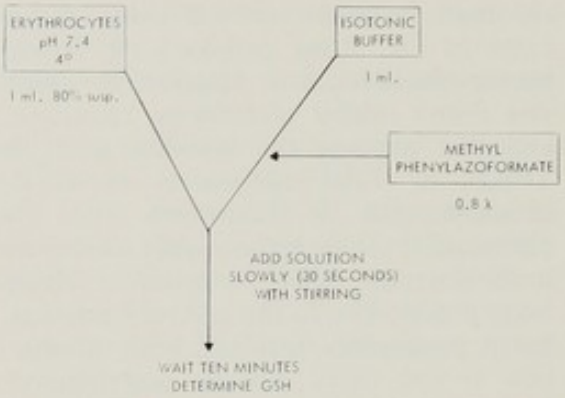


FIG. 2. Procedure for oxidation of GSH in the RBC by azoester.

tetraacetate in methylene chloride. The product is purified by distillation under vacuum. We usually refer to methyl phenylazoformate as "the azoester." It is a brown-red liquid, soluble in all organic solvents and, to a limited extent, in water. About 5 micromoles can be dissolved in 1 ml of water.

When a solution of the azoester is mixed with a suspension of erythrocytes, a rapid disappearance of GSH ensues. An outline of the procedure is shown in Figure 2. The usual procedure is to take a red blood cell suspension, kept at 4° C, and add an azoester solution with constant stirring. The azoester solution is

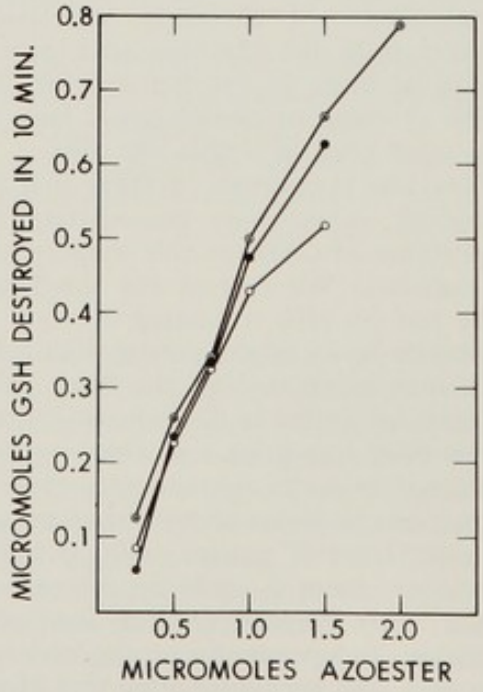


FIG. 3. Micromoles of GSH destroyed plotted against micromoles of azoester used.

*Kosower, E. M., and Huang, P.-k. C.: Phenyldiimide (a letter to the editor). J. Amer. Chem. Soc. 87: 4645, 1965.

prepared just before use. After about 10 minutes, GSH is determined by Beutler's DTNB method. The results are shown in Figure 3 in the form of a plot of the micromoles of azoester used against micromoles of GSH lost. The relationship is almost linear over the range studied and shows that a stoichiometric reaction involving two molecules of azoester and one molecule of GSH has occurred. This is the first time that a simple stoichiometric relationship between GSH lost and reagent added to a red blood cell suspension has been demonstrated. On the basis of this relationship and other information on the chemistry of phenyldiimide, it is possible to propose a detailed mechanism for the

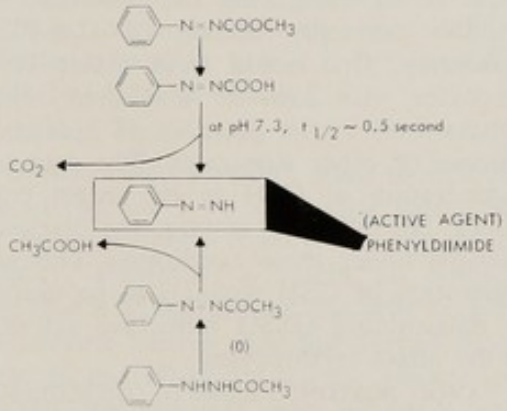


FIG. 4. Pathway from azoester to phenyldiimide.

destruction of glutathione within the red blood cell.*

The pathway from azoester to phenyldiimide is shown in Figure 4. The ester is hydrolyzed to the carboxylic acid, phenylasoformic acid, which is known to lose carbon dioxide very rapidly at any pH below 10 with an estimated half-life of 0.5 seconds at pH 7.4. The product of decarboxylation is phenyldiimide, which can also be derived from acetylphenylhydrazine.

The mechanism of GSH oxidation proceeds through a number of steps. In

*Kosower, N. S., Vanderhoff, G. A., Kosower, E. M., and Huang, P.-k C.: Decreased glutathione content of human erythrocytes produced by methyl phenylazoformate. Biochem. Biophys. Res. Comm. 20: 469, 1965.

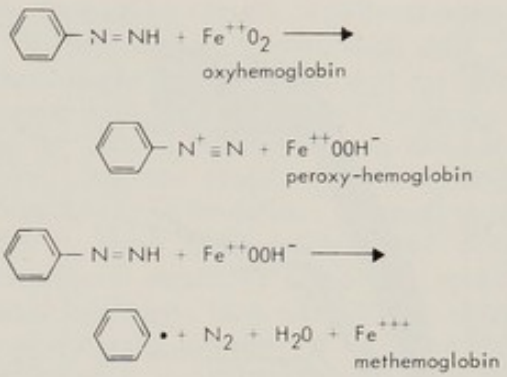


FIG. 5. Mechanism of GSH oxidation.

Figure 5, we see that phenyldiimide reacts with oxyhemoglobin to form peroxyhemoglobin. A second molecule of phenyldiimide reacts with peroxyhemoglobin to yield a phenyl free radical and methemoglobin. In Figure 6, we show that the phenyl free radical reacts with GSH, and that the resulting GS-radical dimerizes to GSSG.

The formation of GSSG is readily shown through the regeneration of GSH upon incubation of the erythrocytes with glucose. As shown in Figure 7, the use of the azoester permits a measurement of the rate of regeneration of GSH in intact erythrocytes. We have found that normal erythrocytes can regenerate about 40 per cent of the initial GSH in 10 minutes and over 80 per cent of the initial amount in 30 minutes. Glucose-6-phosphate dehydrogenase-deficient red blood cells from males regenerated little or no GSH under the same conditions. The erythrocytes of heterozygote females regenerated intermediate levels of GSH. It is important to emphasize that regeneration of GSH in the intact erythrocyte and G-6-PD activity in the lysate do not show

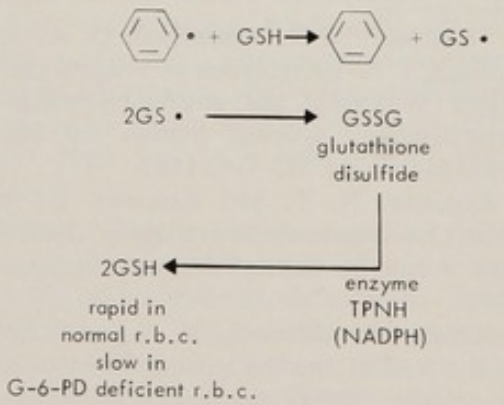


FIG. 6. Mechanism of GSH oxidation.

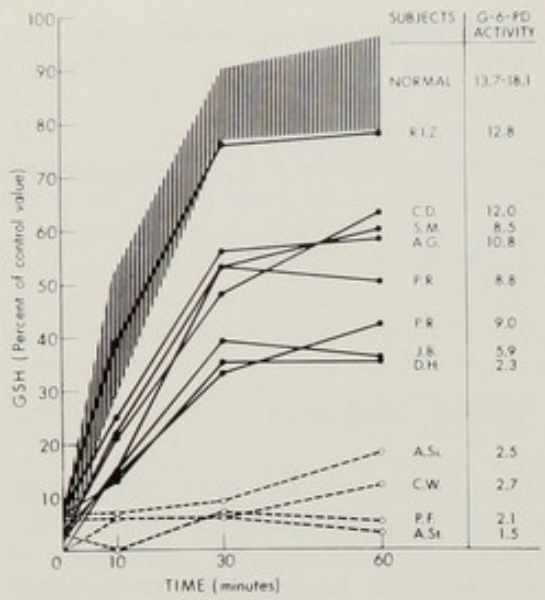


FIG. 7. Regeneration of GSH in erythrocytes of normal, G-6-PD-deficient males, and heterozygote females. //// Normal range. ●——● G-6-PD-deficient heterozygote. ○——○ G-6-PD-deficient males.

a good correlation. Please note the shape of the GSH-regeneration curves for the heterozygous females and the fact that only a fraction of the initial GSH content is regenerated. This shows quite clearly that the cells belong to at least two distinct populations, one which is normal in G-6-PD and another which is deficient. In Figure 8, we can see that the curve for the heterozygous female can be simulated by following the GSH-regeneration of a mixture of normal and male G-6-PD-deficient red blood cells.

We believe that the azoester procedure is a valuable new approach to various aspects of red blood cell metabolism and physiology as related to GSH in vitro and in vivo.*

*Kosower, N. S., Vanderhoff, G. A., and London, I. M.: Regulation of red cell glutathione in normal and glucose-phosphate-6-dehydrogenase deficient human red blood cells. Blood 29: 313-319, 1967.

Kosower, N. S., and Kosower, E. M.: Does, 3,4 dihydroxyphenylalanine (DOPA) play a role in favism? Nature (in press), 1967.

Rieber, E., Kosower, N. S., and Jaffe, E. R.: NADH and the reduction of oxidized glutathione (GSSG) in human erythrocytes (abstract). Fed. Proc. 26: 770, 1967.

PRINS: Well, I would say that obviously this reagent is less harmful to the red cell than N-ethylmaleimide. It might be very worthwhile to repeat many studies now with this reagent. But, of course, one has to check many other things, for example, ATP level, glycolytic activity, etc., before we can conclude that it is less harmful than N-ethylmalemide.

BEUTLER: I would like to ask Dr. Kosower whether this reagent is commercially available, or whether we would either have to make it ourselves or beg it from her husband?

KOSOWER: Well, according to my husband, it is very easy to make, but we are not chemists; he promised me that he would send azoester to anybody who is interested in using it. One possible use occurred to me during the presentation on the different variants of G-6-PD-deficiency. One could treat intact erythrocytes with azoester and then reincubate them in the presence of 2 deoxyglucose or other substrates. The rate of regeneration of GSH in the intact cell might be different among the various variants under these conditions. The regeneration of GSH would also be useful in determining GSSG reductase activity in the intact erythrocytes.

JANDL, BOSTON: I'd like to congratulate Dr. Prins and his associates for this work and to emphasize that there is nothing quite like a specific genetic defect to pro-

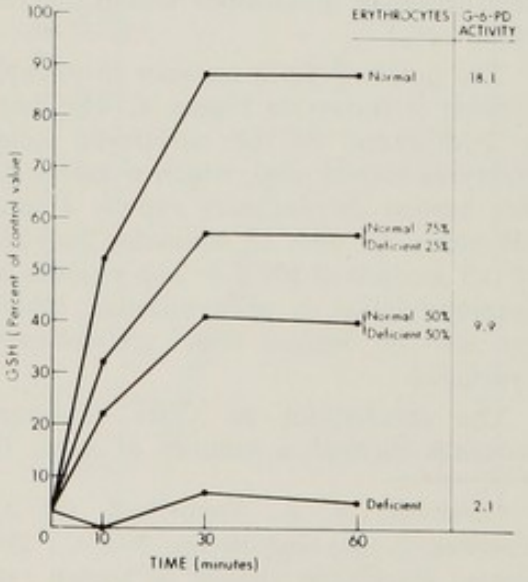


FIG. 8. Regeneration of GSH in mixtures of normal and G-6-PD-deficient erythrocytes.

vide one with final data concerning biochemical regulation and function in cells. Barron has been quoted as remarking that the studies of G-6-PD-deficiency at last proved that the activity of the hexose monophosohate shunt and the oxidation of glucose by red cells did depend upon that enzyme. Similarly, acatalactic (or acatalasemic) red cells are preferable to cells treated with cyanide or azide for investigating the role of catalase. Actually the use of appropriate sulfhydryl inhibitors has provided very valuable information, although none is entirely specific for glutathione. I do wonder about your use of the term, "absence," of glutathione because I believe you reported that there is some glutathione, something on the order of 5 or 6 per cent of normal. As long as some glutathione remains, the cell may be little affected except in that it is hypersusceptible to oxidative "stress."

PRINS: That is true.

JANDL: One wouldn't want to quibble about the last 5 or 6 per cent (although in business that's usually the margin of profit). However, it might be pointed out that it would appear that with respect to enzyme activity and the overall function of the HMP shunt, the amount of glutathione required may be small and the difference between 4 or 5 mg per cent and none may be critical. For example, in the studies by Dr. Jacob and myself of the role of glutathione in regulating the HMP shunt, an actual stimulation of the shunt was observed either when GSH was oxidized to GSSG or when most but not all of the reduced glutathione had been blocked with N-ethylmaleimide. I might point out that when N-ethylmaleimide is used, the GSH is selectively trapped, leaving the oxidized form. In either case the ratio of GSSG/GSH was increased. As I recall, you did measure the GSSG in your patients. Although that level was probably not very high, the "resting" rate of the HMP shunt would probably not be much affected as long as some appreciable glutathione remained in a normal redox balance. The ability of the shunt to maintain a normal rate of activity in the "resting" red cell is well shown in patients with a severe deficiency of G-6-PD. Despite an enzyme activity of only 5 or 10 per cent of normal, such cells, in terms of glucose oxidation, may be quite normal until they are stressed by increasing the rate of oxidation. You have observed that the HMP shunt responded normally to stimulation by methylene blue in these GSH-deficient red cells. Similarly, it might be noted that in our hands methylene blue activates the shunt quite normally in cells that had been pretreated with N-ethylmaleimide. With the hemolytic pre-oxidant, acetylphenylhydrazine, blocking GSH slightly reduces the shunt response to methylene blue. With peroxidation, on the other hand, blocking GSH also blocks the shunt—a reflection, presumably, of the enzyme GSH peroxidase. Indeed this system, in effect, serves as an assay of that enzyme. Have you had a chance to measure the rate of glucose-1-C^{14} oxidation in the cells of these patients and, if so, observed the effects of hydrogen peroxide or of acetylphenylhydrazine and other hemolytic drugs?

PRINS: That is the thing we should do, indeed, but we have not yet done. I have very little comment on this because, at the moment, it is not so easy for us to estimate the difference between no glutathione and the presence of some glutathione. It is very difficult, actually, when you know that there are other sulfhydryl compounds in the red cell—ergothionine, for instance—and so it would require quite a setup to measure accurately the exact amount of reduced glutathione in the glutathione-deficient red cell and even more difficult to measure the ratio of oxidized to reduced glutathione.

MOTULSKY, SEATTLE: Dr. Prins, have you done any studies on glutathione deficiency on platelets, white cells, or any other tissue cell? This might give some further information about the significance of this. In other words, are there cells other than red cells deficient in glutathione?

PRINS: So far, we have studied platelets and white cells, and can say that there is glutathione in those cells. There is furthermore indirect evidence of glutathione content of the liver. The plasma bromosulphalein disappearance rate was normal in the patient we studied. Gluta-

thione is believed to be involved in this reaction, which is used as a liver function test.

CARSON, CHICAGO: I want to add my congratulations to Dr. Prins and his associates for this very nice work, and all that they have accomplished. Dr. Prins has made a point in conversation that perhaps should be made here. He has studied only a very few of these subjects, and in order to retain their cooperation, he has to limit the experiments that are done. Their susceptibility to fava beans was not so clearly brought out in your literature. I believe this has been observed since you published your most recent papers.

PRINS: We advised those people not to use anything that should not be used by G-6-PD-deficient individuals, and they didn't take us seriously. They ate fava beans and had serious hemolytic crises.

LÖHR, MARBURGH: To the question of Dr. Jandl, we have also observed one family with GSH deficiency, a German family. We have estimated, by enzymatic methods reduced glutathione, and oxidized glutathione, and have found, like you, a small amount of reduced glutathione ranging from about 5 per cent of the normal values as oxidized glutathione was virtually absent in our studies.

KAPLAN, DUARTE: I would like to point out that the reagent DTNB does not react with ergothonine. I don't know why, but it is an interesting point to note, so I think when you use reagent, you really measure only the glutathione. Boivin reported that there was a defect in attaching glycine to the dipeptide. Have you had a chance to investigate the actual enzymatic basis of the defect in your family?

PRINS: No, we did not do so. It was Boivin, indeed, who obtained some evidence that it was a defect in the attachment of glycine. But everybody does not agree as yet that there is net synthesis of glutathione in the red cell. And also from this point of view, it is not surprising that we didn't find any incorporation of glycine into glutathione of glutathione-deficient red cells.

VALENTINE: Dr. Prins, your glyoxalase levels were not actually zero, is that correct?

PRINS: That is correct.

ROSS: Could I ask you a question, Dr. Prins? You commented that you x-irradiated GSH-deficient cells with 36,000 rads.

PRINS: That is true.

ROSS: And I believe you said that this decreased the osmotic fragility. Did I understand you correctly?

PRINS: That is right.

ROSS: In other words, these were not susceptible to hemolysis as they had been before they were irradiated?

PRINS: No, at least not to in vitro hemolysis. Schiffer and co-workers irradiated red cells with increasing doses, and they observed an increase in osmotic resistance with 36,000 rads but actually a decreased life span of the erythrocytes. With higher doses, they had a decrease in osmotic resistance and still more red cell destruction. Therefore, there is no parallel between osmotic fragility and susceptibility to in vivo hemolysis in these experiments.

KOSOWER: Of course, decreased osmotic fragility doesn't necessarily mean decreased susceptibility to hemolysis. Erythrocytes of patients with sickle cell anemia, thalassemia, and other anemias show decreased osmotic fragility in in vitro studies but increased fragility in in vivo survival. I would like to ask if you performed any other measurements on these irradiated cells or looked at their morphology?

PRINS: We just looked at osmotic fragility because Schiffer and his coworkers found changes after irradiation in osmotic fragility. We did not regard osmotic fragility as a measure of red cell viability, but rather we wanted to know whether the change in osmotic fragility occurred at lower doses in glutathione-deficient red cells than in normal red cells.

SIMON, ALBUQUERQUE: Have any defects been detected in presumed heterozygotes?

PRINS: No. For instance, with respect to the incorporation of glycine into glutathione, there was no difference with normal individuals. I think we must first measure the rate of synthesis of glutathione from glutamylcystein. As soon as

we can do that with greater accuracy, then we should try possible heterozygotes.

AEBI, BERNE: I think that a thorough study of glutathione compounds in red cells in your cases compared with those of normal individuals would be very informative. As you can see in Figure 9 on the left side, acatalatic red cells are about 80 to 100 times more sensitive to irradiation than normal red cells. If methemoglobin formation is taken as a parameter, the decrease in GSH content in the cells as measured by the DTNB technique is just about the same. There is considerable scatter, I admit, but I think they behave the same way. So this seems to be some indication that the catalase content of the red cell doesn't do anything as far as thiol compounds are concerned. I think a study of radiation sensitivity would be most interesting and challenging.

PRINS: It would be great fun to do it with catalase-free red cells, G-6-PD-deficient red cells, and GSH-deficient red cells in one and the same experiment.

BEUTLER: Dr. Morrison, some years ago you and Dr. Townes described biochemical investigations of an unusual case of methemoglobinemia which was associated with a moderate degree of GSH deficiency. Could you tell us what the current status of this syndrome is?

MORRISON, DUARTE: Some years ago, a case of methemoglobinemia was reported by Townes.* An investigation of the hemoglobin molecule† in this patient showed the hemoglobin to be normal. A number of enzyme systems and factors thought to be involved in methemoglobin reduction were also studied. The pyridine mucleotides, NAD and NADP, were normal, as were glucose-6-phosphate dehydrogenase, lactic dehydrogenase, and triose phosphate dehydrogenase. Of the components in these cells that were studied, only the level of GSH was outside normal limits and low. It was suggested that the biosynthesis of this compound might be involved in the defect. Subsequent work‡ has indicated clearly that low GSH levels could not account for the defect.

During the same period, we were also

*Townes, P. L., and Lovell, G. R.: Hereditary methemoglobinemia: A new variant exhibiting dominant inheritance of methemoglobin A. Blood 18: 18-33, 1961.

†Townes, P. L., and Morrison, M.: Investigation of the defect in a variant of methemoglobinemia, Blood 19: 60-74, 1961.

‡Prins, H.: See text of paper.

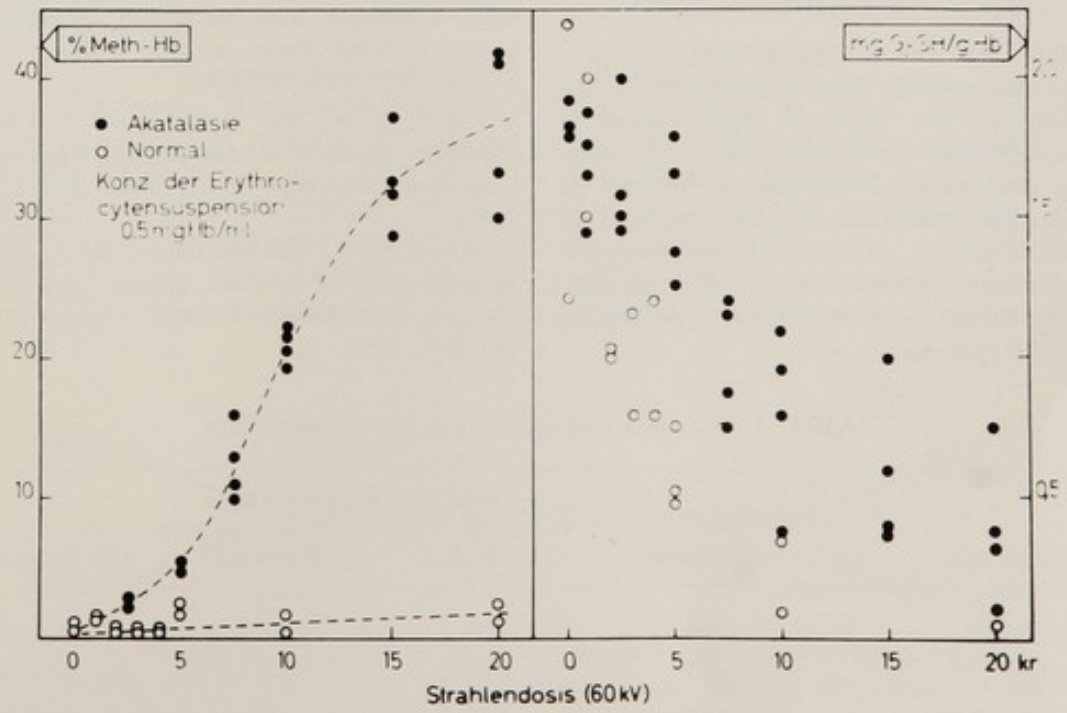


FIG. 9. The effect of x-irradiation on methemoglobin formation in acatalatic and normal red cells.

isolating components of the electron transport system of the red cell, and we reported the isolation of a new hemoprotein which had a unique heme prosthetic group.* Table 1 compares the spectral properties of this component with the likely contaminants of the red cell, clearly distinguishing it from these compounds. Figure 10 shows the spectral properties of this component. Subsequent work has provided further information concerning the heme prosthetic group, which now appears to be a 2,4-di formyl heme.† This component may play a vital role in methemoglobin reduction.

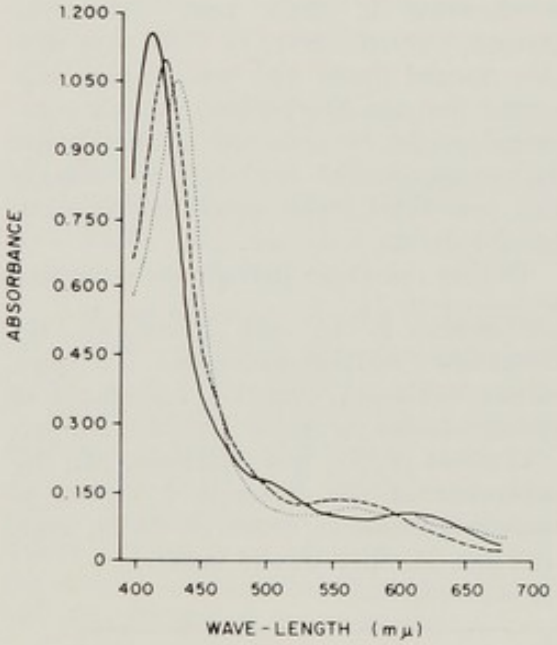


FIG. 10. The absorption spectrum of a new hemoprotein. The hemoprotein is dissolved in 0.1 M *tris* (hydroxymethyl) aminomethane buffer, pH 7.2,—. The solution contained 2.2 mg protein per ml, to which has been added a few crystals of solid $Na_2S_2O_4$, ... ; the hemoprotein preparation dissolved in 0.1 M *tris* (hydroxymethyl) aminomethane containing 0.01 M cyanide, - - - .

In Figure 11, the various dehydrogenases are shown as interacting with an as yet undescribed factor ("Factor X"). This factor, in turn, interacts with the hemoprotein described above, which in turn can interact with hemoglobin or oxygen. The factor may be identical for all dehydrogenases, or it may differ for the varying dehydrogenase. This factor could be a quinone, such as ubiquinone or coenzyme Q; or a non-heme iron protein, for example, might also play such a role. The factor could then interact with the hemoprotein, which in its turn could reduce methemoglobin or interact with oxygen. This would provide a system that would control the level of reduced and oxidized pyridine nucleotide, and also control the rate of reduction of methemoglobin.

TOWNES, NEW YORK: Since our last publication, we have examined glutathione synthesis in the patient that we had described in our earlier paper. Synthesis appeared to occur at about 50 per cent of the normal rate as determined by measurement of the incorporation of radioactive C^{14}-labelled glycine according to the methods of Elder and Mortensen.‡

*Morrison, M.: A new haemoprotein with a unique iron porphyrin prosthetic group isolated from human erythrocytes. Nature 189: 765-766, 1961.

†Reed, D. W., and Hultquist, D. E.: The prosthetic group of a hemoprotein from erythrocytes (abstract) Fed. Proc. 26: 823, 1967.

‡Elder, H. A., and Mortensen, R. A.: The incorporation of labelled glycine into erythrocyte glutathione. J. Biol. Chem. 218: 261-267, 1956.

TABLE 1. *Spectral properties of a new hemoprotein*

Hemoprotein	Absorption maxima (m_μ)			
	Oxidized	Cyanide	Reduced	CO reduced
Preparation	416	424	434	422
Catalase	407	425	—	—
Myeloperoxidase	430	458	475	—
Methemoglobin	405	415	430	418

I don't think these findings really have very great significance. I think that we fell into a trap by trying to relate two abnormal findings into one story—in attempting to relate the relatively low glutathione content in the cell with the presence of the methemoglobinemia.

BEUTLER: The case that Drs. Towne and Morrison reported was typical for methemoglobinemia due to DPNH diaphorase deficiency except for the low GSH and the fact that DPNH diaphorase levels appeared to be normal. Perhaps this finding might be due to the fact that the substrate that we normally use for assay of DPNH diaphorase is, as Dr. Scott has pointed out, DCIP. We use this receptor because it works very fast and very well. Isn't it possible, however, that in a case of a mutation involving the enzyme, the enzyme might still link very well with DCIP but might fail to link with methemoglobin. Then one would obtain methemoglobinemia but still normal assay, using a synthetic acceptor.

MORRISON: You're absolutely correct. That possibility has not been ruled out.

SIMON: I believe there was one other atypical feature. That was the inheritance, which, according to the history, was vertical and dominant and not recessive, so that it is likely to be a different kind of a defect.

TOWNES: We believe that it was transmitted vertically. This should be accepted with some caution because this was not directly documented by ourselves. The patient's father was deceased but, according to numerous good observers, was known to have been cyanotic all of his life. This information was obtained by questioning a number of relatives, including the man's wife, who was quite familiar with the clinical entity.

SMITH, STILLWATER: I wonder if Dr. Morrison has investigated the catalytic activity of the protein that he described. Have you tried to link it in with any of these other receptors?

MORRISON: Yes, we have tried to link this hemoprotein to other components of the methemoglobin reductase system. The isolated hemoprotein is not reduced directly by the partially-purified TPNH or DPNH diaphorases of the red cell. It is reduced, however, if a catalytic amount of methylene blue is added to the reaction mixture. In the scheme that I outlined, I have employed Factor X to designate a component that replaces methylene blue in vivo.

In answer to your first question, we also have attempted to evaluate the possible catalytic activity of this protein. Since the hemoprotein is reducible, it is clearly distinguishable from catalase, which is not reducible. The fact that the hemoprotein will react with carbon monoxide suggests that this protein may act as either a peroxidase or oxidase. However, the protein does not catalyze the usual peroxidase oxidations of dye. Thus, in the scheme I outlined, I have provided it with a central controlling position. If the reducing equivalents are not required for methemoglobin reduction, it may act as an oxidase. In either case, it would provide oxidized pyridine nucleotides for maintenance of cellular metabolism.

MASOUREDIS, SAN FRANCISCO: I wonder if the enzyme activities are additive if deficient cells and normal cells are mixed. Of course, if you expect all of

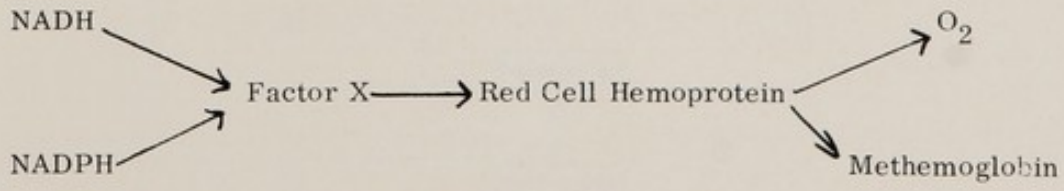


FIG. 11. Schematic diagram of the methemoglobin-reductase system in human red cells. In the case of the Towne patient, Factor X, or the red cell hemoprotein which interacts with it, may be deficient. These components would play a special role in maintaining oxidized pyridine nucleotide for red cell function if the reducing equivalents are not required for reduction of methemoglobin.

this enzymatic activity to be intracellular, you wouldn't expect a cell-to-cell interaction, but I wonder if anyone has done a simple experiment to determine this.

JAFFÉ, NEW YORK: We had the opportunity to do that experiment, Dr. Masouredis, in the course of trying to demonstrate genetic mosaicism in DPNH-methemoglobin-reductase deficiency. Compatible normal cells and cells from a homozygote with DPNH methemoglobin-reductase deficiency were mixed and the activities, as far as we could tell, were additive. There was no evidence of interaction during reduction of nitrite-induced methemoglobin in the intact erythrocytes.

Glutathione Reductase Deficiency

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IN RECENT YEARS investigations of the normal metabolism of glutathione and its inherited defects have underlined the role of reduced glutathione (GSH) in maintaining the stability of red cell structure, hemoglobin, and SH-group-containing enzymes. Spontaneous and drug-induced hemolytic anemias can be caused by deficiency or instability of GSH. Glucose-6-phosphate dehydrogenase deficiency and defects in glutathione synthesis, both inherited defects discussed in this symposium, may lead to GSH deficiency.

The purpose of this paper is to review knowledge of diseases correlated with glutathione reductase deficiency. Most of the results were obtained with the collaboration of Dr. Löhr.

The flavin enzyme glutathione reductase transfers the hydrogen ion from NADPH to GSSG and thus reduces one mole of GSSG to two moles of GSH (Fig. 1).[4,6,17,23] Carson et al.[5] described a mild diminution of glutathione reductase activity in red blood cells. In 1962 Löhr and Waller[14] reported on glutathione reductase deficiency combined with nonspherocytic hemolytic anemia. Fornaini et al.,[8] Punt et al.,[16] and Brittinger et al.[3] described more single cases of glutathione reductase deficiency. We[25] reported on patients with pancytopenia, nonspherocytic hemolytic anemia, or thrombopenia, who simultaneously presented neurological disorders. Red cells of these patients showed a decrease in glutathione reductase activity, and the enzyme was found to have an elevated

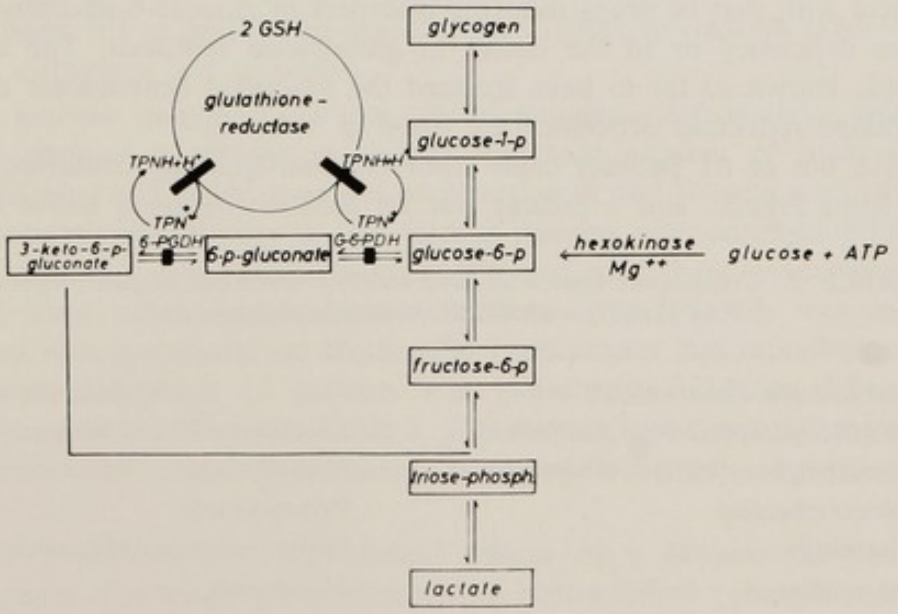


FIG. 1. Scheme of glutathione reduction showing possible sites of enzymatic blocks.

TABLE 1. *Diseases correlated with GSSG reductase deficiency (total 61 cases)*

Diagnosis	Number of patients	Drug exposure unknown	After drug exposure
Pancytopenia	11	3	8
NSHA and thrombopenia	10	8	2
NSHA and leucopenia	3	2	1
NSHA	18	15	3
Thrombopenia	2	2	
Leukemia (acute)	1		
Erythroleukemia	1		
Hemophilia B	1		
Non-hematological disorders	14		

Michaelis constant for GSSG and a shift of the pH optimum to a higher hydrogen ion concentration.

Clinical Findings

Sixty-one carriers of glutathione reductase deficiency in blood cells are listed in Table 1. Fourteen of these had no hematologic disorders at the time of examination. In 3 other patients with leukemia, erythroleukemia, and hemophilia B, no causative connection with glutathione reductase deficiency could be established. In 14 out of 44 patients with various hematologic disorders (nonspherocytic hemolytic anemia with leucopenia or thrombopenia, pancytopenia, or isolated thrombopenia), the manifestation of the disease or the increase of hematologic alterations was shown to be in direct connection with the administration of drug or with the exposure to nitrolacker or thallium. It is of interest that 8 of 11 patients with pancytopenia have been exposed to drugs.

The chemical composition of the drugs that are known to induce hemolytic anemia or pancytopenia on the basis of glutathione reductase deficiency is similar or identical with that of drugs inducing disorders in glucose-6-phosphate dehydrogenase deficiency or in the defect in glutathione synthesis. The chemical compounds known so far to have induced the described hematologic disorders in glutathione reductase deficiency are listed in Table 2.

Fifty-five out of 61 patients came from German-speaking countries, 4 from Italy, 1 from France, and 1 patient was an Indian woman, a native of Peru.

TABLE 2. *Compounds known to have induced hemolysis or pancytopenia in patients with GSSG reductase deficiency*

Azathioprine (Imuran®)?	Acetylsalicylic acid
Salicylazosulfapyridine (Azulfadine®)	Acetophenetidin (Phenacetin®)
Diaminodiphenylsulfone (Dapsone®)	Phenylbutazone (Butazolidin®)
Chloramphenicol	Pyramidone®
Primaquine	Phenprocoumon (Marcumar®)
Chloroquine	Nitrolacker
	Thallium

The frequency of enzymatic deficiency was the same in both sexes. The clinical manifestation of the disease showed a spontaneous onset in most of the patients at ages ranging from early infancy to more than 50 years. Very recently we have also found glutathione reductase deficiency combined with hemoglobin C in a Negro from Ghana.

Hematologic Findings

The patients suffered from normo- to macrocytic nonspherocytic hemolytic anemia. The anemia was often combined with thrombo- or granulocytopenia. One-fourth of the patients developed pancytopenia after drug exposure. Roughly half of the patients showed mild icterus with slight elevation of the indirect bilirubin content of the serum. The liver and spleen were frequently enlarged. Hemorrhagic diatheses were present in patients with more marked thrombopenia. Heinz bodies could be demonstrated in erythrocytes of patients with hemolysis after drug exposure. Reticuloycyte counts were usually slightly elevated; in a few cases there was well-marked reticulocytosis. The osmotic resistance was usually normal or only slightly decreased. Autohemolysis was increased in the few cases examined and could not be corrected by addition of glucose (Dacie type II). Immunologic tests—for instance, the Coombs' test, tests for L.E. factor, or cold agglutinins—gave normal results in all of the patients examined. The serum iron was increased in the majority of patients studied, whereas the haptoglobin concentration in half of the patients was sharply depressed. In the others, the haptoglobin level was normal or even increased. The half-time survival of red cells of 9 patients, determined by the chromium-51 method, was found to be between 7 and 22 days. The lag period between introduction of the drugs and manifestation of the hemolytic signs could not be determined accurately because the patients were admitted only after drug had already been taken for some period of time. In one patient with ulcerative colitis, hemolysis became manifest within a few days after administration of Azulfadine®. Some patients showed an improvement of hematologic symptoms after discontinuation of the drugs. In other patients, no remission of hemolysis occurred, and at last, pancytopenia led to death. The causes of this failure to respond favorably when drug is withdrawn are unknown.

Bone marrow smears of the majority of patients—also in those with pancytopenia—showed a normal or slightly increased number of nucleated cells. Only one-third had lower counts of nucleated cells and showed a transformation of the bone marrow similar to that seen in patients with panmyelophthisis (exhausted marrow). Erythropoiesis was increased, and there were more juvenile cells. In some cases, megaloblasts were seen. Granulopoiesis was only rarely depressed with a concomitant increase in lymphocytes and reticulum cells. The hematologic data from 17 patients with glutathione reductase deficiency and blood disorders are shown in Table 3. The hematologic course of three patients with pancytopenia and glutathione reductase deficiency is represented in Figures 2 to 4.

Figure 2 demonstrates the clinical course of a 60-year-old patient with pancytopenia after long-term treatment with Butazolidin® (phenylbutazone) and Pyramidone® for ischialgia. After discontinuation of the drugs, there was a

TABLE 3. *Hematologic studies on 17 patients with glutathione reductase deficiency*

Name	Age (yrs.)	Number of cells (10^3/mm^3)	Bone marrow r/w* (%)	gr./w† (%)	Reticulocytes (%)	RBC (10^6/mm^3)	WBC (10^3/mm^3)	Platelets (10^3/mm^3)	Cr^{51} ($t_{\frac{1}{2}}$)	Bilirubin (mg %)	Haptoglobin (mg %)
				Pancytopenia							
Ha. Kel.	27	97	60	65	1.0	1.5	1.7	13	—	0.5	251
Pa. Scho.	60	76	143	50	2.7	2.5	1.3	42	—	0.8	11
Wi. Pet.	58	—	150	88	4.6	3.0	2.0	15	8.5	1.2	—
Ka. Rei.	18	211	137	86	23.0	2.1	3.9	99	7.0	1.7	0
Ge. Sie.	69	29	18	20	5.1	1.8	4.3	62	16.4	0.9	0
Ma. Gör.	65	9	20	10	0.4	1.5	2.7	11	—	0.8	425
Ma. Bu.	53	8	47	57	1.8	1.9	1.8	23	—	0.4	104
				NSHA and thrombocytopenia							
Er. Gra.	57	238	95	98	9.8	2.5	8.3	12	17.2	0.8	108
Lo. Bo.	25	81	60	84	2.6	2.1	7.5	61	21.0	3.0	43
El. Ro.	21	35	42	86	1.4	4.8	10.0	17	9.0	—	51
Ru. Mül.	30	48	16	79	4.0	2.1	6.0	44	—	2.5	15
				NSHA and leucocytopenia							
Be. Ma.	44	250	108	99	9.8	2.4	3.3	160	22.0	5.0	11
M. Ost.	50	—	—	—	3.5	3.9	3.5	—	—	0.7	0
A. Mai.	49	101	52	83	1.4	3.4	3.7	107	—	—	158
				NSHA							
K.H. Mül.	18	302	263	62	33.0	2.5	5.5	131	10.0	3.2	6
Ru. Schi.	30	105	33	82	1.9	3.1	9.6	293	—	1.7	6
H. Flät.	34	343	68	99	2.6	5.0	6.2	—	20.0	—	70

*Ratio of red to white cells.
†Granulocytes/white blood cells.

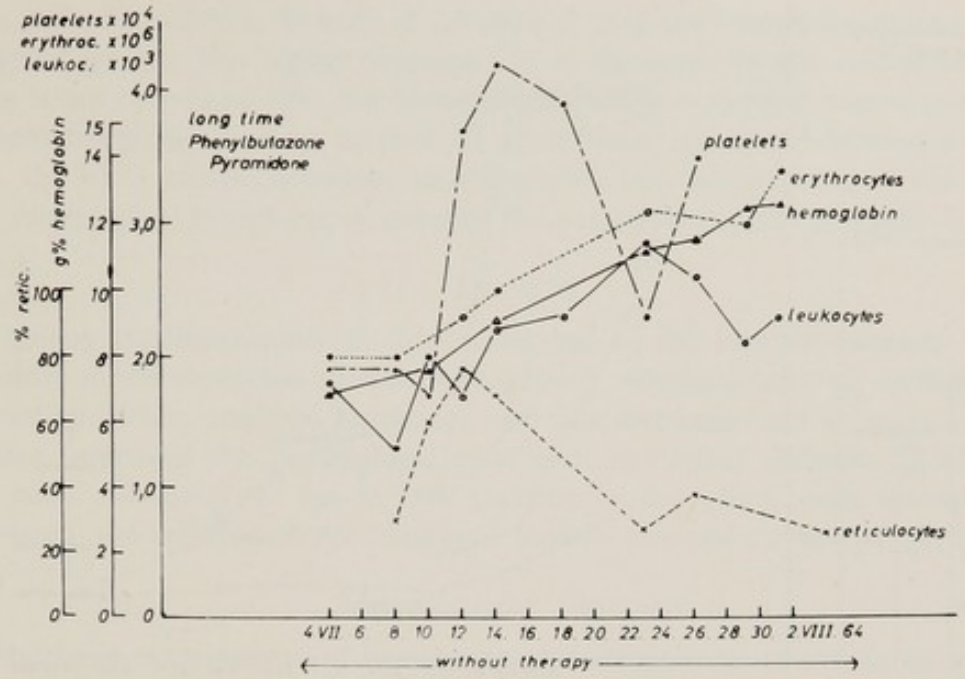


FIG. 2. Clinical course of drug-induced pancytopenia (Pat. Pa. Scho.).

partial remission of the hematologic symptoms. Within one year, the patient took similar drugs again and died subsequently of acute severe pancytopenia.

The hematologic course of an 18-year-old-patient is given in Figure 3. This patient developed pancytopenia after inhalation of nitrolacker (sprayed furniture without mask). The clinical syndrome did not improve after a change of employment. Treatment with corticosteroids in medium doses and anabolic

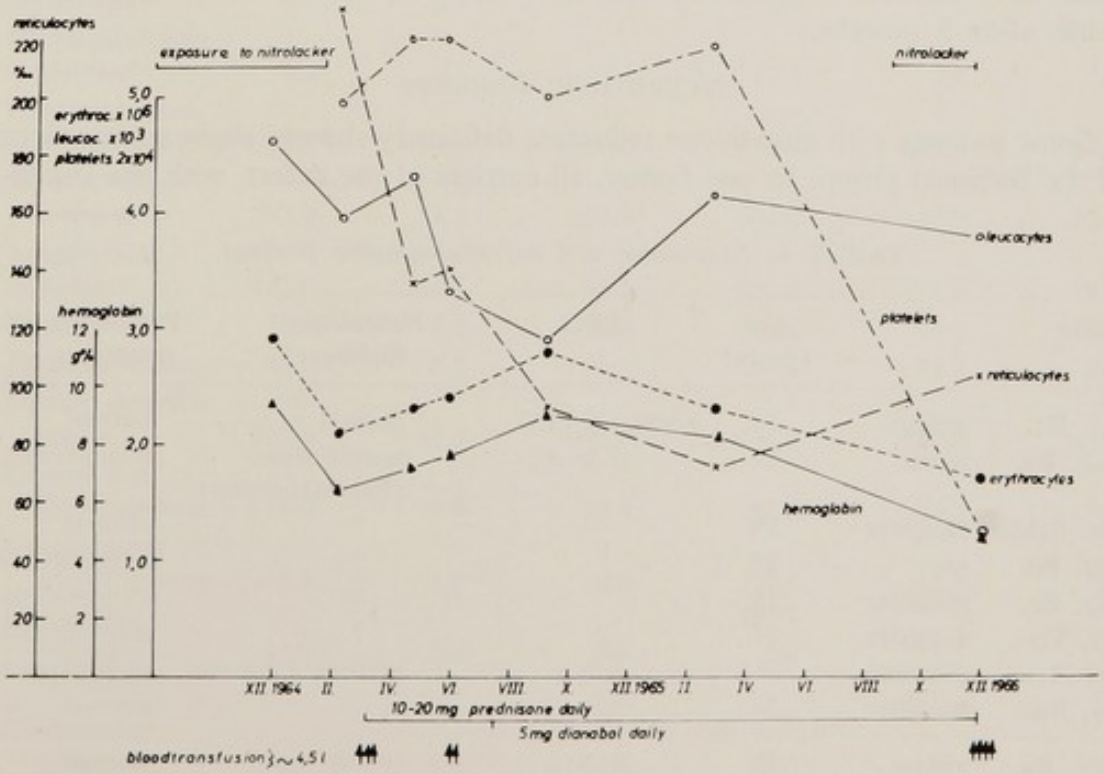


FIG. 3. Clinical course of drug-induced pancytopenia (Pat. Ka. Rei.).

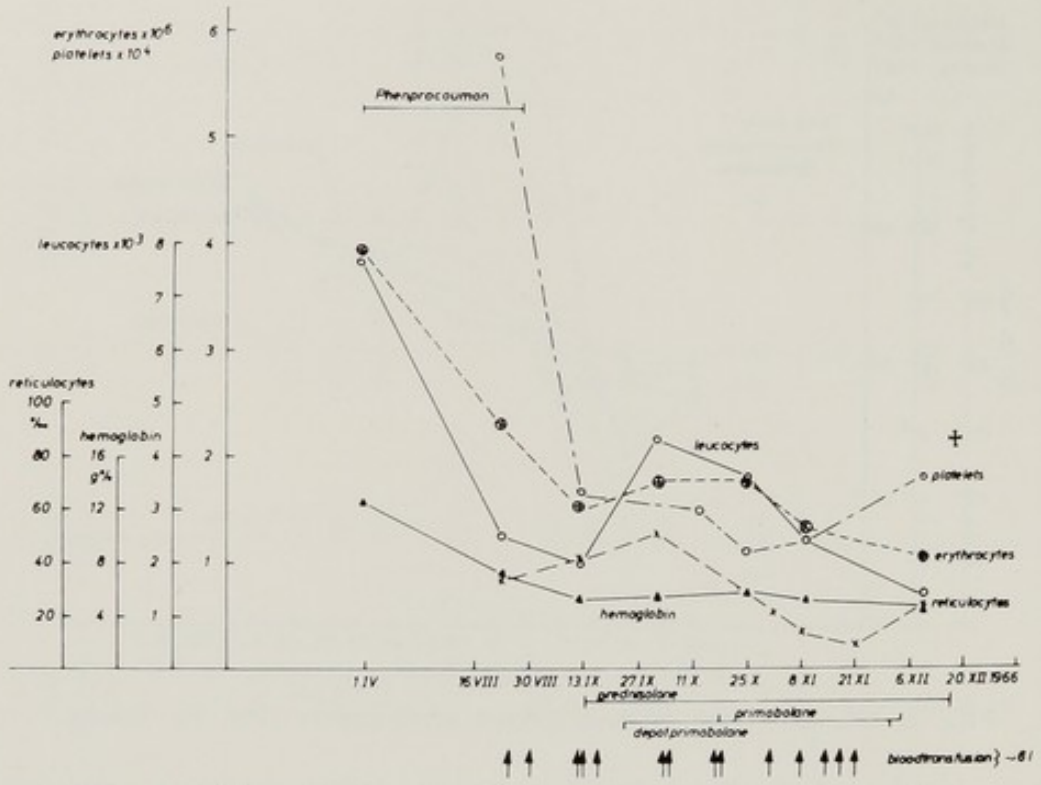


FIG. 4. Clinical course of drug-induced pancytopenia (Pat. Ge. Sie.).

hormone and blood transfusion did not result in long-term improvement. After renewed exposure to nitrolacker, the boy developed a severe recurrence. Figure 4 shows hematologic data for a 69-year-old woman who developed pancytopenia after treatment with Marcumar® (phenprocoumon) for suspected coronary infarction. The severe pancytopenia was refractory to treatment and led to death after 5 months.

Neurologic Findings

Some patients with glutathione reductase deficiency showed slight spastic signs of the Babinski group. In one family, all carriers of the defect, with one excep-

TABLE 4. *Neurologic and encephalographic findings*

Name		Age (years)	EEG	Neurologic findings	Psychiatric symptoms
Fri. Bo.	mother	50	normal α-EEG	normal	normal
Eug. Bo.	father	59	$\theta > \alpha$	spastic signs (Babinski-group)	oligophrenia
Lie. Schla.	daughter	29	"	"	normal
Hel. Bo.	son	28	"	"	oligophrenia
Lor. Bo.	daughter	25	"	"	oligophrenia
Elf. Trei.	daughter	27	?	?	oligophrenia
Rol. Trei.	grandson	4	$\theta > \alpha$	periodic catatonia	oligophrenia
Bri. Bo.	daughter	24	?		normal
Mar. Bu.	widow	53	$\theta > \alpha$	spastic signs	normal
Pau. Scho.	husband	60	"	"	normal

tion, had oligophrenia. Twenty of 22 patients had pathologically-altered electroencephalograms. The higher frequency of theta-waves in the electroencephalogram is not correlated with the hematologic disorders, since it was also observed in hematologically-normal carriers of glutathione reductase deficiency. Therefore, the EEG alteration cannot be interpreted as a consequence of the anemia. The neurological symptoms of some of the patients are given in Table 4.

Therapy

Therapy consists mainly in discontinuation of the causative agents. Further exposure to the chemicals should be prevented. Administration of corticosteroids in medium doses, anabolic hormones, and blood transfusions in cases of severe anemia improved the hematologic picture in individual patients. Splenectomy has been successful so far in one patient; in the other, only thrombopenia improved; the nonspherocytic hemolytic anemia was not influenced significantly.

Morphologic Findings

Histologic examinations of liver biopsy specimens from two patients and from one post-mortem examination permit the diagnosis of hemosiderosis. The patient Ge. Sie. also had hemosiderosis of the spleen and of the pancreas. Her verte-

TABLE 5. *Activities of enzymes in red cells of patients with pancytopenia or NSHA and hereditary glutathione reductase deficiency (IU per 10^{11} erythrocytes, TRA buffer pH 7.5 containing EDTA, 25° C)*

Enzymes	Normals	±	Lor. Bo.	Pau. Scho.	Wil. Pet.	Mar. Bu.
Hexokinase	1.7	0.3	2.7	2.8	3.0	1.6
Diphosphofructo-aldolase	9.0	0.6	17.1	19.5	7.4	10.2
Glyceraldehyde-3-p dehydrg.	212.0	31.2	278.0	570.0	286.0	398.0
3-p-glycerate-1-kinase	297.0	33.2	352.0	600.0	650.0	398.0
3-p-glycerate mutase	102.0	19.1	94.2	362.0	111.0	95.3
Enolase	27.0	4.6	29.8	38.8	43.1	30.6
Pyruvate kinase	45.0	6.5	56.3	40.6	32.4	43.2
Lactate dehydrogenase	367.0	62.5	476.0	725.0	280.0	371.0
Glucose-6-p dehydrogenase	18.0	3.6	33.4	33.0	29.2	21.5
6-p-gluconate dehydrogenase	5.9	1.0	5.3	11.9	17.8	8.2
Glutathione reductase (NADPH)	11.7	2.3	5.4	5.4	5.9	5.2
Glutathione reductase (NADH)	1.3	0.4	1.9	0	—	1.1

bral bone marrow was transformed to adipose tissue, marrow from the femur had numerous nucleated cells,[2] revealing active erythroporesis.

Biochemical Findings

Enzymes

The determination of the activities of glycolytic enzymes, the hexose monophosphate pathway, and glutathione reductase demonstrated only a decrease in glutathione reductase activity. The average value of the spectrophotometric method of Racker[17] was 5.9 IU (range 1.0 to 8.7 IU) per 10^{11} erythrocytes in 61 patients. This is half of the normal value. Most of the other enzyme activities that were measured were found to be increased. This may be due to the population of younger cells as demonstrated by 4 patients in Table 5.

Metabolism

The glycolytic flow-rate and concentration of energy-rich phosphates were normal or increased according to the normal or increased activities of glycolytic enzymes. Cations and methemoglobin concentration were also normal. The results are shown in Table 6. GSH concentration was normal or increased in erythrocytes of patients with pancytopenia or NSHA; GSH stability was also normal or slightly decreased, whereas the Heinz body formation in the Beutler-test was highly increased. The average concentration of GSH was 18.6 micromoles (range 4.8 to 35.0) per 10^{11} erythrocytes in the 53 patients that were examined. The GSH stability of 46 carriers of the defect averaged 24.1 per cent (range 0 to 92 per cent). The Heinz body formation test was abnormal in all cases.

Methemoglobin Reduction

The velocity of methemoglobin reduction in nitrited red blood cells of the

TABLE 6. *Biochemical findings in erythrocytes of patients with glutathione reductase deficiency (micromoles per 10^{11} cells, K^+ and Na^+ in mEq. per L erythrocytes)*

	Normals	±	Lor. Bo.	Pau. Scho.	Wil. Pet.	Mar. Bu.
ATP	19.6	2.1	24.4	19.5	20.3	23.7
ADP	1.9	0.2	3.5	—	2.8	—
AMP	0.8	0.1	1.0	—	3.0	—
Lactate formation/h	36.2	8.2	67.0	49.8	—	26.8
Pyruvate formation/h	1.9	0.4	0.4	2.4	—	4.3
K^+	92.5	4.2	90.0	99.5	—	94.0
Na^+	19.0	4.2	24.0	15.0	—	20.0
Methemoglobin %	< 1		< 1	< 1		< 1
GSH	21.0	3.3	22.9	28.0	18.1	27.5
GSH stability (% decreased)	< 25.0		33.0	24.0	5.0	31.0
Heinz body test	+		+++	+++	+++	+++

patient, Kä. Los, was normal after the addition of glucose and lactate. Using glucose as substrate, 20.6 microequivalents methemoglobin were reduced per hour per 10^{11} erythrocytes (normal 23.1 $\pm$ 8.7); using lactate as substrate, 27.7 microequivalents were reduced per hour per 10^{11} erythrocytes (normal 24.7 $\pm$ 7.4). After addition of toluidine blue to a concentration of 5 x 10^{-4} M, methemoglobin reduction increased to two or three times that of the controls in the presence of glucose; whereas in the presence of lactate, the increase of methemoglobin reduction increased to two or three times that of the controls in the presence of glucose; whereas in the presence of lactate, the increase of methemoglobin reduction was smaller than that in the controls. Using glucose plus toluidine blue as substrate, 542.0 microequivalents were reduced per hour per 10^{11} erythrocytes (normal 204.8 $\pm$ 77.9). Using lactate plus toluidine blue, 67.5 microequivalents were reduced (normal 113.1 $\pm$ 31.4) (Fig. 5). It is possible that in glutathione reductase-deficient cells more NADPH is available for methemoglobin reductase. The ratio of NADPH to NADP was found to be 2 to 3 times higher than in normal cells.

Experiments with Red Cell Stroma

It was possible that the decreased glutathione reductase activity in red cells of our patients was due to lack of a stromal factor or formation of an inhibitor. Therefore, we measured the glutathione reductase activity in digitonin hemolysates of normal individuals with stromata of a patient (Mar. Bu.), and vice versa with the appropriate controls. We found no evidence for the absence of an activator in the stroma nor for the presence of an inhibitor (Table 7).

Properties of Glutathione Reductase

A decrease in enzyme activity can be the result of a reduced concentration of a normal active enzyme protein or can be caused by an altered enzyme protein as is known to be the case, for instance, in polymorphism of glucose-6-phosphate dehydrogenase. Therefore, the pH optimum of glutathione reductase reaction was sought, and Michaelis constants of GSSG and NADPH in lysates of ery-

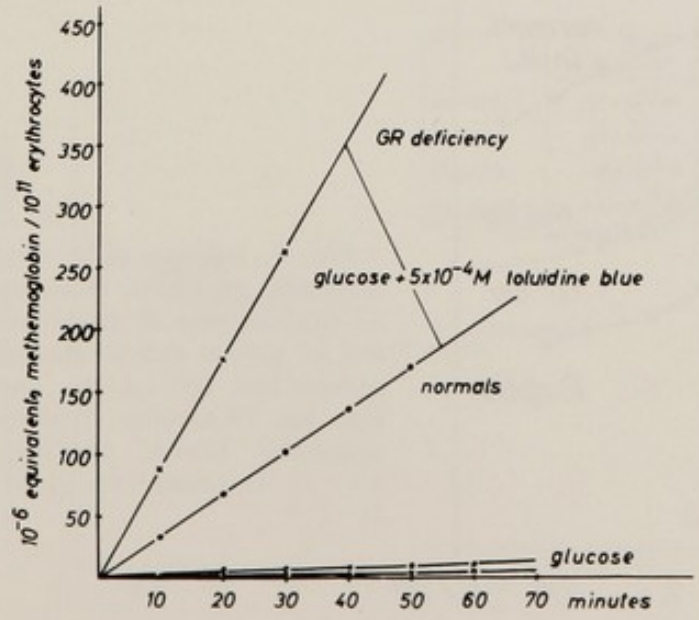


FIG. 5. Methemoglobin reduction in nitrited erythrocytes of normal individuals and patients with GSSG reductase deficiency. Determination of carbon monoxide uptake in phosphate ringer solution pH 7.4 at 37° C[11].

TABLE 7. *Activities of glutathione reductase of digitonin hemolysates after incubation with stromata of erythrocytes*

Stromata	Hemolysate (normals)	Hemolysate (pat. Mar. Bu.)
	Stromata of normal individuals	
without	10.0	5.6
0.15 ml	10.8	5.4
0.30 ml	8.3	5.1
0.45 ml	8.7	4.9
0.60 ml	—	4.5
	Stromata of pat. Mar. Bu.	
without	10.6	
0.15 ml	11.4	
0.30 ml	9.9	
0.45 ml	8.9	
0.60 ml	8.6	

throcytes, leucocytes, or platelets were determined. For convenience, the data for only a few patients are given in Figures 6 to 8. In all types of blood cells the pH optimum for glutathione reductase reaction was shifted regularly to higher hydrogen ion concentration from pH 6.8 for normal subjects to pH 6.4 in defect carriers. The Michaelis constant for NADPH was normal, the half-saturation constant, however, for GSSG was increased two- to three-fold above normal values in digitonin-lysed red cells and platelets. These data are given in Table 8.

On the basis of these findings one can conclude that in glutathione reductase deficiency, the enzyme protein is altered. Only the glutathione reductase of

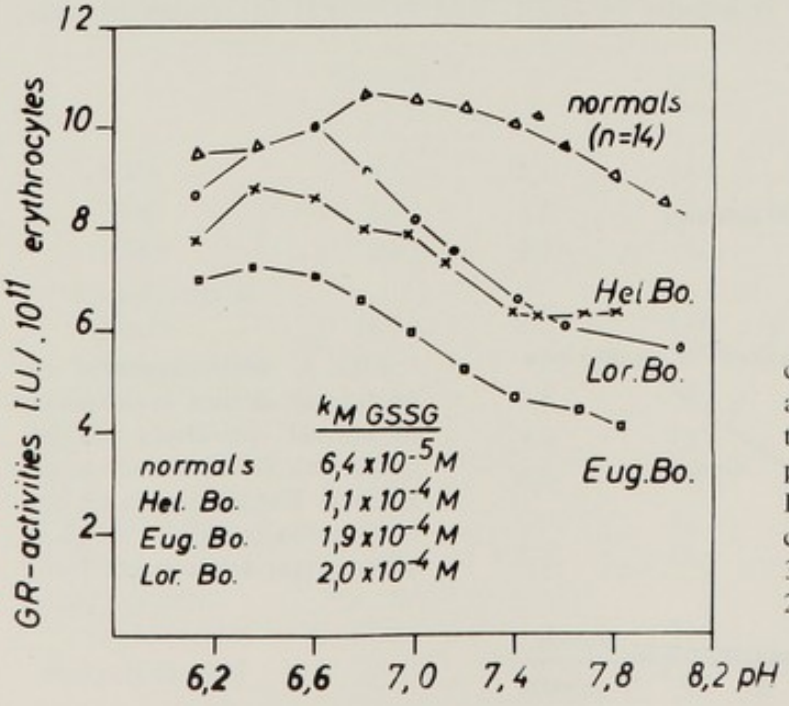


FIG. 6. Influence of H^+ concentration on GSSG reductase activity (enzyme of erythrocytes) of normal individuals and patients Hel. Bo., Lo. Bo., and Eug. Bo. TRA-buffer (0.05M) containing EDTA, NADPH. 3.3 x 10^{-4}M, GSSG 6.5 x 10^{-3}M, 25° C.[25]

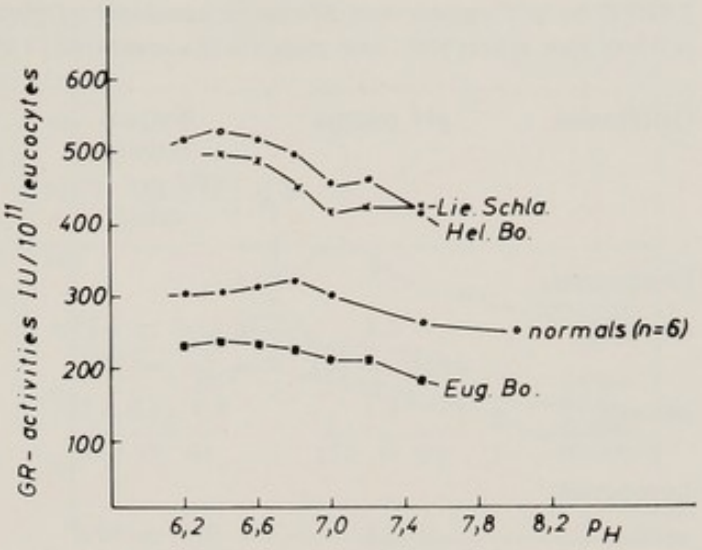
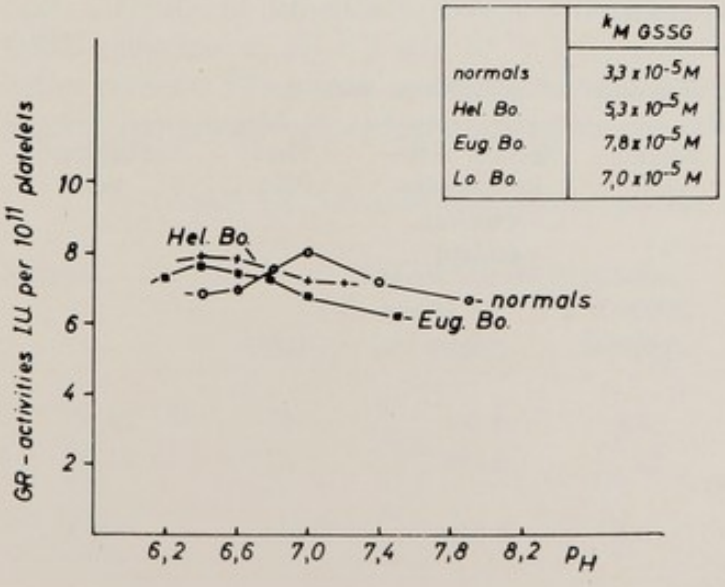


FIG. 7. Influence of H^+ concentration on GSSG reductase activity (enzyme of leucocytes) of normal individuals and patients Hel. Bo., Eug. Bo., and Lie. Schla. TRA-buffer (0.05M) containing EDTA, NADPH 3.3 x 10^{-4}M, GSSG 6.5 x 10^{-3}M, 25° C.[25]

the Peruvian Indian woman (Se. Mon.), who was admitted to our hospital with gastroduodenitis, had a normal pH optimum and a normal Michaelis constant for GSSG. The total activity of the enzyme was only 4.1 IU per 10^{11} erythrocytes. We purified the enzyme of normal and glutathione-reductase-deficient carriers to exclude secondary factors that could change the physicochemical behavior of the enzyme in the crude preparation.

Purification of Red Cell Glutathione Reductase

Eighty ml of citrated blood were obtained by venipuncture. The erythrocytes were washed three times with physiological saline. The pellet of red cells was lysed by addition of four volumes of ice-cold distilled water with 0.1 per cent β-mercaptoethanol and 4.5 x 10^{-4} EDTA. Subsequently, the solution was brought to pH 6.0 with N HCl, and the cell stroma were removed by centrifugation at 4° C. The proteins were precipitated with $(NH_4)_2SO_4$ at 50 per cent saturation. The solution was kept at 4° C overnight, and the sediment was separated by centrifugation and redissolved in distilled water with β-mercaptoethanol and EDTA. After partial removal of salt ions on a



	k_M GSSG
normals	$3{,}3 \times 10^{-5}$ M
Hel. Bo.	$5{,}3 \times 10^{-5}$ M
Eug. Bo.	$7{,}8 \times 10^{-5}$ M
Lo. Bo.	$7{,}0 \times 10^{-5}$ M

FIG. 8. Influence of H^+ concentration on GSSG reductase activity (enzyme of platelets) of normal individuals and patients Hel. Bo. and Eug. Bo. TRA-buffer (0.05M) containing EDTA, NADPH 3.3 x 10^{-4}M, GSSG 6.5 x 10^{-3}M, 25°C.[25]

TABLE 8. *pH optima and Michaelis constants of glutathione reductase in cytolysates of erythrocytes, leucocytes, and platelets (k_M measured in triethanolamine buffer pH 7.5, 25° C)*

Cytolysates	pH optima	Enzyme activity (IU per 10^{11} cells)	k_M GSSG (μM)	k_M NADPH (μM)
Erythrocytes				
normal	6.8 (n* = 14)	11.7 ± 2.3† (n = 50)	64 (n = 14)	16 (n = 14)
patients	6.4 (n = 27)	5.9 (1.0-8.7) (n = 61)	130 (n = 13)	18 (n = 4)
Leucocytes				
normal	6.8 (n = 6)	299 ± 19.0 (n = 6)	41 (n = 6)	14 (n = 6)
patients	6.35 (n = 9)	297 (126-418) (n = 9)	66 (n = 2)	—
Platelets				
normal	7.0 (n = 8)	8.2 ± 1.1 (n = 8)	33 (n = 8)	17 (n = 8)
patients	6.4 (n = 9)	9.2 (3.7-18.3) (n = 9)	67 (n = 3)	

*Number of samples examined. †Standard deviation.

Sephadex G 25 column, the eluate was passed over a CM-cellulose column in a 0.01 M phosphate buffer, pH 6.0. The enzyme was eluted from the column with 0.05 M phosphate buffer, pH 6.4. The highest purification that was obtained with this method was 2000-fold. For determinations of pH optima and Michaelis constants, a large volume of enzyme solution was needed, and therefore 400- to 800-fold purified preparations were used. A scheme of the preparation is presented in Table 9. The final enzyme preparations had a protein concentration of 0.1 to 0.3 mg per ml and contained no hemoglobin. Some preparations had a faint yellow color.

TABLE 9. *Purification of glutathione reductase*

Fraction and treatment	Volume (ml)	Specific activity (μmoles /min/mg. protein)	Yield (%)	Purification
1. Destromatized hemolysates (pH 6.0)	250	0.0063	100	—
2. $(NH_4)_2 SO_4$ 0.5 saturated	8.8	0.184	67	29 x
3. Sephadex G 25	22	0.194	57	31 x
4. CM-cellulose (pH 6.0) (elution pH 6.4)	10.6	3.800	7.6	604 x

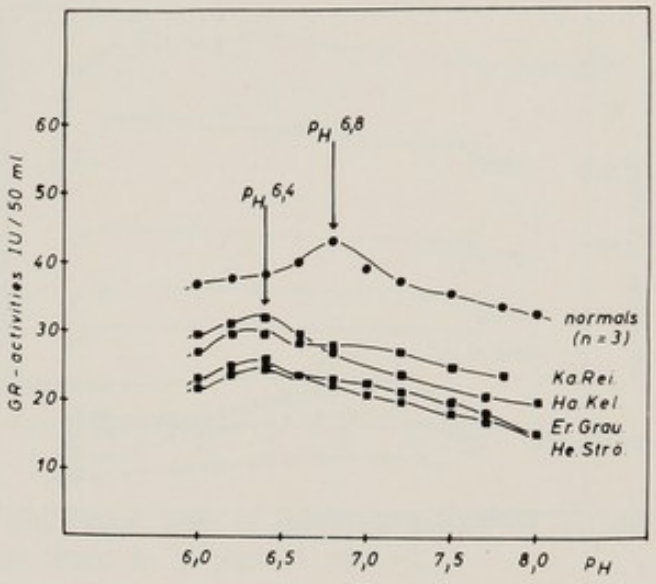


FIG. 9. Influence of H^+ concentration on glutathione reductase activity (purified enzyme of erythrocytes) of normal individuals and patients with glutathione reductase deficiency. Triethanolamine buffer (0.1M) containing EDTA, NADPH 3.5×10^{-4} M, GSSG 2.5×10^{-3} M, 25° C.

NADPH-GSSG Reductase: The pH optimum was determined in 0.1 M triethanolamine buffer with 4.5×10^{-4} M EDTA at 25° C. The pH optimum of the purified enzyme of normal individuals was 6.8, and of defect-carriers it was 6.4. The Michaelis constants for GSSG and NADPH were slightly smaller in purified preparations than in crude normal preparations. In purified as well as in crude preparations from glutathione reductase-deficient red cells, however, the Michaelis constant of GSSG was 2 to 3 times higher than that in normal cells. The Michaelis constant for NADPH was not changed. (Fig. 9 and Table 10).

NADH-GSSG Reductase: The pH optimum for NADH-GSSG reductase was found in purified enzyme preparations of normal cells to be 6.3. In three carriers of the defect it was shifted to pH 6.6, whereas in one patient there was no alteration. Michaelis constants for GSSG and NADH were not changed significantly. The data are given in Fig. 10 and Table 10.

The ratio of NADPH to NADH-dependent glutathione reductase activities, as assayed at pH 7.5 and 25° C, was 8:1 in purified preparations of normal cells and 10.4: 1 in purified preparations of glutathione-reductase-deficient carriers. The ratio of activities for NADPH-GSSG reductase at pH 6.8 and NADH-GSSG reductase at pH 6.3 was 10.7: 1 for normal individuals and 12.2: 1 for defect carriers. There seem to be no clear-cut differences of these ratios between normal and glutathione reductase-deficient red cells.

TABLE 10. *Michaelis constants of purified glutathione reductase*

Subjects	NADPH-GSSG reductase		NADH-GSSG reductase	
	K_M GSSG (μM)	K_M NADPH (μM)	K_M GSSG (μM)	K_M NADH (μM)
Normal (n=4)	42	11	48	270
Ha. Kel	96	10	45	250
Ka. Rei.	87	9	—	270
He. Stro.	97	10	270	400
Er. Grau.	84	8	36	400

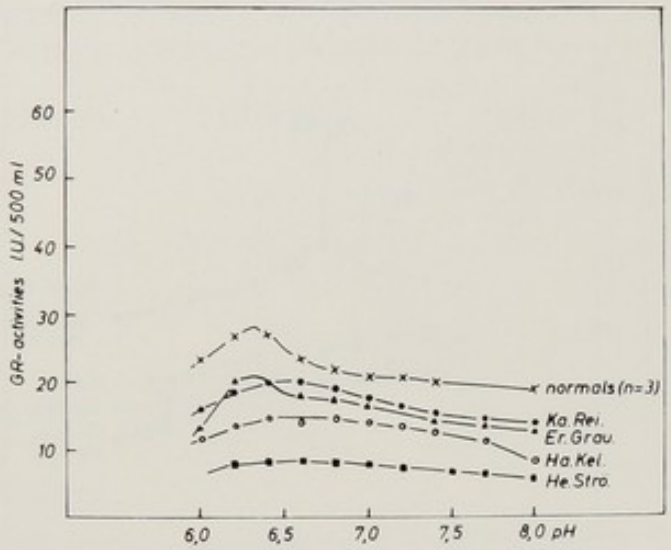


FIG. 10. Influence of H^+ concentration on glutathione reductase activity (purified enzyme of erythrocytes) of normal individuals and patients with glutathione reductase deficiency. Triethanolamine buffer (0.1M) containing EDTA, NADH 4.2 x 10^{-4} M, GSSG 2.5 x 10^{-3} M, 25° C.

Effect of GSSG Concentration on Glutathione Reductase Activity: Glutathione reductase activity reached its maximal velocity in normal individuals at 1.3 x 10^{-3} M GSSG and in defect carriers at 4 x 10^{-4} M GSSG. Similarly, substrate inhibition occurred at lower GSSG concentrations for the enzyme of defect carriers (Fig. 11).

In view of the differences in the pH optimum, of the half-saturation constants for GSSG, and the lower GSSG concentration for the substrate inhibition, it is concluded that an altered enzyme protein is involved in glutathione reductase deficiency. An underlying polymorphism of glutathione reductase with regard to the increased number of defect carriers is a possibility. There may be two different enzymes responsible for GSSG reduction, one dependent on NADPH

TABLE 11. *Michaelis constants and pH optima for GSSG reductase activity (NADPH-GSSG reductase of erythrocytes)*

Authors	Assay conditions	K_M GSSG (μM)	K_M NADPH (μM)	pH optima
	purified enzyme			
Scott et al.[23]	phosphate buffer pH 6.8, 30° C	96	16	6.75
Beutler and Yeh[3]	phosphate buffer pH 7.0	25	.066*	6.7-7.4
Eifler and Wagenknecht[7]	phosphate buffer pH 6.4, 37° C	25	13	6.4
Waller	triethanolamine buffer pH 7.5, 25° C	42	11	6.8
	hemolysates			
Waller, Löhr et al.[25]	triethanolamine buffer pH 7.5, 25° C	64	16	6.8
Löffler et al.[12]	phosphate buffer pH 6.6, 25	24	10	6.4

*See discussion p. 207.

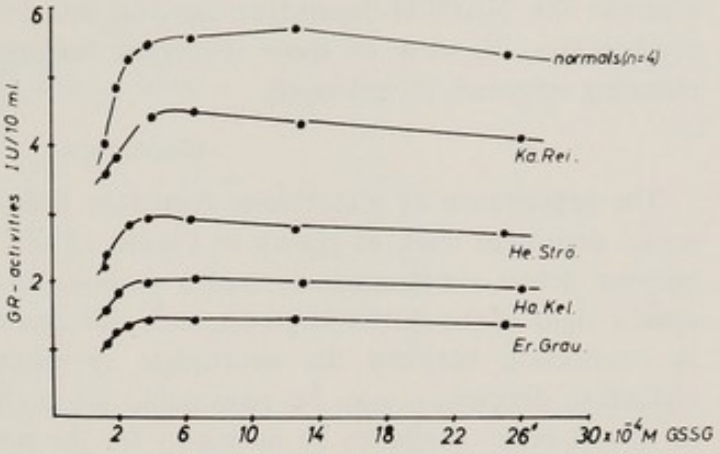


FIG. 11. Influence of GSSG concentration on glutathione reductase activity (purified enzyme of erythrocytes). Triethanolamine buffer (0.05M) pH 7.5 containing EDTA, NADPH 4.2×10^{-4} M, 25° C.

and the other on NADH. The published data of other investigators for the both GSSG reductase reactions are listed in Tables 11 and 12.

The data in Tables 11 and 12 are not completely comparable because of differences in assay conditions. Nevertheless, the Michaelis constants are in a close range, with one exception[1] for K_m NADPH and K_m NADH Löffler et al.[12] and Eifler and Wagenknecht[7] have found the pH optimum for NADPH-GSSG reductase to be 6.4; the majority of authors have found it to be pH 6.8. The pH optimum for NADH-GSSG reductase was commonly observed to be between 6.1 and 6.3. We agree with Beutler and Scott and their collaborators[1,23] that we probably are dealing with one enzyme that can react with both coenzymes since the ratios of both activities were the same in purified preparations of normal and deficient cells.

Eifler and Wagenknecht[7] proposed two different enzymes because by purification they could separate the NADH-GSSG reductase from NADPH-dependent enzyme. Rapoport[19] found no NADH-dependent enzyme in rabbit reticulocytes,

TABLE 12. *Michaelis constants and pH optima for GSSG reductase activity (NADH-GSSG reductase of erythrocytes)*

Authors	Assay conditions	K_M GSSG (μM)	K_M NADH (μM)	pH optima
	purified enzyme			
Scott et al.[23]	phosphate buffer pH 6.2, 30° C	17	190	6.2
Beutler and Yeh[1]	phosphate buffer pH 7.0	18	1.5*	—
Eifler and Wagenknecht[7]	phosphate buffer pH 6.1, 37° C	25	170	6.1
Waller	triethanolamine pH 7.5, 25° C	48	270	6.3
	hemolysate			
Löffler et al.[12]	phosphate buffer pH 6.2, 25° C	22	310	6.2

*See discussion on p. 207.

whereas the NADPH-dependent enzyme was four times as high as in rabbit erythrocytes. In view of these divergent findings, the question of two GSSG reducing enzymes is still open.

Genetics

The inheritance of glutathione reductase deficiency seems to be as an autosomal dominant trait, as shown in Figure 12 for one family. All carriers of the enzyme defect simultaneously had a pathologic electroencephalogram (EEG), spastic signs of the Babinski group, and, with one exception (II/2), oligophrenia. A connection between the neurologic symptoms and a general glutathione reductase deficiency may be reasonable since the high concentration of GSH in neural cells[18] seems to be necessary for the normal function of these cells.

In cases 1 to 4 in the third generation of the pedigree, we have not determined the properties of glutathione reductase. Therefore, it is impossible to say that there were no carriers of the enzyme defect. Further examinations may clarify this point.

Chromosomal Analysis

Schroeder[22] in Heidelberg has done chromosomal analyses of leucocyte cultures of 6 patients with glutathione reductase deficiency and hematologic disorders, using the method of Moorhead et al.[15] The results are given in Table 13. Five to seventeen per cent of mitotic figures in cell cultures of patients with pancytopenia showed single chromosomal breakage and achromatic regions. Patients with nonspherocytic hemolytic anemia exhibited no significant increase of breakage frequency. Endoreduplications and polyploid cells were rarely seen.

Cytologic examinations of bone marrow revealed a 15- to 60-fold atypical increase in cell nuclei, which Schroeder interpreted as originating from mitotic disorders. It seems conceivable that some of these cells die in the bone marrow.

The frequency of chromosomal breakage is considerably higher than in panmyelopathies of unknown etiology.[9,21,22] Also, the high frequency of the atypical nuclei was not observed in idiopathic panmyelopathies or in the bone marrows

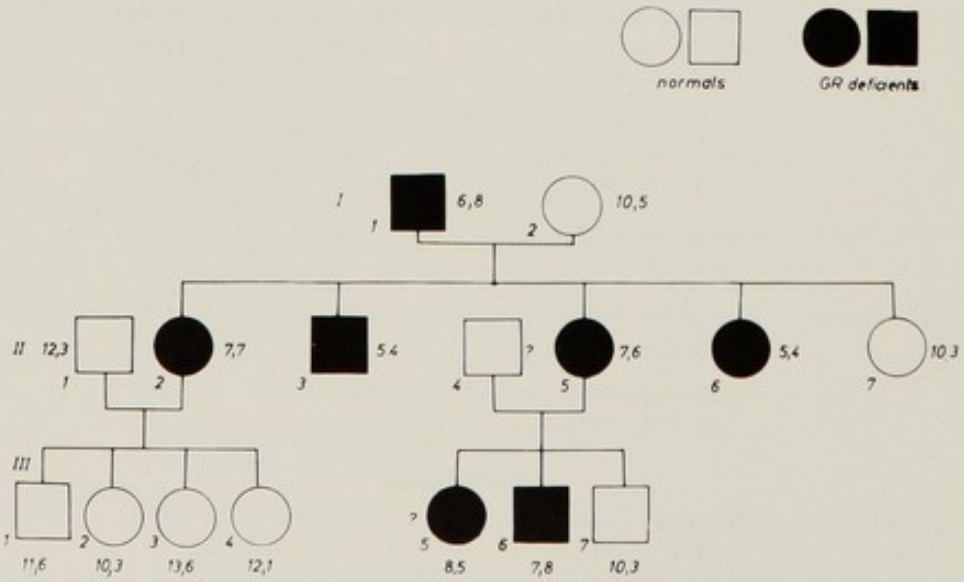


FIG. 12. Pedigree of the families Bo.-Schla.-Trei. with glutathione reductase deficiency.

TABLE 13. *Chromosomal abnormalities in cultured lymphocytes of patients with pancytopenia or NSHA and GSSG reductase deficiency*[22]

Number	Name	Diagnosis	Enzyme deficiency	Number of metaphases	Number of chromosomal aberrations	%	Achromatic regions	Chromosomal breakage
75	Mar.Bu.	pancytopenia	GR	38	5	17	1	5
107	O.K.	pancytopenia	GR	65	10	15	—	11
118	K.R.	pancytopenia	GR	57	3	5	1	4
115	H.H.	NSHA	GR	52	1	2	—	1
116	B.M.	NSHA	GR	50	—	—	—	—
205	R.Mü.	NSHA	GR	50	—	—	—	—
112	M.D.	panmyelopath.	unknown	19	3	16	3	—
119	M.F.	panmyetopath.	unknown	51	1	2	—	1

Chromosomal breakage on 1417 mitoses of patients without hematological disorders; in Heidelberg at the same time was 1.2%.

of patients with iron-deficiency anemias.[22] A connection between the observed mitotic disorders and glutathione reductase deficiency may be considered in light of the results of the work of Sakai and Dan,[20] who studied the influence of SH-groups on cell division.

What kind of mechanism correlates the enzyme defect and its metabolic consequences and the life-span of the blood cell? Jacob and Jandl[10,11] presented some evidence for the essential role of SH-groups in maintaining the structure of the cell membrane. They could demonstrate that red cells treated with small doses of p-hydroxymercuribenzoate were eliminated rapidly by the spleen after reinjection. Waller and Feine[24] have found that after incubation of red cells with 2-bromomercurihydroxopropan in a concentration of 5×10^{-5} M, the half-life of erythrocytes is shortened to 12 to 16 days as judged by the chromium-51 method. This treatment leads to a 70 to 100 per cent inhibition of glutathione reductase. The GSH concentration of glutathione reductase-deficient cells was normal, but Heinz body formation was regularly high. The shortening of the red cell life-span is therefore to be considered partly as a consequence of an impaired protection of essential SH-groups in glutathione-reductase-deficient cells. We suppose that the actual activity of glutathione reductase within the cells is lower in vivo than under the optimal assay conditions with regard to decreased substrate affinity of the enzyme. Furthermore, we have seen a further decrease of enzyme activity in the defect carrier who is being treated with drugs. The chemical structure of the hemolysis-inducing drugs is quite different, and no uniform mechanism can be proposed for being the cause of this hemolytic action. It is possible that the metabolites of these drugs interact with unprotected SH-groups, with reduced pyridine nucleotides, and with free hydrogen bondings in the presence of oxygen, the absence of which is assumed to be one of the causes leading to drug-induced hemolysis in glucose-6-phosphate-dehydrogenase-deficient cells.[13] It is by no means clear why the discontinuation of drug does not interrupt the fatal course of disease. We have some explanations for glutathione reductase deficiency and correlated hematologic and neurologic disorders, but many problems await further clarification.

Summary

A syndrome including deficiency of glutathione reductase activity in blood cells and other hematologic disorders as well as neurologic disturbances was studied in 47 patients. The spleen and liver of defect carriers were usually enlarged, and thrombopenia, nonspherocytic hemolytic anemia, with or without thrombopenia, or pancytopenia was observed. In 14 patients, most of whom had pancytopenia, manifestation of the disease or an increase in hematologic aberrations was directly related to treatment with certain drugs or to exposure to nitrolaker or thallium.

Fourteen of the total of 67 relatives of the patients who were defect-carriers had no hematologic disorders at the time of examination. Erythrocytes, leucocytes, and platelets were all deficient in glutathione reductase activity. The GSH content of erythrocytes was normal in patients with the enzyme defect. GSH stability to acetylphenylhydrazine was normal or only slightly pathologic. The Heinz body formation test was always markedly pathologic.

The K_m GSSG of crude preparations of glutathione reductase from all blood cells was two to three times higher than the value for normal individuals. The pH optimum for the NADPH-dependent enzyme reaction was shifted from 6.8 to 6.4. The K_m NADPH was the same for defect-carriers and normal persons. Red cell glutathione reductase of normal individuals and defect-carriers was purified by a new method. The differences in the physicochemical behavior of the purified enzyme from normal and defective subjects were the same as those in crude preparations. The cause of the decrease in activity of glutathione reductase cannot be attributed to an inhibitor or a deficient stroma-bound activator.

Chromosomal analyses of lymphocyte cultures of six patients with glutathione reductase deficiency revealed a marked increase in chromosome breakage in those who had pancytopenia. Cytologic examination of the bone marrow revealed a 15- to 60-fold increase of atypical cell nuclei.

The enzyme defect and neurologic and hematologic findings were found to be autosomally dominant when the pedigree of a large family was examined.

Acknowledgments

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DISCUSSION

ROSS, LOS ANGELES, CHAIRMAN: The opportunity to discuss this very interesting paper is now provided to you, ladies and gentlemen.

CARSON, CHICAGO: I wanted to congratulate Drs. Waller and Löhr on the very interesting and extensive piece of work that they have done with so many cases. The sum total of our cases was two, and both of these are now lost to us. Furthermore, on neither of our cases were we able to do family studies. It is of some possible interest that both of these individuals were of German ancestry.

Some years ago we reported an apparent activation of glutathione reductase in normal hemolysates incubated with normal ghosts. We are now doing some fairly extensive studies with membranes prepared free of hemoglobin, and we have been able easily to reproduce this phenomenon, using normal glutathione reductase. We are able to cross ghosts from normal cells with ghosts from G-6-PD-deficient cells, both from Negro and Caucasian A- and B- forms, and find that the ghosts from the G-6-PD-deficient cells normally activate (if that is the word) glutathione reductase in normal hemolysates. Both types of ghosts fail to activate glutathione reductase from G-6-PD-deficient subjects. This suggests that there is something in the secondary elevation of glutathione reductase that occurs in G-6-PD-deficiency which may be intrinsic to the hemolysates or to their glutathione reductase rather than to membrane differences.* I am very aware of the possibility that we might be eluting glutathione reductase from these ghosts. They do retain a little bit of glutathione reductase activity but the amount appears to be insufficient to account for this. However, in view of the experiences of many others with respect to G-6-PD, we are not saying that this is not an elution phenomenon. Even if it is, the crossed ghost experiments suggest a definite difference of some kind with

*Carson, P. E., Scharrer, B., Morgan, B., and Ajmar, F.: In vitro activation of glutathione reductase (abstract) J. Clin. Invest. 46: 1044, 1967.

reference to G-6-PD-deficiency.* We were able to show, before we lost our cases, that the general carbohydrate metabolism of patients with GSSG-R deficiency as determined using carbon-14-labelled glucose, had a depression similar to that which we found in G-6-PD-deficiency.

I am interested in the idea of primaquine susceptibility being an integrated phenomena involving the first portion of the pentose phosphate pathway, including G-6-PD-deficiency, glutathione reductase deficiency, glutathione deficiency, and 6-GPD-deficiency. Some time ago, Dern and Brewer and their associates† did attempt to induce primaquine hemolysis in a study with a partially 6-P-GD-deficient patient who was also, unfortunately, for this purpose, a heterozygote for G-6-PD deficiency. We have recently had the opportunity to obtain some blood, through Dr. Parr in London, with virtually complete 6-P-GD-deficiency. We labelled it and gave it to compatible recipients. This blood was indeed primaquine-susceptible. The patient was an elderly lady with an elevated red cell G-6-PD, but no frank hemolytic anemia. So this represents another instance where the enzyme deficiency is virtually complete without hemolytic anemia. In contrast, glycolytic enzyme deficiencies always appear to be associated with hemolytic anemia.

WALLER, TUEBINGEN: Your observation that the ghosts from G-6-PD-deficient cells activate glutathione reductase in normal hemolysates is very interesting. We couldn't find an activation with ghosts of normal cells.

JAFFÉ, NEW YORK: There is a question that is probably disturbing to a number of people in this audience. A decrease in the activity of an enzyme of approximately 50 per cent seems to be too small a reduction to be responsible for a clinical disease of the severity that has been described by Dr. Waller.

I would like to mention some observations that we have made in the last few years while looking at the activity of NADPH-linked glutathione reductase. One finding for which I have no good explanation is that hemolysates prepared from the erythrocytes of four of five patients with hereditary methemoglobinemia associated with NADH-methemoglobin reductase deficiency have, in addition, approximately 20 to 30 per cent less GSSG reductase activity when determined by the rate of disappearance of NADPH.‡ Now, conversely, when the system described by Schrier and Carson and co-workers§ has been used, the capacity of such hemolysates to reduce exogenous GSSG to GSH also is demonstrably slower during the initial part of the reaction. After two hours, the hemolysate with an apparent decrease in GSSG reductase activity does generate as much GSH as does the normal control hemolysate assayed at the same time. The initial concentration of GSH and its stability upon incubation of such erythrocytes with acetylphenylhydrazine is normal. Hence, the physiologic significance of a 20 to 30 per cent decrease in enzyme activity is open to question.

Recently, we have had an opportunity to study the blood of a woman of Puerto Rican origin who had experienced an acute hemolytic episode about six years ago and who, since that time, has been known to have a persistent, fairly well compensated hemolytic disorder, with persistent unconjugated hyperbilirubinemia and a reticulocytosis of 7 to 10 per cent. She also was found to be homozygous for hemoglobin C disease. Dr. Helen Anderson felt that this patient's hemolysis could not be explained by her hemoglobin C disease alone. We found

*Carson, P. S., Okita, G. T., Frischer, H., Hirasa, J., Long, W. K., and Brewer, G. J.: Proc. Ninth Cong. Europ. Soc. Haemat., Lisbon, 1963. Basel and N. Y., S. Karger, 1963, pp. 655-665.

†Dern, R. J., Brewer, G. J., Tashian, R. E., and Shows, T. B.: Hereditary variation of erythrocytic 6-phosphogluconate dehydrogenase. J. Lab. Clin. Med. 67:255, 1966.

‡Jaffé, E. R.: Hereditary methemoglobinemias associated with abnormalities in the metabolism of erythrocytes. Am. J. Med. 41:786-798, 1966.

§Schrier, S. L., Kellermeyer, R. W., Carson, P. E., Ickes, C. E., and Alving, A. S.: The hemolytic effect of primaquine. IX. Enzymatic abnormalities in primaquine-sensitive erythrocytes. J. Lab. Clin. Med. 52:109-117, 1958.

a 50 per cent decrease in the activity of NADPH-linked glutathione reductase in the hemolysate of her erythrocytes. G-6-PD activity and pyruvate kinase activity were increased, reflecting the young cell population. The concentration of GSH was normal. With intact erythrocytes and, using the technique described by Dr. Kosower, in which endogenous glutathione was oxidized to GSSG by treatment with azoester, the rate of regeneration of GSH upon incubation with glucose was found to be indistinguishable from that observed with normal erythrocytes. I, therefore, raise the question about the mechanism by which an apparent 50 per cent decrease in enzyme activity can lead to disease of the major proportions that were described here. Perhaps, the decreased GSSG reductase activity was not the primary effect of an abnormal gene, but was secondary to some other defect.

WALLER: Let me respond to the comment of Dr. Jaffé. We suppose that the actual activity of glutathione reductase within the cells of defect-carriers is lower than under the optimal test conditions with regard to decreased substrate affinity to GSSG of the enzyme. Furthermore under drug administration (chloroquine) we have seen in the erythrocytes of defect-carriers a glutathione reductase activity about 10 per cent of normal cells. After discontinuation of drug, the glutathione reductase activity increased to 40-50 per cent of normal cells. It is possible that the drug-induced inhibition of the altered glutathione reductase is higher than that of the normal enzyme.

Just before we left Germany, we examined the erythrocytes of a patient from Ghana, a homozygote with hemoglobin C disease and glutathione reductase deficiency. The glutathione reductase activity was only about 10 per cent and the GSH level, 40 per cent of normal. The GSH stability test of Beutler and the Heinz body formation test also were markedly abnormal. The substrate affinity to GSSG and the optimum pH for the reaction were typically changed in the purified enzyme.

VALENTINE, LOS ANGELES: It has also been our experience that frequently, in the hemolysate assay system for glutathione reductase, the enzyme doesn't 'take off,' so to speak, for a little while. It has a bit of a lag period. I wonder if some of these situations, such as the ones with methemoglobin, are related to the functional SH group that this enzyme appears to have. It is very susceptible to NEM, as Dr. Waller showed, and we have had the same experience. Glutathione reductase will be substantially diminished in activity with amounts of NEM that fail to eliminate the glutathione from the cell. It is very sensitive to iodoacetate. It is peculiarly sensitive to chromate in a way that has been mentioned previously, and it is possible that as one measures this enzyme in the general population of red cells, there is more erraticism in the way it behaves than is true for most of the things that we measure.

CARSON: The induction period, if you want to call it that, for glutathione reductase is something that we have always noticed but cannot explain. One of my colleagues, Robin Powell, told me that if he changed the order of addition of reagent, starting the reaction with GSSG instead of hemolysate or TPNH, he didn't get an induction period. We came upon another interesting thing when we thought we had a disaster because all our reductases went to about half of what we had been getting. We found that the supply of GSSG that we had been using had been changed. On checking this with several oxidized glutathione substrates, even though they were chromatographically pure, we got quite different activities. Since then we have observed far more often than we did during the previous five-year period or longer, erratic glutathione reductase activities that I attribute, in part, to differences in some of the substrates. We have thus had to revert to comparing values from a known G-6-PD-deficient and a known normal subject when checking an unknown, to make sure that our relationships are maintained. I know an individual who happens to be heterozygous for hemoglobin C, and his glutathione reductase is about half normal. As far as I know, he has shown no evidence of hemolytic susceptibility. The study of this kind of a coincidence might be interesting.

JAFFÉ: I want to agree with both Drs. Valentine and Carson on the occasionally

erratic nature of the GSSG reductase assay and to emphasize that we were aware of the problems. We have used the Gilford recording spectrophotometer, which will easily follow any variation in the assay. In expressing enzyme activities, we have used the maximal rates observed. We have had an opportunity to assay erythrocytes from the patient with hemoglobin C three times with essentially identical results, but I am still unwilling to make the diagnosis of hemolysis due to GSSG reductase deficiency. This discussion has emphasized the difficulty in evaluating these enzyme deficiencies and has brought to light the possibility of the very interesting association between hemoglobin C and altered enzyme activity. We have been speaking, primarily, of the system in which NADPH oxidation was followed. Sometimes, it might appear to be more reasonable to look at the other side of the reaction coin, i.e., the rate at which GSSG is reduced to GSH in either hemolysate or an intact cells, as a means of evaluating the significance of decreased enzyme activity.

CARSON: When we determined glutathione reductase by measuring the GSH formed as compared with determinations by reading it at 340 mμ with the TPNH conversion to TPN, we didn't get the same answers. We found, however, that we could get exactly the same answer if we used exactly the same concentrations of GSSG and of NADPH. At 340 mμ you are limited by the amount of TPNH you can use but not limited in the amount of GSSG. Doing it the other way, you can put in a lot of TPNH if you can afford it, but you can't, unless you want to go through laborious dilutions, use as much glutathione. As far as assigning 50 and 10 and 34 per cent of normal, we have always been worried about this, but I trust we have been sufficiently careful because we have demonstrated primaquine susceptibility in our first case repeatedly over a number of years. His glutathione reductase, though only 50 per cent of normal, was three standard deviations or more from the mean. It is quite different from that of other people. Our other case had hemolysis after Gantrisin and had only 34 per cent of normal enzyme activity. We need to study more cases from many more laboratories to work this out.

BEUTLER, DUARTE: First of all, I would like to take this opportunity to correct an error in our 1963 paper on glutathione reductase (ref. 1 in paper). The K_m values for both TPNH and DPNH were unfortunately miscalculated, and this explains why our values don't match those of other authors. I regret any inconvenience this error has caused.

I should like to ask Dr. Waller to comment on one point regarding the mechanism of formation of abnormal enzymes in his families. If the abnormal gene results in production of any enzyme and the normal gene were directing production of a normal quantity of qualitatively normal enzyme, one would expect more than one-half the normal amount of enzyme activity in his subject. It was rather surprising to me to learn that the kinetic characteristics of the enzyme were abnormal, even though the amount of gene product was what one would expect a normal gene acting alone to produce.

I should also like to comment on the matter of the DPNH and TPNH activities being due to a single enzyme or to different enzymes. Dr. Irwin in our laboratory gathered a large number of

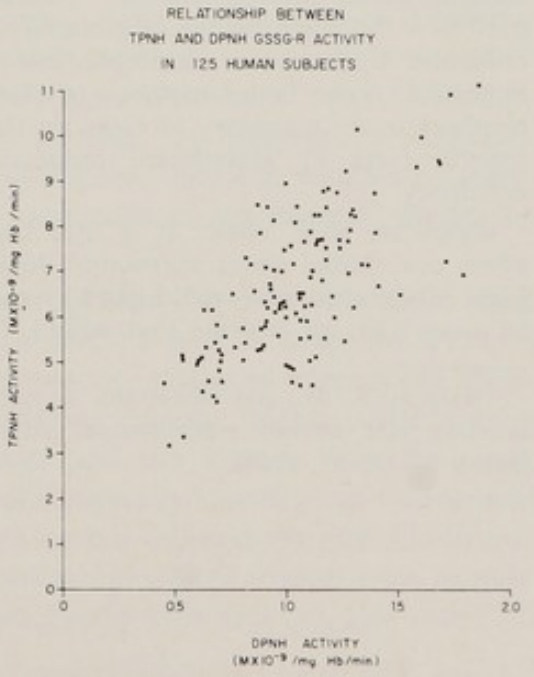


FIG. 1. The relationship between TPNH and DPNH GSSG-R activity in 125 human subjects.

human blood samples and assayed the activity of glutathione reductase both with DPNH and TPNH as substrates. Figure 1 shows that there is quite a good correlation between the two enzymatic activities. This supports the idea that they are under single control. It is obviously not conclusive. It may not be nearly as impressive as Dr. Scott's failure to separate the enzymes through a very high degree of purification, but I think it is one additional piece of evidence suggesting that only a single enzyme molecule is present.

At the same time Dr. Irwin also compared the glutathione reductase activities of Negroes and Caucasians and found no significant difference. This is of some interest because of the recently-reported difference in glutathione reductase activity of the skin in Negroes and Caucasians to which has been ascribed the darker skin of Negroes.*

WALLER: I thank Dr. Beutler for his very important comment. Until now, all examinations on normal TPNH-dependent glutathione reductase demonstrate until only one enzyme. The same is true on electrophoresis. Therefore we suppose that the enzyme of defect-carriers is altered. But I think that it is very difficult to interpret the results of electrophoretic determinations, because until now the performance of glutathione reductase separation in electrophoresis is imperfect. Some better methods of electrophoresis are necessary to clear up the genetic facts of glutathione reductase deficiency.

KOSOWER, NEW YORK: It is true that when one thinks about autosomal dominant inheritance, one would like to have, let's say half, an enzyme that would, in quantity, behave in a normal fashion. It is possible that if the other chromosome produces some altered enzyme, that this part of the altered enzyme would, together with the half-normal enzyme, give a different qualitative aspect as far as K_m is concerned?

BEUTLER: It is also possible, I suppose, that the abnormal gene products repress the normal allele and therefore the bulk of abnormal enzyme is produced. There are a number of interesting possible explanations, and I think that is one of the things that makes these observations so provocative and important.

IRWIN, LOS ANGELES: Over the past two years, Dr. Rubinstein at the City of Hope found in his neurology service a number of patients with abnormal electroencephalograms. His findings are the same as those described in Dr. Waller's paper. We had occasion coincidentally to measure the glutathione reductase in a number of these patients who were found to have normal or slightly elevated values. The one common denominator amongst these patients was elevated serum iron or evidence of iron storage disease. Now it is known that there is increased iron deposition in the basal ganglia in patients with iron storage disease. Dr. Waller mentioned that there was elevated serum iron or evidence of hemosiderosis when liver biopsy was done in his patients. I would like to suggest that perhaps these changes were due to increased iron deposition rather than to low glutathione reductase activity.

WALLER: I am not a neurologist. It is possible that you have seen similar alterations in EEG's of patients with hemosiderosis, but our enzyme-defect carriers, with and without hematological alterations, both show the described EEG disorders. I think that there is no relation between hemosiderosis and EEG alterations.

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*Halprin, K. M., and Ohkawara, A. Glutathione and human pigmentation. Arch. Derm. 94:355-57, 1966.

Hereditary Spherocytosis

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Thorndike Memorial Laboratory and Second and Fourth (Harvard) Medical Services, Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston

HEREDITARY SPHEROCYTOSIS (HS) has been recognized as a disease entity for almost 100 years and as an inborn disorder of red cells since before the turn of the century. Despite its relative frequency and its relatively uniform and easily recognized morphological expression, the exact biochemical nature of this presumed inborn error of red cell metabolism has yet to be defined. This failure to establish a clear genetic mechanism is attributable to two features of the disorder: first, that the primary abnormality appears to involve a subtle defect in membrane structure, and thus relates to an as yet poorly understood area of knowledge; second, expression of the defect involves an essential interaction with the spleen, the functions of which also are incompletely understood.

The cardinal features of HS are (1) an increased rate of red cell destruction, (2) spherocytosis, (3) splenomegaly, (4) familial occurrence, and (5) invariable benefit from splenectomy. It is relatively frequent as a cause of congenital hemolytic anemia, particularly among those of Northern European descent. Its prevalence in the United States and England is of the order of 200-300 per million,[31] and its inheritance has been established repeatedly to follow a "dominant" pattern[39] with a family incidence very close to 0.5.[31] In many instances—perhaps as many as a quarter of random cases—prior familial history is lacking or negative and the disease is believed to have arisen as a sporadic mutation.[36] Although suspicions have been expressed from time to time that HS may encompass genetic subgroups,[48] there is no clear evidence for heterogenity. A rather similar red cell disorder has been observed in the deer mouse in which a homozygous inheritance is required for "clinical" expression of the disease.[1] Examples of homozygosity for HS in man are rare.[2]

The clinical intensity of this hemolytic disorder varies moderately. Most commonly HS causes a moderate hemolytic anemia with hemoglobin levels in the range of 8 to 12 grams per cent, reticulocytosis, erythroid hyperplasia, moderate splenomegaly, and persisting or relapsing icterus. Many patients have little or no anemia, despite the hemolysis, and the disease may be occult ("compensated hemolytic anemia") or only clinically manifest as "congenital hemolytic jaundice." A minority of patients have a relatively severe, life-threatening hemolytic process. Splenomegaly is almost invariably evident even in well-compensated cases, and in adults the weight usually falls between 500 and 2000

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grams. The spleen weight reflects in part trapped red cells and red cell debris, but most of the enlargement is due to true hyperplasia, the number of spleen cells being increased on the average about eight-fold.[26] Characteristically, red cells tend to leak from the sinuses, but not from the cords of the red pulp during preparation of spleen specimens for hematologic examination.

Shape of the Hereditary Spherocyte and its Interactions with the Spleen

The characteristic feature of this disorder is the spheroidal appearance of the red cells and their corresponding increase in osmotic fragility. Although the degree of spheroidicity is quite variable from patient-to-patient, there is a rough correlation between spheroidicity and the severity of hemolysis. The spherocytosis and increased osmotic fragility reflect a slight geometrical abnormality of the cell: although usually normal in volume, the HS red cell is below normal in surface area, thus being deficient in the surface-to-volume ratio which determines spheroidicity.

An important feature of the spherocytosis that is evident both on microscopic examination and in the osmotic fragility curve (Fig. 1) is that there tends to be two populations of cells: a minor population of dark-staining "microspherocytes" that create a very fragile "tail" on the osmotic fragility curve and a major population, the fragility of which may be only very slightly greater than normal. In their series of classic studies of the relationships between osmotic fragility and the spleen, Emerson et al.[12-14] and Young et al.[49] showed that corresponding to the minor, most fragile population of red cells in the peripheral blood, there is a similar but larger population in the splenic red pulp. After splenectomy and cure of the hemolytic anemia, spherocytosis persists, but the

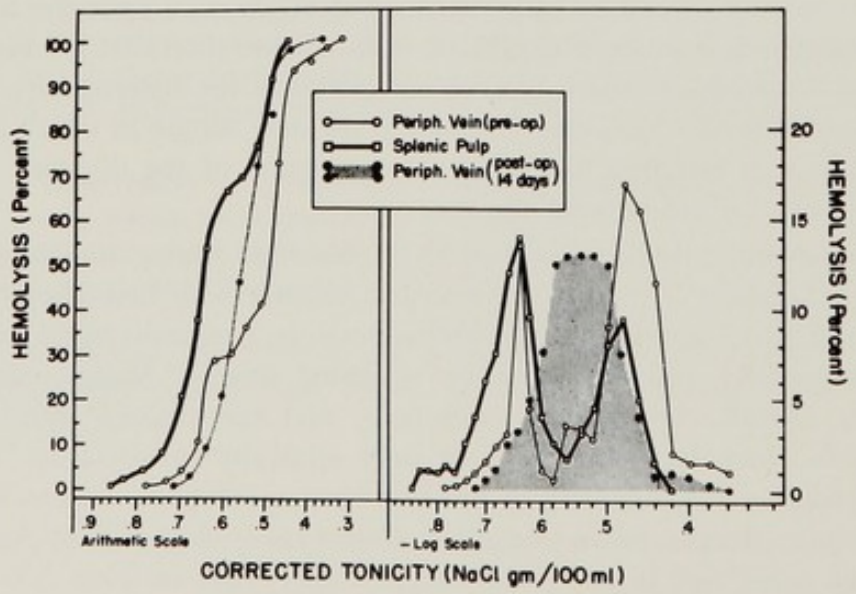


FIG. 1. Replacement of two populations of spheroidal red cells in HS preoperatively by a single, intermediate population postoperatively. The "conditioned" microspherocytes constitute the more fragile cells seen in the bimodal curves of untreated patients. (Reprinted from Emerson, C. P., Jr., et al.: Arch Intern. Med. 97:1, 1956.)

two populations of cells are replaced by a more homogeneous, single population of intermediate osmotic fragility (Fig. 1). From these and other data it was deduced that the spleen repetitiously detains and "conditions" the circulating HS red cell so as to progressively exaggerate the spheroidicity and ultimately to destroy the cell.[14] Removal of the spleen eliminates the dual role of inducing and of destroying spherocytes, thereby eliminating the double population of cells. Later direct evidence in vivo was provided, using Fe^{59}-labeled cells, substantiating that after their release from the marrow, HS red cells gradually shift from the major, less fragile population to the more fragile "conditioned" population.[15] Other studies in vivo, using Cr^{51}-labeled cells, confirmed that HS cells are actually destroyed specifically in the spleen both in patients and in normal recipients.[27]

Increased Susceptibility of HS Red Cells to "Erythrostasis" and Metabolic Stress

In investigating the effect of the spleen on HS red cells, Ham and Castle[16] and Emerson, Shen, Ham and Castle[13,14] studied their behavior during prolonged sterile incubation, or "erythrostasis," in vitro. In the first direct demonstration of an intrinsic metabolic abnormality, these workers showed that HS cells undergo an abnormally rapid increase in osmotic fragility and later autohemolysis during sterile incubation. The increased "incubation fragility" and autohemolysis that have proved to be the standard means of diagnosis were found to be exaggerated by packing the cells prior to incubation (Fig. 2) and to be counteracted by periodic additions of fresh serum[16] or by supplements of glucose.[44] Although not realized by many subsequent workers, it was demonstrated at this time (Ham, T. H. and Castle, W. B., personal communication) that the initial change in HS cells during incubation in vitro is an abnormally rapid rate of cellular swelling, most evident during the first 10 or 12 hours (Fig. 3). Thereafter an abnormally rapid loss of volume and of surface was observed (Fig. 3). The net effect of these consecutive changes is that osmotic fragility is

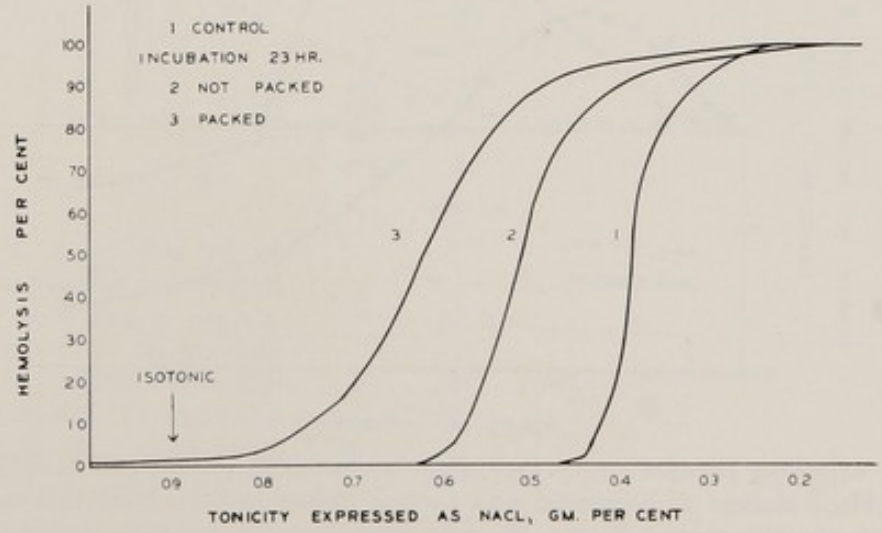


FIG. 2. Effect of preliminary packing of red cells (curve 3) on their subsequent "incubation fragility." (Reproduced through the courtesy of Dr. T. H. Ham and Dr. W. B. Castle.)

most striking at 18 to 24 hours, whereas autohemolysis does not become extensive until about 48 hours.

The observations of Ham and Castle[16] noted above and those of Dacie and Selwyn,[7,8,43,44] showing that fresh serum or simply glucose protected HS cells against autohemolysis in vitro, led to a number of attempts to demonstrate a defect in energy metabolism. Some initial studies with P^{32}-labeled orthophosphate suggested a deficient capacity to generate ATP. However, a number of investigators eventually concurred that there were no abnormalities in the levels or relative distribution of phosphorylated intermediates[11,45,50] and no overall deficiency in glucose consumption.[44] Nevertheless, the rapid morphologic breakdown of HS cells during incubation was found to be accompanied by rapid declines in the levels of ATP and 2,3 DPG[38,41] and by abnormal increments in cell water and total cation.[38]

In re-examining their general metabolic behavior Jacob and I[22] were surprised to find that HS red cells were actually hypermetabolizing. In red cells from splenectomized patients, in whom red cell age distribution was essentially normal, the rates of glucose consumption and of lactate production were increased by 20 to 30 per cent above those of normal red cells (Fig. 4). This hypermetabolism was evident at both low and high concentrations of phosphate (Fig. 4), but did not affect the rate of glucose oxidation via the hexose monophosphate shunt.

The finding of increased glycolysis in a cell undergoing metabolic decompensation was reminiscent of previous observations with red cells exposed to low concentrations of sulfhydryl inhibitors.[20,21] When red cells are exposed to low concentrations of the relatively impermeant agent, p-mercuribenzoate (PMB), the cell membranes become more permeable to cations, Na^+ accumulates in the cell and K^+ is lost, and spherocytosis occurs; nevertheless, as this break-

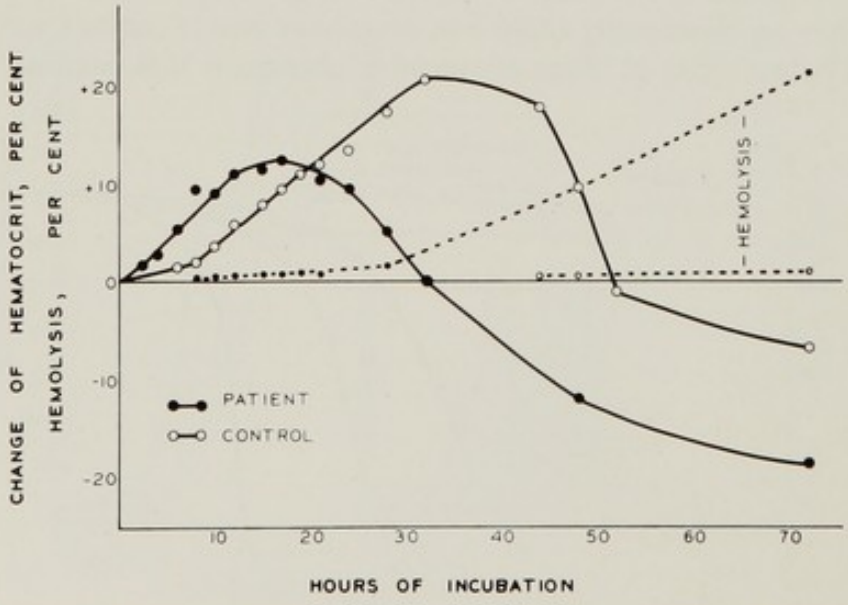


FIG. 3. Sequential changes during prolonged incubation in volume and hemolysis of HS red cells (black circles) as compared with normal (white circles). Note that the patterns differ principally with respect to rate. HS cells swell abnormally rapidly during the critical first 10 to 12 hours. (Reproduced through the courtesy of Dr. T. H. Ham and Dr. W. B. Castle.)

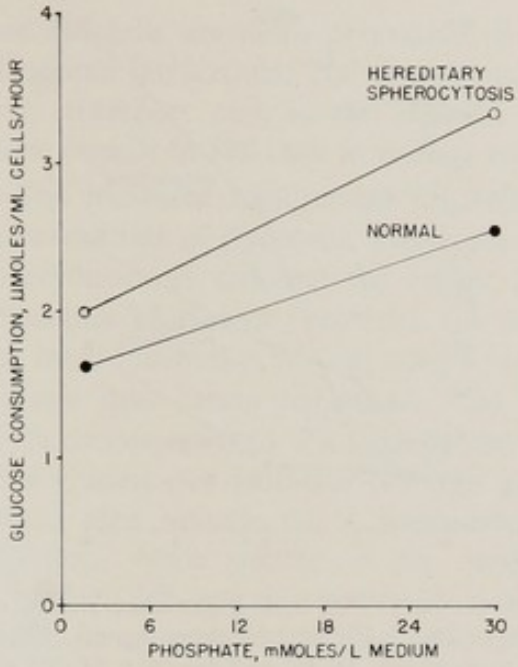


FIG. 4. Increased glucose consumption by HS red cells at low and at high concentrations of phosphate. Each point represents the mean of 7 or 8 experiments.[22] (Reprinted from Jacob, H. S.: Sem. Hemat. 2:139, 1965.)

down in osmotic regulation and volume control proceeded, glycolysis was actually increased (Fig. 5). During the subsequent hours of incubation cell swelling, spherocytosis, and finally hemolysis occurred, in the classic sequence of colloid osmotic hemolysis (Fig. 6). As in most, if not all, forms of colloid osmotic hemolysis caused by membrane injury, there was a concomitant diminution in cell surface area and thus in the so-called "critical hemolytic volume."[20] The

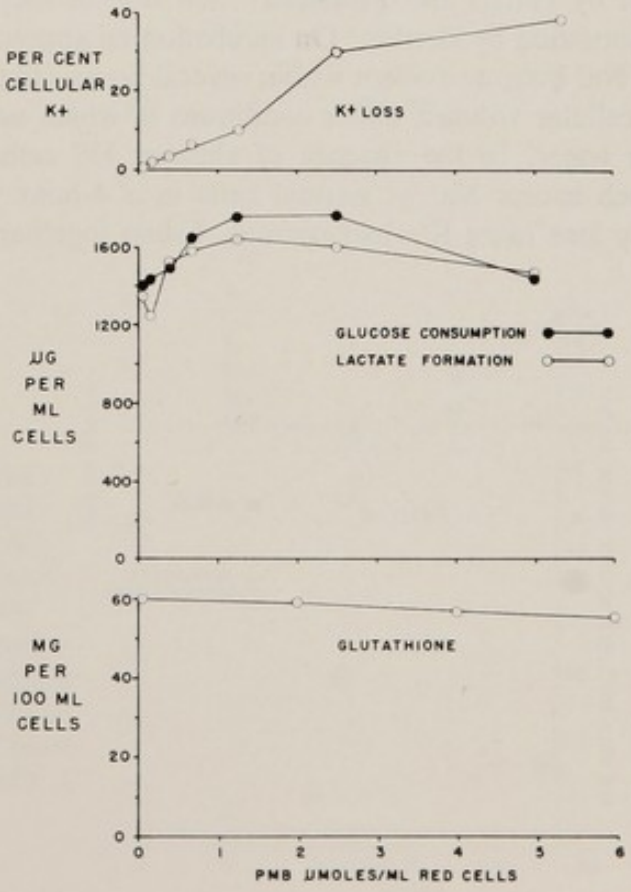


FIG. 5. Association of increased membrane permeability with K^+ loss and a stimulation of glycolysis in red cells exposed to small amounts of p-mercuribenzoate. (Reprinted from Jacob, H. S., and Jandl, J. H.: J. Clin. Invest. 41:788, 1962.)

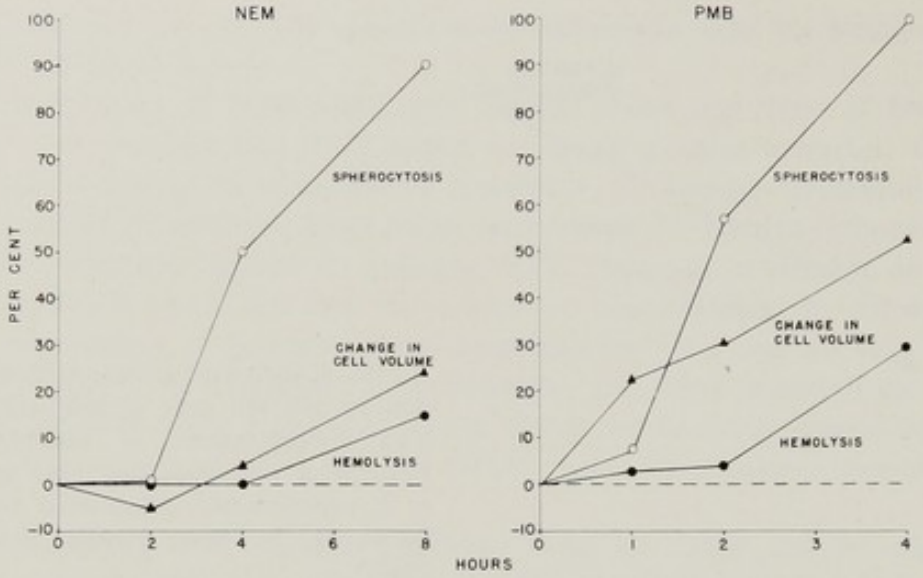


FIG. 6. Sequential changes in spherocytosis (hemolysis in 0.50 g per cent NaCl), cell volume, and hemolysis in red cells exposed to the sulfhydryl-blocking agents, N-ethyl maleimide and p-mecuribenzoate. (Reprinted from Jacob, H. S., and Jandl, J. H.: J. Clin. Invest. 41:779, 1962.)

similarities were so striking that studies of cation metabolism were made in HS cells. Under similar steady state conditions, HS cells were found to transport Na^+ outward at rates from 30-40 per cent greater than that of normal cells (e.g., Fig. 7). The rate of Na^+ influx was also found to be excessive. The finding of increased rate of outward (largely active) transport confirmed a prior observation by Harris and Prankerd[18] and that of increased inward flux confirmed an observation by Bertles.[3] On incubation an appreciable accumulation of intracellular Na^+ became evident within several hours (Fig. 8) accompanied by an increase in cellular volume, under conditions in which normal cells gained neither in Na^+ nor water. In the absence of glucose, HS cells accumulated about 3 times as much excess Na^+ as normal cells in a 4-hour period. At no time interval did they lose more K^+ than normal. Taken together these observations demonstrate

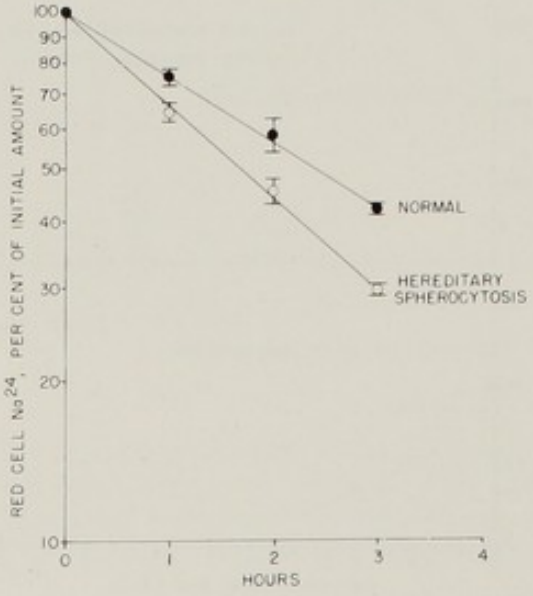


Fig. 7. Outward, largely active, transport of intracellular Na^{24} by normal and HS red cells. (Reprinted from Jacob, H. S., and Jandl, J. H.: J. Clin. Invest. 43:1704, 1964.)

that HS cells are more permeable to Na^+ than normal cells, that maintenance of normal intracellular Na^+ concentrations involves an increase in outward (active) transport and, in the absence of glucose, Na^+ regulation breaks down more rapidly in HS than in normal red cells.

The increased requirement for active transport of Na^+ tallied well with the increased rate of glycolysis, and it was concluded that the increased susceptibility to erythrostasis reflected the earlier exhaustion of glucose. Prior to complete utilization of glucose, however, the heightened accumulation of lactic acid in the usual incubation system, with a resulting fall in pH, tends to inhibit glycolysis and thus cation regulation. The settling out and packing of cells during erythrostasis in vitro also accelerates the decompensation of active transport. In a glucose-rich medium, HS cells gained little Na^+ (2.7 mEq/L) and maintained their volume for 4 hours when the cells were kept in suspension by agitation. When packed at the outset, Na^+ gain was appreciable (+ 12.0 mEq/L), cell volume increased and spheroidicity was more pronounced, as would be observed in normal cells only after more prolonged incubation (Fig. 2). The deleterious effect of cell packing on the subsequent viability of HS cells, as demonstrated in vivo, is shown in Figure 9. These various observations emphasized that depletion of substrates and accumulation of products may cause injury to cells on the basis simply of inadequate mixing. Further evidence that the heightened transport of Na^+ underlay the heightened glycolytic rate was obtained by selectively inhibiting a major portion of the ATPase system essential to active transport in human red cells. In concentrations having no effect on normal red cells in a 4-hour period of incubation, ouabain accelerated the rate at

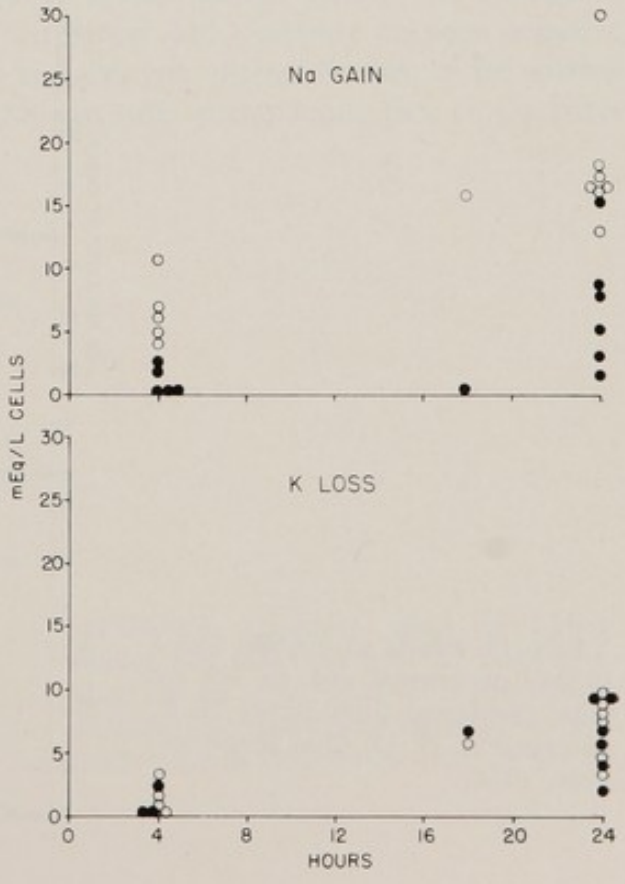


FIG. 8. Comparison of HS red cells (white circles) and normal red cells (black circles) with respect to intracellular cation concentrations after various periods of incubation. Differences are seen only in the accumulating of Na^+ and are most striking in the 4-hour samplings (refer to Fig. 3). (Reprinted from Jacob, H. S., and Jandl, J. H.: J. Clin. Invest. 43:1704, 1964.)

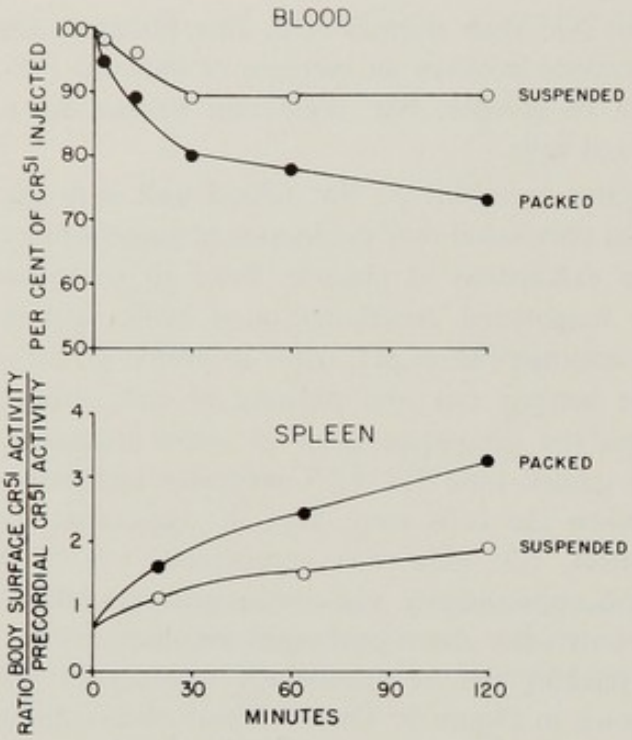


FIG. 9. Blood survival (above) and splenic sequestration (below) of HS red cells that had been incubated for 3½ hours, then labeled with Cr^{51} and injected into normal recipients. Packing clearly had a deleterious effect on cellular viability even during this short period of incubation. (Reprinted from Jacob, H. S., and Jandl, J. H.: J. Clin. Invest. 43:1704, 1964.)

which HS cells accumulated Na^+, but had no abnormal effect on losses of K^+ (Fig. 10). The suppression of active transport by ouabain inhibited glucose consumption by an average of 18.5 per cent in HS cells but had no appreciable effect upon normal red cells during the 4-hour interval studied.[22] This heightened effect of cardiac glycosides on glycolysis in HS cells is accompanied by an

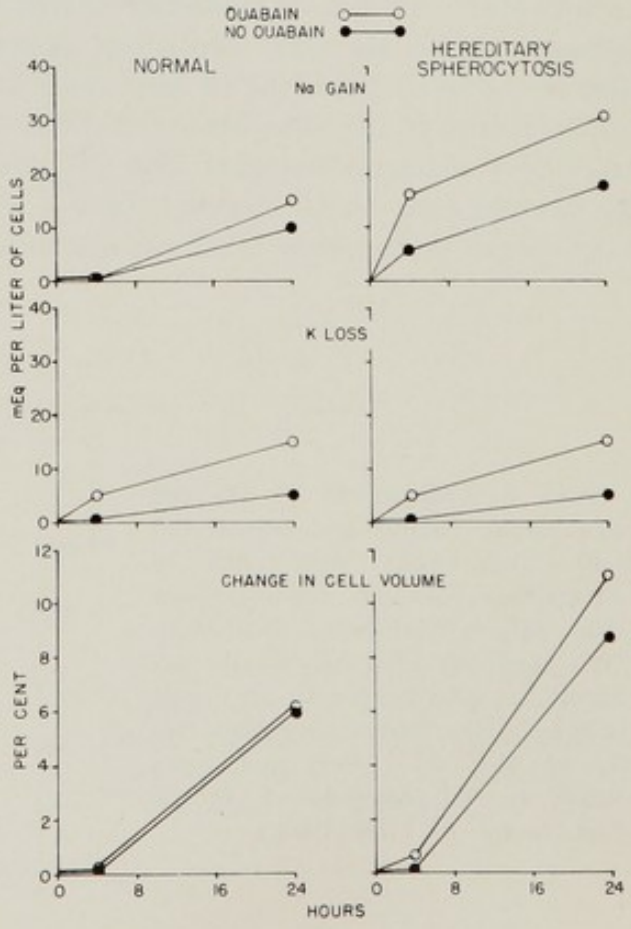


FIG. 10. Effects of ouabain (3.5×10^{-5}M) on normal and on HS red cells. (Reprinted from Jacob, H. S., and Jandl, J. H.: J. Clin. Invest. 43: 1704, 1964).

appropriately greater inhibition of ATP utilization[29] and of Na^+ flux.[22] When HS cells were suspended in a low-Na^+ medium, in which most of the Na^+ was replaced with equimolar choline, the deleterious effects of incubation in vitro were largely prevented. That the swelling and hemolysis that followed decompensation of the sodium pump represented colloid osmotic hemolysis was confirmed by the demonstration that an impermeant molecule, sucrose, was highly protective (Fig. 11). This group of experiments substantiated the thesis that the heightened glycolysis and increased rate of metabolic deterioration during erythrostasis can be attributed to the increased energy required to compensate for the abnormal permeability of the HS cell membrane for Na^+, the products of the accelerated breakdown of ATP (ADP and phosphate) acting as stimulants to glycolysis.[22,42] Based upon the Na^+ flux data in HS, the calculated increment in glycolysis required is 16 per cent; this is in good agreement with the amount inhibited by ouabain (18.5 per cent) and accounts for most of the difference in glycolytic rates between HS and normal cells.

Splenic Pooling and the Mechanism of Conditioning

Implicit in the reasoning of Castle's group in their studies of erythrostasis in vitro was that the normal spleen is a site of vascular stasis, thus accounting for the similarities between red cells from the splenic pulp and red cells from the peripheral blood that had been incubated for many hours. A number of studies, mainly of anesthetized or tranquil animals, had revealed marked erythroconcentration and evidence for slow blood flow. Thus it was rather surprising to find that red cell mixing kinetics in the normal human subject indicated a rate of transit not greatly slower than in most other organs.[17] The finding that HS red cells "mix" slowly in the normal spleen,[17] is difficult to interpret, for it is not possible to distinguish with certainty between selective sequestration of abnormal red cells and delayed mixing. Studies of the mixing of normal Cr^{51}-labeled red cells in patients with splenomegaly has, on the other

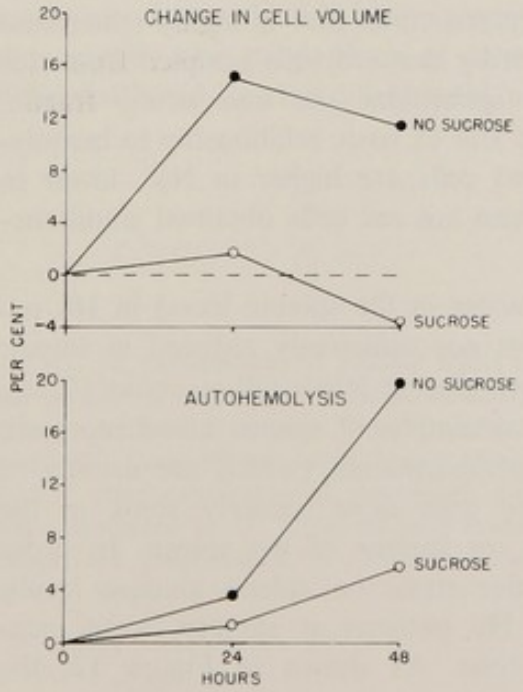


FIG. 11. Protective effect of adding sucrose (80 mM) on HS red cells incubated for up to 48 hours in a glucose-free medium. (Reprinted from Jacob, H. S., and Jandl, J. H.: J. Clin. Invest. 43:1704, 1964.)

hand, provided clear evidence for the existence of a second, slow-transit vascular pool. In a recent analysis of spleen-mixing data from various sources,[25] it has been concluded that the slow-transit pool, even though it may hold as much as 20 to 25 per cent of the total red cell mass, has an input representing no more than 1 to 2 per cent of the total splenic blood flow. Accordingly, it becomes detectable only when escape from this pool is abnormally retarded, usually by at least several minutes. Based upon present-day knowledge of the microanatomy of the intermediate circulation of the spleen, it is almost certain that the major, rapid-transit pathway involving about 98 per cent of the arterial blood is through the sinuses of the red pulp, whereas the minor, slow pathway is through the cords. As the cords are discontinuous vascular spaces, the only escape for cordal red cells is by passing through the intervening screen-like basement membrane into a venous sinus. This escape necessitates squeezing through the fenestrations of this unique membrane, the diameters of which are about 3 μ. As was proposed years ago,[47] it is probable that the slightly greater thickness-to-diameter ratio in HS red cells impairs their ability to undergo deformation and thereby to squeeze through tight openings. Indeed, by use of inert (Millipore) filters, it is possible to selectively detain HS red cells from mixtures of normal and HS cells;[28] this separation with Millipore filters is facilitated by lowering the pH of the perfusate.[35]

The observations above indicate that in each passage through the spleen, there is a 1 or 2 per cent chance of a red cell entering the cordal compartment. The HS cell is slowed in its transit through this compartment by a slight physical impediment in escaping through the openings in the enclosing basement membrane and may be subjected to many minutes, perhaps as long as an hour, of stasis. With each return to the spleen there is a random chance of repeating this stress with an insufficient intervening period for "recovery." Thus with time, there accumulates a minor population of red cells that have undergone repetitious stasis to the point that metabolic decompensation has occurred with increasing spheroidicity; this in turn makes each subsequent passage more difficult and a population of cells accumulates in the spleen cords that is highly spheroidal and on the verge of hemolysis. It is noteworthy that red cells sampled from this splenic compartment are not only more spheroidal and osmotically fragile, but, in keeping with the permeability defect and its basic relationship to hemolysis as proposed above, the spleen pulp red cells are higher in Na^+, lower in ATP and higher in inorganic phosphate, than are red cells obtained simultaneously from the splenic artery.[38]

It has been objected that the level of glucose in the splenic blood in HS patients—approximately 50 mg per cent[38]—is not sufficiently reduced to impair red cell metabolism, which is unaffected until glucose levels fall to about 10 mg. per cent. This objection ignores the fact that samples of splenic blood represent mixtures of cells from the two vascular compartments (which are usually of about equal size in HS). Presumably the cells most securely stuck in the cords are the least likely to leak from a cut surface of the spleen. In order to observe the effects of complete vascular stasis on spleen glucose levels, sections of spleen freshly obtained from HS patients at surgery, were incubated at 37° C for various periods of time. As shown in Figure 12, the

glucose concentration of whole spleen fragments is only about one-third that of peripheral blood, an amount corresponding to the relative content of blood. It would appear that there is essentially no glucose apart from that present in the perfusing blood. On incubation the glucose is entirely consumed within about 30 minutes. As there is a marked lactate deficit during this period, it is likely that much of the glucose, or of the intermediates, is consumed by the contiguous spleen cells (which are capable of oxidative phosphorylation) at the expense of the less active, glycolytic red cells. It can be concluded that the deleterious effects of erythrostasis in the spleen are greatly aggravated and speeded up by the competitive action of the metabolically-active spleen cells. It appears quite likely that the transit time of HS red cells through the slow (cordal) compartment is sufficiently protracted to result in intracellular depletion of substrate; indeed an individual red cell in apposition to a spleen RE cell may become deprived virtually instantaneously.

Abnormal Behavior of Membrane Lipids in HS Red Cells

Although the sequence outlined above explains very nicely the interrelations between membrane permeability, increased glycolysis, spheroidicity, and splenic conditioning, it does not provide an explanation for the fact that highly conditioned cells are "microspherocytes" that have lost considerable surface and tend to have a high MCHC. This membrane loss is not inconsistent with the observations that have been made during glucose deprivation in vitro, where the "second phase" involves loss of cations and loss of surface just prior to hemolysis (Fig. 3), nor is it inconsistent with a colloid osmotic mechanism, for most if not all forms of colloid osmotic hemolysis due to membrane or metabolic injury involve a diminution in the critical hemolytic volume.[24] However, the basic nature of this membrane alteration is not as yet fully explained. A number of recent studies have called attention to the behavior of membrane lipids in HS cells but certain points of disagreement exist.

Although abnormalities in membrane lipid composition have been reported,[29] a number of investigators have agreed that there is no appreciable abnormality in the amount and proportions of membrane lipids in HS cells.[9,10,37] However, Prankerd[38] and Reed and Swisher[40] have found that during prolonged incubation

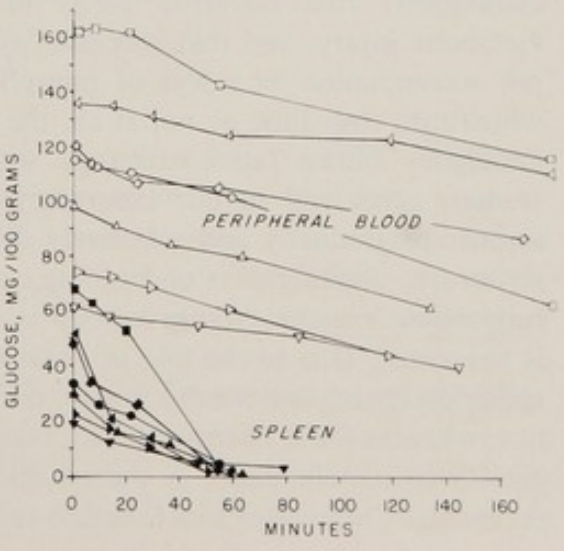


FIG. 12. Comparison of glucose levels in the peripheral blood with those in blocks of spleen obtained at surgery from 7 patients with HS. Comparisons were begun immediately after extirpation of the spleen and were continued at 37° C for up to 3 hours. (Reprinted from Jandl, J. H. and Aster, R. H.: Amer. J. Med. Sci. 253:383, 1967.)

and glucose depletion, HS red cells lost lipids at an increased rate. Reed and Swisher[40] noted that this excessive loss of lipids was partially inhibited by glucose, the beneficial effect of which was not blocked by ouabain; noting the fact that all lipid moieties were lost proportionally (or "symmetrically"), they concluded that the process represented the loss of whole fragments of membrane. Subsequently, Weed and Bowdler[46] presented direct evidence for piecemeal fragmentation of HS cells during prolonged incubation in serum or plasma and showed marked crenation and beginning budding and fragmentation of the membranes after only 5 hours' incubation without glucose (followed by washing in saline). These observations appear to explain the extreme surface loss encountered on prolonged (24 hours or more) incubation; in addition, the investigators suggest that fragmentary degeneration, rather than colloid osmotic hemolysis, is the primary mechanism of hemolysis in vivo.

Using a different incubation medium, Jacob[19] confirmed the abnormal "symmetrical" loss of membrane lipids by HS red cells. In this and another previous report,[23] an increase in phosphate incorporation into phospholipids was described in HS red cells. As these cells are characterized by increased transmembrane sodium flux, studies were carried out that demonstrated in various red cells an association between Na^+ flux, phospholipid incorporation of phosphate, and lipid loss. From this Jacob[19] concluded that the loss of membrane lipid in HS cells is attributable to an effect of active transport on the turnover and thereby the "stability" of the phospholipids. Although a functional relationship probably does exist between membrane injury, cation leak rate, and lipid loss, there are reasons for doubting that the rate of active transport per se is a necessary feature. A dependence on active transport is not evident in the fact that lipid loss is maximal in the absence of energy metabolism (including active pumping of Na+) or in the fact that it is unaffected by ouabain.[40] Furthermore the rate of phosphate turnover in the phospholipid fraction is well below the rate of ATP turnover or of cation transport.

Quite independently of the issues raised above, Murphy[32,33,34] has reported evidence that the amount and distribution of free cholesterol constitutes a major determinant of red cell shape. In contrast to the process noted above, Murphy observed a process of spherocytosis and lipid loss (i.e., involving free cholesterol) that occurred early during incubation, that was unrelated to metabolic injury, and that was not "symmetrical"—that is, cholesterol loss was not accompanied by a loss of phospholipid. Unlike the various phospholipids, which turn over little or not at all, the free cholesterol of the red cell membrane undergoes rather rapid exchange with serum free cholesterol. In Murphy's studies, confirmed in our laboratory, [4,5] normal red cells incubated in fresh serum are gradually and selectively depleted of cholesterol due to the fact that serum free cholesterol is undergoing continued "loss" by esterification. When the responsible enzyme "transesterase" is destroyed by heat,[32] cholesterol depletion is prevented. Due to the loss of cholesterol in unheated serum, red cells become spherocytic and osmotically fragile, despite maintained glycolysis and cation control; whereas these changes do not occur in heated serum (or saline), as long as glycolysis is maintained. A remarkable feature of this phenomenon is that it is reversible. Cholesterol was found to reaccumulate in the red cell membrane when

the depleted, spheroidal cells were resuspended in heated serum containing normal levels of cholesterol.[32,33] Furthermore, red cells rendered osmotically fragile by cholesterol depletion were found to rapidly recover normal fragility if returned to the circulation in vivo.[5]

In exploring the mechanism by which red cells acquire a flattened "target" shape in obstructive jaundice, Cooper demonstrated that the change in shape was due to an extension of surface associated with a specific accumulation of cholesterol.[4] This increase in membrane surface area and the decrease in osmotic fragility—changes opposite from those seen in HS—was demonstrable when normal red cells were incubated in vitro in jaundiced serum or in serum fortified with bile salts or were injected and studied *in vivo* by means of the Cr^{51}-osmotic fragility technique. Conversely, target cells lost their redundant surface when injected into normal recipients. The pathogenesis of the target cell appears to result from a bile salt-mediated mechanism that both inhibits transesterase and shifts the cell: serum equilibrium for cholesterol toward the cell.

Since the bile salt-mediated mechanism for extending red cell surface represents in essence the antithesis of HS, studies were then made of the behavior of Cr^{51}-labeled HS red cells in patients with obstructive jaundice and target cells (Fig. 13). In each of three jaundiced recipients, the osmotic fragility of HS red cells diminished progressively, reaching normal values within 2 days. Attending this remarkable transformation, the survival of the HS cells was virtually normalized.[5] In three "paired," normal recipients, on the contrary, osmotic fragility changed little and red cell survival was very poor (Fig. 13). Thus, in contrast to the unsuccessful attempt to improve HS by the "flattening" effect of iron deficiency,[6] these studies indicate that the underlying membrane defect can be circumvented by factors that favor the accumulation of cholesterol in the membrane.

These observations shed new light on some confusing aspects of cell "conditioning" and the primacy of various changes reported, for certainly a cell that has become spheroidal or conditioned by loss of whole membrane fragments is

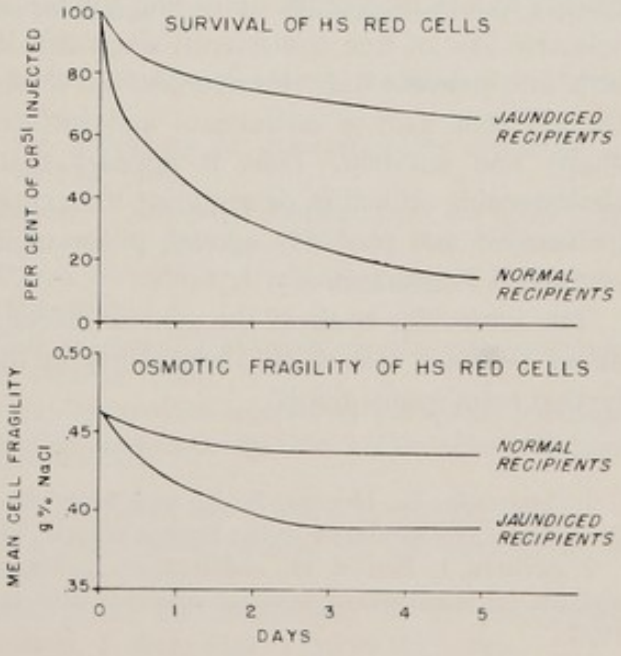


FIG. 13. The survival and osmotic fragility pattern of Cr^{51}-labeled HS red cells in a patient with obstructive jaundice as compared with that of a normal recipient. Within the first 24 hours or so of transfusion into the jaundiced recipient, the HS cells acquired a normal fragility and thereafter survived almost normally. This experiment is representative of three "paired" studies. (Unpublished data from Cooper, R. A., and Jandl, J. H., 1967.)[5]

not likely to be benefited by the selective uptake of free cholesterol. It would appear that a phase of splenic conditioning, perhaps a critical phase, involves a reversible loss of membrane cholesterol; the factors during stasis that impair glycolytic metabolism may well predispose to a negative cholesterol balance. Only later, possibly as an agonal event in red cells undergoing the final phase of splenic conditioning, is there an actual loss of whole fragments.

As stated at the beginning, the underlying genetic defect in the HS red cell is unknown, but it affects the metabolic and physical properties of the cell membrane. In the presence of a spleen this subtle defect is evident in 3 phases: (1) the constant and most "primary" aberration is that the membrane is slightly deficient in area and is hyperpermeable to Na^+, as the result of which the requirements for active transport and maintained glycolysis are increased. The membrane defect predisposes the HS cell to metabolic injury during normal splenic cordal pooling, as the result of which (2) a minor population of hyperspheroidal cells is acquired, characterized by a reversible impairment of active Na^+ transport and a reversible loss of membrane due to a selective loss of membrane cholesterol. Finally, (3) a third, prelytic phase supervenes in which the cells become microspherocytes which undergo irreversible metabolic failure, fragmentation, and, eventually, lysis.

Summary

Hereditary spherocytosis (HS) appears to arise from a subtle intrinsic defect in the red cell membrane which leads to secondary changes involving the loss of cell surface, spherocytosis, and eventually hemolysis. The most "primary" and "constant" abnormality thus far established is that the membrane is hyperpermeable to Na^+; in order to survive, the HS red cell requires an increased rate of active sodium transport and must consume glucose and regenerate ATP at an accelerated rate. When active transport is hindered, HS cells swell and accumulate Na^+ at an abnormal rate. In the patient, these changes occur during repetitious passage through the slow (cordal) vascular compartment of the spleen. A secondary alteration of these cells in vivo and in vitro is their propensity to lose membrane lipids and surface area. A reversible phase of this is the selective loss of free cholesterol; when this loss is prevented by injecting HS red cells into patients with elevated bile salt levels and obstructive jaundice, however, the HS cells gain in cholesterol and surface area and become normal both in shape and survival. Thus it appears that the serum:cell partition of free cholesterol is critical in determining the expression of the primary defect. A later, irreversible and probably agonal, phase of lipid loss is piecemeal fragmentation of the cell membrane.

The molecular basis of the genetic defect in HS has not been established. Indirect evidence favors an abnormality in the stromal protein, possibly one affecting membranes thiols.

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DISCUSSION

ROSS, CHAIRMAN, LOS ANGELES: Dr. Jandl's excellent paper is open for discussion.

WAYS, SEATTLE: An observation that fits very well with Dr. Jandl's hypothesis is one made by Dr. Reed. He has compared the red cell lipids of patients with HS before and after splenectomy, and I think he has enough patients now to say with a fair degree of certainty that the lipid per cell is indeed higher after splenectomy than before. Since most of the nonsplenectomized patients have a younger-than-average red cell population, a priori, you would expect that the nonsplenectomized patient should have higher lipids per cell. The higher level, in the splenectomized patients, does, however, include *both* the cholesterol and the phospholipids.

We became interested in Dr. Jandl's work with target cells, and recently had the opportunity to see a patient with a florid megaloblastic anemia secondary to a total gastrectomy performed 12 years before, at which time his spleen was also removed. This patient had about 50 per cent target cells, some of them really huge "macrotarget" cells. Over the years we have assayed red cells of many different sizes, and there seems to be a very good correlation between the amount of cholesterol and phospholipid per cell and the mean corpuscular volume. This patient, interestingly enough, was well off the regression line. His MCV was 160, and we would expect such a patient to have about 1.65×10^{-11} mg of lipid phosphorus per cell. His cells contained 1.95×10^{-11} mg.

I would like to argue gingerly with the point that you might correct the HS abnormality as you have done in these patients simply because of mass action. We don't know how much of the exchange of cholesterol depends on energy, perhaps none of it does. When the HS cells are transfused into an environment that contains high levels of free cholesterol, it is at least conceivable to me that the equilibrium is such that you are actually able to force enough cholesterol into the membrane (even though it may be defective) to correct the defect. It has been observed that many patients with hemolytic anemia have low plasma cholesterol levels. We have never systematically measured the plasma cholesterol in patients with HS. Do you have any data on that?

JANDL, BOSTON: We have not measured serum cholesterol levels in patients with hereditary spherocytosis and anemia. All of the studies thus far have involved blood from splenectomized patients so as to avoid the confusing effects of a young, heterogeneous cell population. As for the general influence of splenectomy per se on red cell shape, we are inclined to think that in effect "conditioning" by the spleen diminishes the red cell surfaces, whereas cholesterol and bile acids extend it.

BEUTLER, DUARTE: I am particularly fascinated by the prospect that gall stones are actually good for patients with hereditary spherocytosis. It would seem, from your studies, that a common duct stone could help to correct their red cell defect.

JANDL: I don't have enough personal experience with the effect of obstruction by gall stones in unsplenectomized patients with hereditary spherocytosis. However, I have discussed this point with Dr. Louis K. Diamond, who has seen this rather common complication a number of times. Dr. Diamond confirmed making the comment (attributed to him in the past by others) that when patients with hereditary spherocytosis and gallstones develop obstructive jaundice, there may be a dramatic reduction in the spherocytosis and osmotic fragility and an improvement in the anemia.

BEUTLER: In your studies of sodium flux pumps, I noted that you carried out some very careful studies of potassium flux of HS cells. If I remember correctly, your findings were that there were no abnormalities in potassium flux, but there was an abnormal leak of sodium from the plasma into the cell. The potassium-sensitive ATPase is supposed to work on both sodium and potassium. One mole of sodium is extruded while one mole of potassium is pumped into the cell. I would think this would result in an accumulation of potassium in the cell. Perhaps the pump really doesn't work the way it is supposed to, or perhaps the

pump is abnormal in these particular patients. What is the explanation for this discrepancy between the way the pump is supposed to work and the way it appears to work in these cells?

JANDL: That is a good question and arises from the conclusion by Glynn and others a decade ago that active transport outward of Na^+ is stoichiometrically coupled to the transport of K^+ inward. I think it is now generally believed that coupling is incomplete and only involves part of the active transport mechanisms. As reviewed recently by Hoffman*, about one quarter of the ATP-dependent, ouabain-inhibitable transport of Na^+ is independent of K^+. It is also doubtful that Na^+ and K^+ transport are coupled in "Pump II," which accounts for about 30 per cent of the active transport of Na^+. Thus only about half of the total outward transport of Na^+ is known to be coupled to K^+ transport.

Certainly, in addition, it is possible experimentally to alter selectively the flux of potassium and sodium in red cells. Thus, it is not essential that potassium be affected by a change in membrane permeability that affects the turnover of sodium, and indeed there is no evidence for any abnormality in potassium transport in hereditary spherocytosis red cells.

MASOUREDIS, SAN FRANCISCO: I wonder if Dr. Jandl would care to speculate with regards to the primary defect. Apparently we now have difficulty with sodium flux and loss of cholesterol. Is the cholesterol loss secondary to the electrolyte difficulty or is it the other way around?

JANDL: No, I didn't mean to imply that HS cells have a primary defect on cholesterol turnover, although the primary membrane defect may well result in an excessive lability of membrane cholesterol. Our studies to date only indicate that factors inducing an increase in the cholesterol content of hereditary spherocytosis red cells tend to offset the underlying membrane defect remarkably well. In studying cholesterol acquisition by red cells, Cooper has noted two entirely different reaction rates. After red cells are depleted of cholesterol (but not phospholipid) by incubation in fresh serum in vitro, they can recover thereafter their normal content of cholesterol and their shape very rapidly in vitro; in vivo, such cells recover normal osmotic fragility very rapidly, with a half-time of recovery of about an hour. On the other hand, acquisition of "excess" cholesterol (additional to that normally present) by either normal or HS red cells takes longer: about 24 hours or so, as shown in Figure 13 of my paper. Presumably two different processes are involved.

LÖHR, MARBURGH: Have you determined the composition of individual fatty acids in the phospholipid faction?

JANDL: Although we haven't measured these, others have reported phospholipid and fatty acid composition to be perfectly normal in amount and in relative concentration.

KOSOWER, NEW YORK: Has anybody found more about what happens to the cholesterol in patients with pyroxidine deficiency anemia?

JANDL: Cholesterol is low in a number of anemic disorders and classically it is very low in untreated patients with pernicious anemia. It is also low in folic acid deficiency, in pyridoxine-responsive anemia, and in many hemolytic and at least some aplastic anemias. In patients with pernicious anemia who are treated with B_{12}, there may be a very rapid rise in the serum cholesterol. I think it should be emphasized that it is not the high serum level of cholesterol per se that causes red cells to expand their surfaces. The partition of free cholesterol between serum and cells presumably is determined in large part by equilibria between the binding sites on the serum lipoproteins and on the cells. If there is little or no binding protein in the serum, there may be a relatively normal red cell cholesterol despite a marked reduction in serum cholesterol, as exemplified in acanthocytosis. Presumably for the same reason, red cells that are incubated in buffered saline won't lose cholesterol (at least not until they undergo fragmentation) for free cholesterol is poorly and very slowly soluble in non-protein media. Patients who have target cells with excessive cholesterol and increased surface area usually have supranormal serum levels of free cholesterol. However, the

*Hoffman, J. F.: The red cell membrane and the transport of sodium and potassium. Amer. J. Med. 41:666-680, 1966.

gain in red cell cholesterol cannot be related simply to the serum level, for this ignores the presumed role of the binding proteins. An important influence in vitro and by inference in vivo is the level of bile salts. These were found to have a marked effect on the partition of cholesterol between serum and cells, shifting the equilibrium toward the cell. Bile salts, when added in rather high concentrations to serum in vitro, also inhibit the enzyme, transesterase, as noted by Glomset.* This favors cholesterol retention by cells in vitro, but the true role of the mechanism in vivo is uncertain. I will defer to Dr. Ways who knows much more about lipids than I.

WAYS: In acanthocytosis, the cells actually do lose cholesterol as they age, and according to the best evidence that we can obtain, they lose it more rapidly than a normal cell. These patients have a free cholesterol level of 20 to 25 mg per 100 ml so again this may be a matter of shifting the equilibrium.

JANDL: When normal red cells are brought down to that level by incubation in serum depleted of cholesterol, the cells become very spheroidal within a few hours; this is not the case in acanthocytosis, wherein the low serum cholesterol levels result from the deficiency of β-lipo-protein.

WAYS: Yes, and the situation is complicated by the abnormal phospholipid distribution. It is of interest that we have now transfused acanthocytes into normal recipients, and the cells survived perfectly normally. If the loss of lipid has anything to do with the decreased survival times seen in patients with acanthocytosis, we may have shifted the equilibrium back again and prevented the lipid loss.

NEERHOUT, LOS ANGELES: We have performed red cell stromal lipid profiles on several spherocytotic and congenital nonspherocytic patients and found these to be within normal limits, including fatty acid distribution. We have been interested in the red cell lipid patterns of patients with liver disease, primarily obstructive liver disease, and have, indeed, noted some changes in the quantitative aspects. However, our studies show an increase in all lipid fractions in obstructive jaundice so that the total lipid phosphorus and cholesterol values are all increased. Possibly, there is some tendency toward more cholesterol than other lipids, but in general, all seem to be fairly well increased. Our experience has been similar to that of Dr. Ways in that when you correct stromal lipid content for the red cell volume, you do, in general, tend to get back to a more standard figure, although in some of the patients with obstructive jaundice this has not been the case. In these patients, lipid content seems to be in excess of what one would have anticipated for their cell size. If cholesterol is, in fact, increased over the phospholipids in the membrane in certain situations, it would be most interesting since there are concepts of membrane structure that require for the integrity of the membrane a fairly constant ratio of phospholipid to cholesterol. Our findings would be in agreement more with this philosophy. If your findings are the more proper ones, this might explain possibly the abnormal permeability of the cells.

JANDL: Again, I don't want to imply that the ratio is inherently abnormal in HS, because it is not. It is abnormal in targeting in that there is more cholesterol than there should be, with a proportional increase in surface area. The conversion of a normal red cell to a flat, osmotically-resistant cell (or of an HS cell to a normally-shaped cell) involves a specific increase in cholesterol. There is no measurable increase in phospholipid in these cells. Target cells are not increased in volume, incidentally, only in surface area and thus in diameter.

PRINS, AMSTERDAM: Dr. Jandl, is the increased rate of pumping in any way reflected in the amount of ATP or the ratio ATP/ADP in the abnormal red cell?

JANDL: Yes, there is an abnormally rapid decline in the ATP/ADP ratio

*Glomset, J. A., and Wright, J. L.: Some properties of a cholesterol esterifying enzyme in human plasma. Biochim. Biophys. Acta 89:266-76, 1964.

Glomset, J. A.: The mechanism of the plasma cholesterol esterification reaction: plasma fatty acid transferase. Biochim. Biophys. Acta 65:128, 1962.

when HS cells are deprived of glucose in vitro, and the levels of ADP and AMP are reported as somewhat increased in fresh HS red cells pre-splenectomy. Red cells recovered from the spleen pulp at splenectomy are considerably higher in ADP and inorganic phosphate, and lower in ATP. As long as they are properly suspended in a physiological, glucose-replete medium at the proper pH, etc., however, HS cells can maintain a normal ATP/ADP ratio for many hours in vitro, according to measurements of others.

KOLER, PORTLAND: Dr. Jandl, targeting is also perhaps most characteristic of homozygous C disease. Have you had an opportunity to study the relations that you described in this entity?

JANDL: About ten years ago I explored this very question by transfusing normal red cells into a patient with hemoglobin C disease, using chromium-51 as a method of measuring changes in osmotic fragility in vivo. It was surprising to find that there was a gradual shift in the osmotic fragility of the transfused normal cells toward the patient's decreased fragility pattern. The shift was small and of uncertain significance and had a "half-time" of over 30 days. An even smaller and still less certain, but rather similar, shift occurred when normal cells were transfused into a patient with sickle cell anemia and somewhat less-flattened red cells. In normal individuals there was no detectable shift. In patients with immunohemolytic anemia and spherocytosis, however, as we reported in 1958, there was a marked and progressive increase in the osmotic fragility of transfused normal cells, which attained the extremely-increased fragility of the patient's cells in 3 or 4 days. The shift in the homozygous C patients, I would reiterate, was relatively slow and incomplete as compared to the dramatic shift that can be seen in the patients with obstructive jaundice. At present, I would interpret these data to suggest that the targeting in hemoglobin C disease may be partly an acquired change in the red cell; it is doubtful, however, that it is the same mechanism so readily and rapidly demonstrable in patients with obstructive jaundice.

ROSS, CHAIRMAN: I should like to pay tribute to Dr. Jandl, who has cast a spell that has kept all of you here, I'm sure, far beyond the time that you ordinarily sit in a scientific meeting.

Pyruvate Kinase Deficiency

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THE CONGENITAL nonspherocytic hemolytic anemias represent a heterogeneous group of diseases that have certain features in common. Among these are an absence of appreciable spherocytosis, a normal osmotic fragility when fresh red cells are tested, no abnormal hemoglobin, and persistence of hemolysis after splenectomy. These disorders were first discussed in detail in 1953 by Dacie and his colleagues.[12] The following year Selwyn and Dacie[36] categorized them as Type I and Type II on the basis of different behavior in an in vitro autohemolysis test. When sterile blood was incubated for 48 hours, blood from Type II patients had marked autohemolysis which was not correctable by addition of glucose to the incubation system. Further, Type II red cells were found to have impaired glucose utilization, and a defect in glycolysis was suggested. de-Gruchy and his associates[14,15] confirmed these findings and, in addition, found correction of increased autohemolysis by ATP, reduced erythrocyte ATP, and an accumulation of certain phosphorylated glycolytic intermediates. On the basis of their studies, they postulated a defect in the glycolytic mechanism of Type II erythrocytes.

Our group, therefore, began a study of glycolytic enzymes in congenital nonspherocytic hemolytic anemia, and this led to the demonstration in 1961 by Valentine and his associates[45] of a specific deficiency of the enzyme, pyruvate kinase, in the red cells of three patients with congenital nonspherocytic hemolytic anemia conforming generally to the Type II category. These findings and corollary studies were soon extended.[38,39] Similar patients were soon reported from various parts of the world.[19,44] It is clearly apparent that pyruvate kinase (PK) deficiency hemolytic anemia is a metabolic and clinical entity definable in terms of an inborn error involving the main pathway of glycolysis.

The major clinical and metabolic features and genetics of PK deficiency, based upon our studies and reports in the literature, are reviewed herein. In addition, further data are presented. The biochemistry and kinetics of erythrocyte and leucocyte pyruvate kinase are described elsewhere in this symposium. Two reviews of pyruvate kinase deficiency have been published recently.[19,44] The metabolic features in particular are discussed thoroughly by Keitt.[19]

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Clinical Aspects

Incidence

It appears that PK deficiency and G-6-PD deficiency are the most common deficiencies associated with the syndrome of congenital nonspherocytic hemolytic anemia.[19] PK deficiency, which represents the first specific, heritable enzymatic defect discovered involving the main pathway of glycolysis, has been found in many patients described previously in the literature as atypical or familial, or as having Dacie's Type II nonspherocytic anemia (W. W. Zuelzer, personal communication, 1964).[11,13,33] Other inborn defects of the Embden-Meyerhof pathway, such as triose phosphate isomerase deficiency[35,43] and hexokinase deficiency,[42] which have been described subsequently, are uncommon compared with the incidence of PK deficiency.

Since the initial report by Valentine and his associates in 1961,[45] at least 95 cases of PK deficiency (K. R. Tanaka and W. N. Valentine, unpublished data)[9,10,17,19,32,40,41] verified by assay have been found primarily in the United States and Europe. The disease is most common in people of Northern European origin,[44] but has been noted in Italians[6] and Japanese,[26] a Mexican child,[39] an American Negro, and a Syrian girl.[17] Both sexes are affected equally.[44]

Clinical Features

PK deficiency does not have distinguishing or pathognomonic clinical features. It has the usual hallmarks of most chronic hemolytic processes, such as jaundice of varying degree, exacerbation of anemia by infection or other stress, slight to moderate splenomegaly, and a high incidence of gallstones. Considerable variation exists in the severity of the clinical disease. The spectrum ranges from severe neonatal anemia,[4] requiring multiple transfusions, at one extreme, to a fully compensated hemolytic process in healthy adults at the other.[39,45] The onset is usually noted in infancy or early childhood, but some patients apparently escape detection until adulthood. Most of the reported cases have been infants, children, or young adults, but the deficiency has even been detected in cord blood,[20] as well as in a woman of 65 years.[29] In spite of a moderately severe hemolytic anemia in most instances, survival to adulthood is common. However, an unusually severe form of PK deficiency has been described in an Amish isolate[3] in which family histories indicate a high mortality rate in the first few years of life.

Prominent frontal bossae may be observed in severely affected children.[4] Chronic leg ulcers may occur, though rarely.[40] The majority of PK-deficient patients have been transfused, but a significant number have maintained a comfortable hemoglobin level without transfusions, particularly after splenectomy, and have been able to lead active lives. Women with PK deficiency seem to tolerate pregnancy with no unusual complications (K. R. Tanaka and W. N. Valentine, unpublished data).[10,19,26]

Hematologic Findings

The levels of hemoglobin and packed red cell volumes vary widely, but generally fall in the range of 5 to 11.5 g per 100 ml and 15 to 35 per cent respectively. Morphologically, the disease is characterized by the presence of a fairly

uniform macrocytosis of slight to moderate degree in adults.[44] This macrocytosis is probably due to the increased reticulocyte count and not to folic acid deficiency.[30] The erythrocytes are normochromic, with only slight anisocytosis and poikilocytosis. Irregularly contracted red cells may be seen in varying numbers. In infants or young children with severe anemia, greater degrees of morphologic change may occur. However, marked red cell spiculation, described in a patient with PK deficiency,[28,31] probably connotes a common type of erythrocyte destruction and in vitro behavior and is not specific for PK deficiency.[29]

Reticulocytosis of a moderate to marked degree is the rule. It is of interest that reticulocyte counts after splenectomy are considerably higher than before splenectomy in PK-deficient patients.[16] Extremely high values are seen only after splenectomy (Fig. 1). In our 18 patients, the hemoglobin values are quite similar in the two groups, whereas the reticulocyte percentages are much higher after splenectomy. This phenomenon and its possible relationship to the pathophysiologic mechanism of the beneficial effect of splenectomy is thoroughly discussed by Keitt.[19]

There is severe shortening of the life span of the red cell in most patients. However, the variability of the half-life of Cr^{51}-labeled erythrocytes is apparent from a review of the literature and is demonstrated by four patients assayed for pyruvate kinase in our laboratories (Table 1).

Effect of Splenectomy

Although at first it was generally felt that splenectomy is not indicated in PK deficiency, there is now sufficient evidence[19] that some degree of benefit usually occurs, especially in the severe cases. The hemolytic process clearly persists, but hematocrit levels may increase slightly, transfusion requirements may be

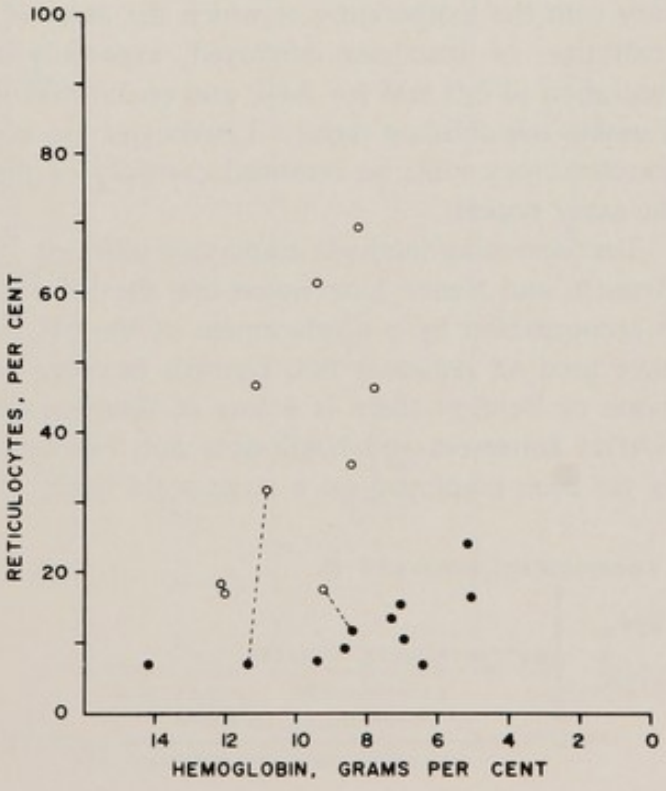


FIG. 1. Reticulocyte counts in splenectomized (open circles) and non-splenectomized (solid circles) patients with PK deficiency compared with hemoglobin levels. The same patient before and after splenectomy is connected by a dashed line.

TABLE 1. *Red cell survival in PK-deficient patients*

Patient	Hemoglobin (g%)	Packed cell volume (%)	Reticulocytes (%)	T Cr^{51} (days)
*E.T.	9.9	32	34.2	3.0
*J.L.	10.8	35	32.0	6.5
*M.V.	8.5	—	35.0	11.0
T.M.	10.1	32	5.8	21.0

*Splenectomized

decreased or eliminated, growth and development in children may be accelerated, and osseous changes may revert toward normal. The paradoxical rise in reticulocyte count following splenectomy has been mentioned already.

Metabolism in PK Deficiency

PK Assay

Pyruvate kinase catalyzes the conversion of phosphoenolpyruvate to pyruvate with regeneration of ATP (Fig. 2). The assay procedure employs phosphoenolpyruvate as substrate, buffer, ADP, KCl, $MgSO_4$, crystalline lactic dehydrogenase, NADH, and red cell hemolysate, and is quantitated in terms of the linked reaction by which pyruvate is transformed to lactate. The conversion of NADH to NAD is measured spectrophotometrically at 340 mμ. Activity is expressed in terms of micromoles of NADH converted to NAD per 10^{10} red cells per min. This spectrophotometric procedure has been utilized widely and has proven to be a fairly reliable means of distinguishing homozygous, heterozygous, and normal subjects. The absolute activities observed in different laboratories vary with the temperature at which the assay is performed as well as with concentration of reactants employed, especially ADP and PEP. A final concentration of 2.0 mM for ADP and of 3.0 mM for PEP is about optimal (K. R. Tanaka, unpublished data).[8] Leukocytes are not affected in PK deficiency and therefore they must be removed carefully or their contribution corrected for in the assay system.

Two screening methods employing different "endpoints" have been described. Brunetti and Nenci[5] have noted that the PK reaction in the absence of a buffer is accompanied by a displacement of the pH toward alkalinity and hence they have used an indicator that changes in color during the reaction. In the technique of Beutler[1] there is a loss of fluorescence during the PK reaction since NADH fluoresces and NAD does not. Neither of these screening methods has as yet been employed on a large scale basis.

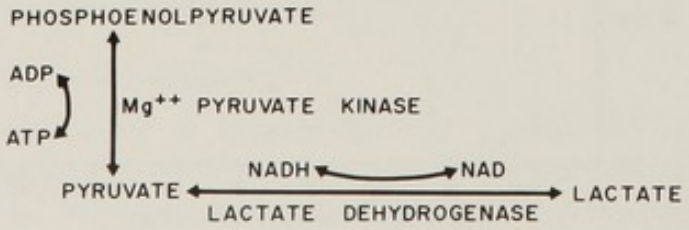


FIG. 2. Schematic illustration of the reaction catalyzed by pyruvate kinase and the subsequent conversion of pyruvate to lactate.

PK Activity

Review of the literature indicates that most PK-deficient patients have about 5 to 20 per cent of the normal (mean) enzyme level, and heterozygotes have about half the normal activity. Red cell PK values obtained by the spectrophotometric method, employing the assay conditions as described previously[39], are depicted in Figure 3 for 17 clinically-affected subjects, 56 apparent heterozygotes (mean 1.15 with a range of 0.35 to 1.69), and 59 normal individuals (mean of 2.77) examined in our laboratories. It is to be noted that there is considerable variability in activity among the homozygotes and heterozygotes. The values among the homozygotes do not correlate well with the clinical severity of their disease. Excluding three of the apparently high values among the homozygotes, who were not completely free of transfused blood at the time of assay, there were three patients with PK values well within the lower range of heterozygous values. Koler et al.[20] have reported two clinically-affected patients with PK values in the heterozygous range. The possibility of double heterozygosity for two enzyme defects, of course, must be excluded in these situations.[31] A variant form of enzyme must also be considered.

It should also be noted that several heterozygous subjects had red cell enzyme levels comparable to those seen in homozygous PK deficiency, but had normal hemograms and no evidence of clinical disease. Red cell life span is normal[31] or perhaps slightly decreased[26] in heterozygotes. There may be some disturbance of

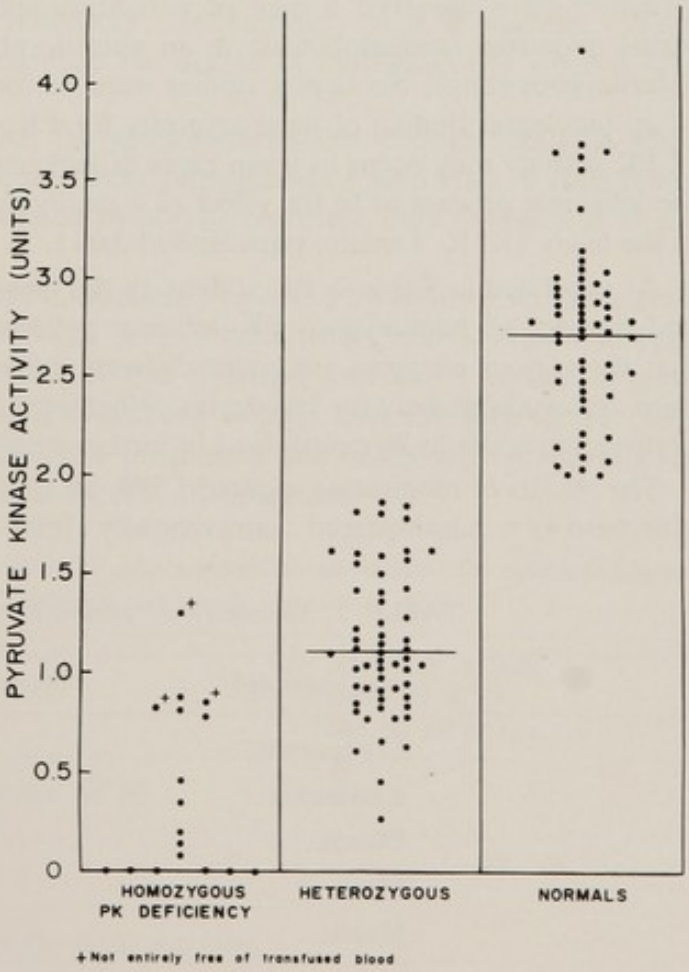


FIG. 3. Scattergram of PK assay values in 17 patients, 56 presumed heterozygotes, and 59 normals. The mean of the heterozygous group and of the normals is indicated by a bar.

red cell metabolism in these individuals with intermediate PK values. For instance, autohemolysis may be minimally increased[30,39] and certain biochemical parameters may be abnormal (discussed later). Further detailed studies of heterozygotes should be helpful in revealing the normal limits of red cell metabolism as well as the precise metabolic derangements leading to hemolysis in homozygous individuals.

Although no overlap between heterozygotes and normal individuals is shown in Figure 3, it should be recognized that the upper limit of PK value for heterozygotes was selected arbitrarily on the basis of family studies.[39] We have recently found an obligatory heterozygote (child of mother with clinical disease) with a PK value in the low normal range. There is no assurance that a complete or accurate separation of some normal subjects at the lower PK range from heterozygotes is possible by the assay technique utilized.

In spite of the limitations delineated here, it is apparent from the literature as well as from our own experience that the PK assay is extremely valuable and provides a good means of separating homozygotes, heterozygotes, and normal individuals from each other in most instances. The precautions are mentioned so that no attempt is made to translate in vitro assay values directly into metabolic derangements within the red cell on a quantitative basis.

Erythrocyte pyruvate kinase has not been found to be reduced markedly (in homozygous range) in any other condition in over 600 assays (K. R. Tanaka, unpublished data).[27,39] In a study of over 70 patients with anemia, Loder and de Gruchy[22] have observed a case of hereditary spherocytosis and one of paroxysmal nocturnal hemoglobinuria in an aplastic phase to have PK values in the heterozygous range. No family studies were performed to exclude the possibility of an incidental finding of heterozygosity for PK. A slight to moderate decrease of PK activity may occur in some cases of leukemia[27,39] which may be related to the leukemic process or to the effect of a relatively high white cell contamination in the assay (K. R. Tanaka, unpublished data).

As tabulated in Table 2, PK activity in the leucocytes[6,20,39,45] plasma,[8] liver,[6,24] and muscle[6] of homozygous PK-deficient patients has been normal, suggesting that the various enzymes are under different genetic control. However, adequate data are available only for leucocytes. Whether the defect is truly specific for the erythrocyte needs to be determined in further studies.

The results of monitoring expired $C^{14}O_2$ as a measure of the metabolism of D-glucose-6-C^{14} administered intravenously into two pyruvate-kinase-deficient

TABLE 2. *Specificity of pyruvate kinase deficiency*

Tissue examined	Pyruvate kinase activity
Erythrocytes	Deficient
Leucocytes	Normal
Plasma	Normal
Liver	Normal
Muscle	Normal

patients are shown in Table 3. There was no apparent difference between normal subjects and PK-deficient patients. This is an additional bit of evidence for specificity of the defect for erythrocytes, but it should be recognized that this is a gross evaluation and that the great reserve capacity of tissues such as hepatic cells may certainly obscure partially decreased levels of an enzyme. There may be evidence[21] for decreased content of at least one type of liver pyruvate kinase.

As yet, the nature of the red cell enzyme defect remains unknown. Biochemical aspects of red cell and white cell pyruvate kinase are considered elsewhere in this symposium[21] and will therefore not be discussed further here.

Autohemolysis

Most cases of PK deficiency demonstrate clearly increased hemolysis after 48 hours of sterile incubation, uncorrectable by glucose (Type II as originally defined by Selwyn and Dacie[36]), but correctable entirely or to a major degree by ATP.[16,39] Marked Type II autohemolysis seems to correlate somewhat with clinical severity and reticulocyte count. However, it is clearly evident from our experience and from the literature about PK patients that the autohemolysis pattern is quite variable. Autohemolysis may be mild and may be partially prevented by glucose (Type I),[7,9,10,26,32] or essentially unchanged by glucose.[8,10,16]

The increased autohemolysis of PK-deficient red cells is usually prevented by the addition of ATP, but the mechanism of this effect is still unclear. A variety of other compounds, such as AMP, ADP, NAD and coenzyme A also may reduce autohemolysis.[39] Recently, Twomey and his associates[41] have reported that incubation with ATPase inhibitors (ethacrinic acid, ouabain, and di-isochloromercuribenzoate) reduced the degree of autohemolysis when it was increased.

As Dacie[11] has re-emphasized, the autohemolysis test, though retaining some usefulness in practice, is nonspecific. Any impairment of glycolysis, if sufficiently severe, is likely to result in increased hemolysis corrected only partially, if at all, by the addition of glucose.

In the clinically severe case of PK deficiency reported by Keitt,[19] autohemolysis was 27 per cent without glucose and 45 per cent with glucose. Autohemolysis was increased in Case 1 and Case 5 and, perhaps, in Case 7 of our original series[39] of patients by the addition of glucose. Similar data are seen in the cases studied by Grimes et al.[16] Keitt[19] has postulated that inhibition of reticulocyte respiration by glucose may account for this phenomenon.

TABLE 3. *Metabolism of D-glucose-6-C^{14} administered intravenously in pyruvate-kinase-deficient patients**

	Per cent of 6 μc dose expired as $C^{14}O_2$ within 60 minutes
Normals (mean of 4)	2.3
Patient J.L.	2.4
Patient E.T.	3.0

*These studies were performed by Dr. A. D. Schwabe.

Red Cell Metabolism in PK Deficiency

As shown in Figure 4, the specific deficiency of pyruvate kinase in the erythrocytes occurs near the final step in the main pathway of glycolysis. All other reactions of the Embden-Meyerhof pathway as well as of the hexose monophosphate shunt are normally active.[6,22,23,39,45]

Pyruvate kinase catalyzes an important step in the regeneration of ATP from ADP and provides the pyruvate for the subsequent conversion to lactate. Thus, PK-deficient red cells would be expected to have difficulty maintaining normal levels of ATP and NAD. Indeed, most patients with PK deficiency have low red cell ATP levels[6,7,16,23,30,33,37,44] which may be unstable on incubation.[13,30] In addition, the content of NAD and NADH, determined together[16] or separately,[7,22] has been found to be reduced, and Oski and Diamond[30] have noted a marked fall in erythrocyte NAD during an eight-hour incubation. These observations would be in keeping with the nature of the red cell defect. However, the level of ATP may be in the normal range[19] or even increased.[18,41] Red cell ATP, assayed by the firefly method of Beutler and Baluda[2] in four of our PK patients and demonstrating the spectrum of ATP values reported in the literature, is shown in Table 4. It should be noted that patient E.T., with the highest reticulocyte count, has the highest ATP value. This is consistent with the capacity of the reticulocytes to utilize mitochondrial oxidative phosphorylation and thus to have a selective advantage in the presence of a glycolytic enzyme defect as pointed out by Keitt.[19] These normal or high ATP values are not incompatible with the presence of the PK defect in the red cells. It is of some interest that T.N., a PK heterozygote, had a reduced amount of red cell ATP (Table 4). Miwa and Nagata[26] have noted a somewhat decreased ATP content in two heterozygotes (one of whom also had a slightly increased level of 2, 3-DPG). Keitt[19] has also found a slightly low ATP

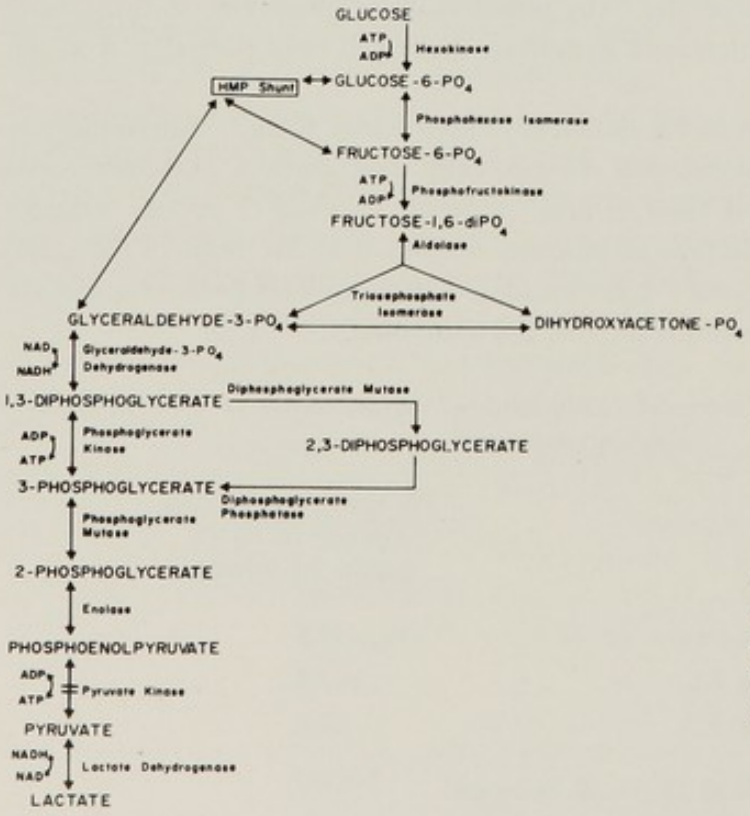


FIG. 4. Pathways of glucose metabolism in the red cell. The metabolic block near the end of the Embden-Meyerhof pathway at the level of pyruvate kinase is indicated.

TABLE 4. *Red cell ATP*

Diagnosis	Reticulocytes (%)	ATP (μmoles/g Hb)
Normal (27)	—	4.42±0.57
W.N. PK deficiency	13.6	2.77
S.H. PK deficiency	12.4	4.32
S.R. PK deficiency	15.5	5.95
E.T. PK deficiency	56.0	6.38
E.T. PK deficiency	61.6	7.03
T.N. PK heterozygote	1.6	2.86

level in one of three heterozygotes. ATP decreased more than normal in an 8-hour incubation of a sample from a heterozygote studied by Oski and Diamond.[30]

As might be expected with a metabolic handicap late in the glycolytic sequence, the concentration of intermediates above the PK step, such as PEP, 3-phosphoglycerate, and especially 2, 3-DPG, has been shown to be increased, whereas pyruvate and lactate levels have been found to be decreased or normal.[7,16,33,37,46] From their extensive studies, Loos[25] and Zurcher et al.[47] have suggested that the increased steady-state concentration of 3-phosphoglycerate in PK deficient red cells causes an increased turnover of 2, 3-DPG with a resultant decrease in the glycolytic yield of ATP in spite of normal lactate formation. Even glucose-6-phosphate, which is near the beginning of the Embden-Meyerhof pathway, has been found to be increased to twice the normal level.[28] The accumulation of various intermediates probably accounts for relatively low glucose-6-phosphate dehydrogenase activity in the presence of reticulocytosis in PK deficiency.[7,9,16,22,27]

Glucose utilization was noted initially to be impaired in Type II nonspherocytic hemolytic anemia by Selwyn and Dacie.[36] It has now been amply demonstrated that glucose utilization is defective in PK deficiency when the increased percentage of reticulocytes and the extremely high capacity of the reticulocyte for glucose consumption are recognized.[19] As shown in Table 5, glucose utilization by the red cells of E.T. with PK deficiency and a high reticulocyte count was above normal, but less than M. M. who had hemolytic anemia of an unknown type and a reticulocytosis of only 9.9 per cent. The

TABLE 5. *Glucose utilization*

Subject	Reticulocytes (%)	μmoles/ml RBC/hr
Normal (5)	—	1.5-2.4
E.T. PK deficiency	56.0	4.4
W.N. PK deficiency	13.6	2.8
T.N. PK heterozygote	1.6	2.3
M.M. hemolytic anemia, type?	9.9	5.4

erythrocytes of another PK-deficient patient, W. N., with 13.6 per cent reticulocytes, utilized glucose at a rate only slightly greater than normal blood. From his studies, Busch[7] concluded that the PK step is *not* the rate-limiting step in glucose metabolism of PK-deficient red cells and thus postulated the presence of some other critical metabolic block. Rose and Warms[34] have described kinetic studies with pyruvate kinase from normal and mutant cells and steady-state studies with whole glycolyzing cells. They have concluded that pyruvate kinase is operating normally far below saturation with respect to PEP. PK-deficient red cells reach much higher and even saturating levels of PEP in order to utilize a larger proportion of their enzyme. At high glycolytic rates in normal and deficient cells, glyceraldehyde phosphate dehydrogenase becomes rate-limiting as a result of an increased ratio of NADH to NAD and increased 1, 3-diphosphoglyceric acid. Further investigations of this nature should result in a better understanding of normal red cell metabolism as well as aiding in elucidating the exact metabolic sequence of events leading to the premature demise of the PK-deficient red cell. Currently, one must conclude that important observations have been made, but much remains to be learned before a complete understanding is achieved of the relationship between low PK assay values and hemolysis of the red cell.

Genetics

Affected subjects, equally distributed between male and female, are presumed to be homozygotes, though they vary in clinical severity. Their erythrocytes are markedly deficient in pyruvate kinase activity. Intermediate levels of enzyme activity can be demonstrated in the red cells of (1) both parents of affected children, (2) all children of an affected parent, (3) some siblings of affected subjects, and (4) some siblings of parents of affected children. These individuals with partial but detectable enzyme deficiency, not reflected in clinical disease, are presumably heterozygotes. Their hemograms are normal. Two families studied by the authors which demonstrate these findings are depicted in Figure 5. Pyruvate kinase deficiency anemia usually occurs as a single case in a family or as multiple cases in siblings. This disease is not observed in parents or children of affected patients, unless the circumstances described by R. D. Koler (personal communication) exist. He has recently found pyruvate kinase defi-

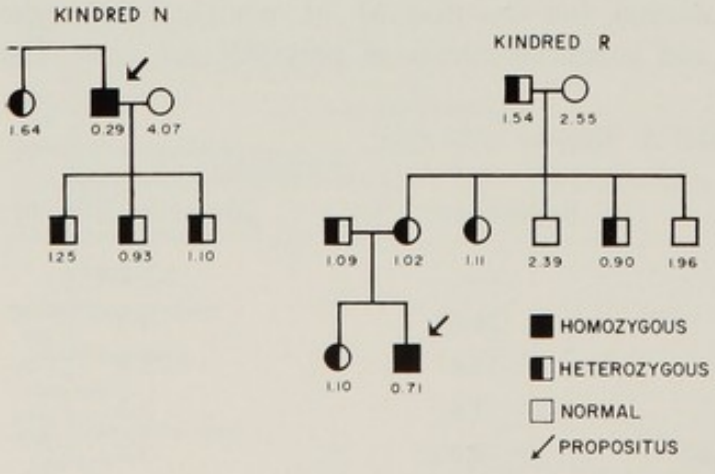


FIG. 5. Pattern of inheritance of pyruvate kinase deficiency in families N and R. An autosomal recessive mode of transmission is demonstrated. Squares designate males, and circles designate females. The chart is constructed on the assumption that intermediate values of pyruvate kinase for a member of the kindred without the disease indicates the heterozygous state and that the disease indicates homozygosity.

ciency in a parent and child. The spouse of the affected parent in this instance had red cell pyruvate kinase activity in the range of presumed heterozygosity. These various observations are most compatible with a recessive, autosomal mode of inheritance of PK deficiency.

Almost all family members may be classified as normal individuals, apparent heterozygotes, or homozygotes on the basis of the erythrocyte pyruvate kinase assay. However, there appears to be some overlap between normal and intermediate values in the red cell pyruvate kinase assay. An overlapping zone also occurs occasionally between intermediate values and those of patients with the clinical disease. Koler et al.[20] have reported two patients with erythrocyte PK values in the intermediate range but who had clinical disease otherwise not distinguishable from other PK-deficient patients. A similar instance wherein the patient with hemolytic anemia had red cell PK values in the intermediate range (like those of his parents) has been noted (K. R. Tanaka, unpublished data). No other concomitant enzyme deficiency could be demonstrated. These cases suggest that there is genetic heterogeneity. The other situation of an apparent heterozytote with a normal hemogram having a red cell PK value well within the usual homozygous range has also been observed.[39] It should be noted that there is considerable variation in pyruvate kinase values in apparent heterozygotes *among* families as well as *within* families.

Hsu et al.[17] have recently reported very low levels of PK in persons with minimal or no hemolysis in two pedigrees in which the propositi with similar levels had severe anemia. They have postulated the presence of two allelic or pseudoallelic "interacting" genes associated with PK deficiency based upon their genetic analysis and biochemical and hematologic findings. Gene "A," corresponding to the PK deficiency as previously described in the literature, in the homozygous state (not represented in their pedigrees) results in lowered ATP production, autohemolysis corrected by ATP, and moderate hemolytic anemia. Homozygosity for gene "B" results in no diminution of ATP levels, no correction of autohemolysis by ATP, and severe hemolysis. Both have "low" PK. Heterozygotes for both abnormal genes (genotype AB), exemplified by their six subjects, showed similarly low values, slight reduction of ATP, correction by ATP of slightly increased autohemolysis, and minimal hematologic abnormalities. Heterozygotes are indistinguishable from one another by PK values, and are normal clinically. The attempts to demonstrate differences in PK structure between A and B have been unsuccessful to date. Genetic polymorphism akin to other enzyme deficiencies is extremely likely in PK deficiency, but further data are needed and the interesting concept postulated by Hsu and her associates requires confirmation.

The presence of normal PK activity in leucocytes of PK-deficient patients and the studies of Koler and his associates,[20] demonstrating differences between leucocyte and red cell PK of normal blood in diethylaminoethyl cellulose chromatography, electrophoresis, solubility in $(NH_4)_2 SO_4$, kinetics, and peptide patterns, would indicate separate genetic control. In their initial studies, no chromatographic differences were detected between the red cell enzyme from PK-deficient patients and normal red cell enzyme. It remains to be determined whether red cell PK deficiency is due to a control gene mutation or a structural

gene mutation, or possibly to some other mechanism. Further data pertaining to this matter are presented by Koler and his associates[21] elsewhere in this symposium.

No linkage of PK deficiency with ABO blood groups, Rh type, and haptoglobin loci has been described,[44] but no definite conclusion may be drawn on the basis of the small number of patients that have been studied. The accumulating reports on PK patients from various parts of the world suggest a rather wide geographic distribution of the gene, but more data are necessary to determine distribution as well as frequency of the mutant gene.

Summary

PK deficiency, the first specific, heritable enzymatic defect discovered involving the main pathway of glycolysis, appears to be the most common of the glycolytic disorders (excluding those involving the hexose monophosphate shunt). It has now been documented in over 95 cases, ranging in age from birth to 65 years, and predominantly in patients of Northern European ancestry. There are no distinguishing clinical features. The usual hallmarks of chronic hemolytic anemia are present. Although the hemolytic process clearly persists, splenectomy frequently provides some degree of benefit, especially in the severe cases. There is a remarkable variation in the severity of the disease in homozygotes. The heterozygotes have about half-normal PK activity in their red cells and are clinically normal, but may have some biochemical abnormalities in their red cells.

An autosomal recessive mode of inheritance has been amply demonstrated. There is suggestive, but not precise, evidence of genetic polymorphism. The nature and exact frequency of the gene mutation(s) remain to be established.

The major clinical and metabolic features of PK deficiency have been reviewed briefly, and additional data from our studies of PK-deficient patients have been presented.

Although much has been learned in recent years, the specific sequence of biochemical events resulting from the metabolic block and leading to destruction of the PK-deficient red cell remains to be elucidated. Further intensive investigations, utilizing the PK-deficient red cell as a model, should lead to a better understanding of the normal as well as abnormal energy metabolism of the human red cell.

Acknowledgments

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DISCUSSION

LÖHR, MARBURGH, CHAIRMAN: We have also investigated about 15 homozygous cases of pyruvate kinase deficiency. The PK activities of our homozygotes were higher than those in your cases, Dr. Tanaka. Only in the homozygotes did we find a decrease of ATP and an increase of phosphoenolpyruvate in the blood cells. We do not have a sufficient explanation for the fact that a decrease in pyruvate kinase activity to 10 or 20 per cent of the normal leads to hemolysis, because the limiting enzyme glycolytic pathway never is pyruvate kinase. It is hexokinase. I think that our, and also the greater part of your, PK activities in the homozygote are higher than the rate-limiting hexokinase reaction. My second question concerns the two-allele method postulated by Hsu, Robinson and Zuelzer (ref. 17 of paper). We have carried out high voltage electrophoresis of pyruvate kinase in red blood cells almost freed of leukocytes and platelets. After high voltage electrophoresis in an acid medium two bands of enzymatic activity were seen (Figure 1). We have found that the rapidly-migrating band is absent but the slow band is present in two families with pyruvate kinase deficiency. We have extracted the pyruvate kinase from these two bands and measured the activity. The fast-migrating band contains about 60 per cent of the total activity, and the slowly-migrating one contains about 40 per cent. In our cases, homozygotes have an average of about 40 per cent normal activity. I think that this may be the explanation that only one band is absent. Perhaps this may represent a demonstration of a two-allele system in erythrocytes.

TANAKA, LOS ANGELES: I believe that Dr. Koler may present some other data about the two bands, and Dr. Townes has also done some electrophoretic work on pyruvate kinase. The values found in laboratories in various parts of the world for pyruvate kinase deficiency generally range between 5 and 20 per cent of the normal value. It is true that in certain individuals the level is higher than 20, 40, or 50 per cent, and in one or two individuals, otherwise conforming to pyruvate kinase deficiency with marked elevation of reticulocytes, etc., the pyruvate kinase activity has been low normal or about normal. Whether this is another situation remains to be proved, but most cases conform to the range of 5 to 20 per cent or at most about 40 per cent of normal. Rose and Warms (ref. 34



FIG. 1. High voltage electrophoresis of red cell PK. Upper strip: normal, Lower strip: PK-deficient.

of paper) have recently done some studies using whole cell systems of both normal and pyruvate kinase-deficient cells. They confirmed that pyruvate kinase is normally operating far below saturation as far as PEP is concerned in the red cell, and that increasing the concentration of PEP does increase the apparent activity. As you initially indicated in 1962, there may be a difference in the Michaelis constant in the pyruvate kinase-deficient group. Two of four of our patients seem to respond the way you have described (increased K_m for PEP), and there may be genetic heterogeneity or some other explanation for this.

Busch indicated that he did not feel that pyruvate kinase was a rate-limiting situation and, therefore, one must look for other metabolic errors in pyruvate kinase deficiency. Rose and Warms used pyruvate kinase-deficient red cells as well as normal cells in a whole cell system. It was shown that when these cells are working at a high glycolytic rate, glyceraldehyde phosphate dehydrogenase may become then a rate limiting step because of the increased ratio of DPNH to DPN (NADH to NAD), and therefore that this might play a role. As I have indicated earlier, the intermediates accumulate as high up as G-6-P. This may play some role in the overall impairment of metabolism, because the concentration of G-6-P that accumulates is of sufficient degree to change the rate of hexokinase activity. This indicates that we need to learn a lot more about what actually is going on in the red cell. We really do not know the mechanism of regulation of the pathway through the Rapaport-Luebering shunt. It is our feeling that the primary defect certainly is at the pyruvate kinase step. The sequence of events, however, that leads to hemolysis needs to be elucidated.

LÖHR: I partially agree, but I think you have found normal glucose utilization. If the accumulation of glucose-6-phosphate produces an inhibition of hexokinase reaction, you never could find normal glucose consumption. I think that we do not have a sufficient explanation for the metabolic block in this system. Yes, we found augmentation of phosphoenol pyruvate levels but I think we cannot explain it by the pyruvate kinase activities. Perhaps physiological activities of red cell are lower than in our test systems where we are measuring enzyme activity under optimal condition.

TANAKA: I certainly agree that if the hexokinase step were really inhibited, glucose utilization would not be normal. I did not mean to imply that accumulation of glucose-6-phosphate would shut it off entirely. I think, again, that one has to keep in mind (and I know Dr. Löhr is well aware of this) what has recently been reemphasized by studies that Dr. Keitt has performed, that the reticulocyte is really able to glycolyze at a very rapid rate. Therefore, when one has a high reticulocyte population, and one is only able to show glucose utilization just at the normal level, that is abnormal since a normal reticulocyte may be able to utilize glucose perhaps at 8 to 10 times the normal rate. Then I think one would have to say that there is a relative deficiency which probably is significant for the premature death of the pyruvate kinase-deficient cell.

BEUTLER, DUARTE: I should like to comment very briefly on our new screening method for PK deficiency that Dr. Tanaka mentioned. We have some new data that I thought might be of interest. We have tested this procedure now for the detection of heterozygotes. With the very kind cooperation of Dr. Koler and Dr. Bowman, we have had blind samples sent to us. We were able, I believe, to identify all of Dr. Koler's heterozygotes and he was able, if I understand correctly, to identify them in his laboratory using this method. We were able to identify, with certainty, all but one of Dr. Bowman's heterozygotes and were uncertain about only one. I think that if carefully done, this test probably does provide a means for screening for the heterozygous state of pyruvate kinase deficiency.

A second comment I would like to make regards our method for ATP determination with the firefly method which Dr. Tanaka used in this study. We have been able to show that the addition of protein to the ATP standard increases the reading. This, I think, represents about half the explanation for the previously unexplained high values that are

obtained. The other reason why the method gives higher results than other procedures, is that extraction of red cell ATP with trichloracetic acid turns out to be incomplete. Furthermore, the loss of ATP from stored heparinized blood is very rapid. We now recommend that when this method is used, bovine albumin be added to the ATP standard, which I think now gives true ATP levels. Finally, I would like to ask Dr. Tanaka if he could comment on the reports in the literature of PK-deficient blood samples that gained activity, not during pregnancy, but merely during storage or shipment.*

TANAKA: Our experience has been that if one takes blood in ACD and leaves it at room temperature, the values remain the same for about five days with perhaps some decrease, but not much. The temperature may vary for shipments that come to us, but we have not been able to detect any increase. We have not studied samples specifically for the effect of shipment because values were not established prior to shipment, and this, perhaps, should be done to verify what you have mentioned.

I might mention that the screening test that Brunetti and Nenci have described is not perhaps as colorful as Dr. Beutler's. However, the other method appears to have the advantage that one can use blood directly from the finger, using a usual hemoglobin pipette or some other capillary pipette. We have modified the test by taking 10 or 20 mm^3 of blood and putting it into distilled water for a minute and then mixing the reagent instead of adding digitonin as Brunetti and Nenci have done. In this way we have been able to show that the leucocytes hold up in this one-minute interval and therefore do not contribute their activity, and therefore there is less interference with high white cell counts in the peripheral blood that would interfere with the heterozygous situation in Brunetti and Nenci's system. But this test, I think, remains to be validated. Brunetti and Nenci report they were able to detect heterozygotes. I think we need more information before this screening test is proven to be as good for the detection of heterozygotes as Dr. Beutler's.

VALENTINE, LOS ANGELES: A couple of comments about the discussion of the rate-limiting aspects of the block: It seems to me, irrespective of the in vitro data, that the fact that the intermediates pile up within the cell as expected behind the block does indicate that there has been an interruption in the proper flow of glycolytic intermediates through the cycle.

The other point that I should like to mention is simply this. I think we should always keep in mind that when we assay these cell populations, whether we are assaying for an enzyme level or whether we are assaying them in terms of their capacity for glucose utilization, we are assaying the cells that are there. These are the cells that are still maintaining acceptable levels of metabolism, and the prima facie evidence of it is that they are there to assay. But this does not tell us what the activity of the moribund cell is or what the glucose utilization of any given cell will be tomorrow or next week. In order to avoid the syndrome of hemolytic anemia, the glucose utilization has to be maintained at an acceptable level over the life span of a cell that approximates normal.

SIMON, ALBUQUERQUE: The mother of a severely-affected homozygote had a normal reticulocyte count prior to and following her pregnancy. In the second and third trimester of her pregnancy, her reticulocytes were 3.9 and 4.6 per cent, respectively; there was no fall in hematocrit. The female infant, also a severely-affected homozygote, required exchange transfusion. I wonder if others have observed hemolysis in heterozygotes with pregnancy or at other times. Secondly, Doctor Tanaka, I wonder whether you have encountered any situation in which the autohemolysis of defibrinated blood was elevated and in which supplemental ATP did not reduce the level of autohemolysis. Finally, is the ATP you used neutralized or acid?

TANAKA: We have not encountered any

*Wiesmann, U., Tönz, O. Richterich, R. and Verger, P.: Dei Erythrocyten-Pyruvat-Kinase bei Gesund un und bei Nichtsphärocytärer hämolytischer Pyruvat-Kinase-Mangel-Anämine. Klin. Wchnschr. 43:1311-1318, 1965.

patient that we consider to be homozygous for pyruvate kinase who had marked hemolysis which is not corrected, at least to a moderate degree, with ATP. I think that when one gets marked hemolysis, one cannot expect full correction or prevention by ATP, but certainly correction for the most part occurs. However, Oski and Diamond (ref. 30 of paper) have described a patient with severe spicule formation that did not correct with ATP. I am not aware of other reports.

Initially, all the studies were done with acid ATP. Then some further studies were done using neutralized ATP, and it still seems to work; but, as I have mentioned previously, we certainly do not know the actual mechanism.

Now as far as the first question is concerned, we have not had the opportunity to examine homozygous patients during pregnancy. She presumably would have to be a heterozygote and, as I have mentioned, the heterozygous PK-deficient red cell could have accelerated destruction as indicated by studies showing some decrease in red cell survival, increase in 2,3 DPG, or slight increase in autohemolysis. Perhaps, the combination of pregnancy and minimal impairment of red cell pyruvate kinase may have caused some degree of hemolysis in your patient. We have not studied that.

WILLIAMS, LOS ANGELES: Dr. Tanaka, have you seen any aplastic crises in these patients? You did mention a marked drop in reticulocytes with infections.

TANAKA: Yes, one of our patients developed a gall bladder attack and had surgery at that time. Otherwise, he had been maintaining essentially normal levels. During that episode of illness his hemoglobin dropped from about 14 to 7.6 g and then recovered spontaneously. There was a reduction during that time in his reticulocyte count, but not really to an aplastic level.

VALENTINE: I might just mention that we have certainly seen the other situation: the patient who, under stress or with an intercurrent infection, has a quite marked drop in hemoglobin levels but at the same time becomes jaundiced and develops more reticulocytes.

What happens when you put ATP into the autohemolysis test is something that has been very puzzling. Certainly, if you do not add it neutralized, you change the pH of the system very profoundly be-

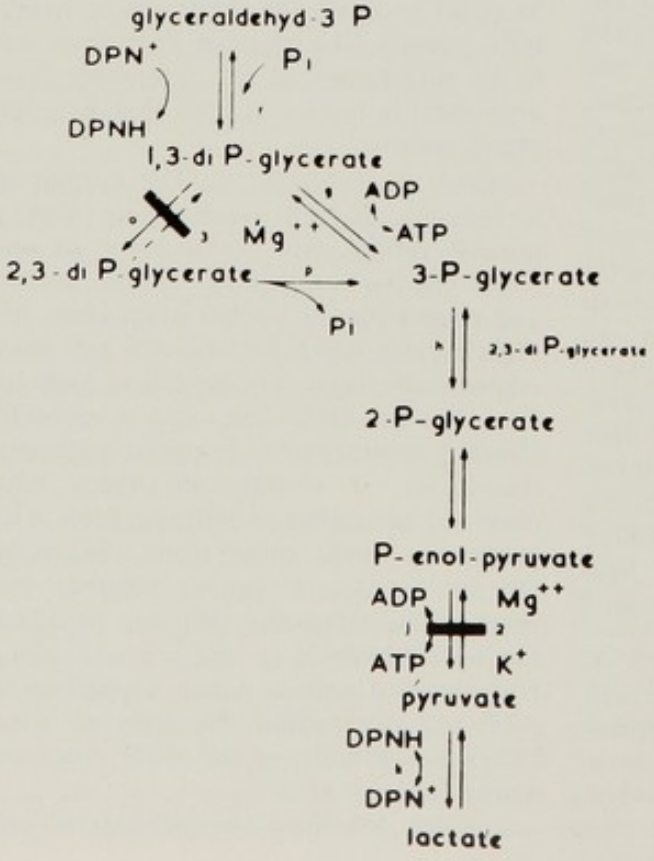


FIG. 2. Schematic representation of the last part of the Emden-Meyerhof pathway in human erythrocytes.

cause it is intensely acid. We have had the experience of ATP correction of autohemolysis in many kinds of hemolytic anemia that appear to be hereditary, but in which we have not been able to find a specific enzyme defect. We presume that we are not measuring the right thing and that ATP has, in a rather ubiquitous way, corrected a lot of different situations. And I think we should recall that ATP complexes magnesium in the external environment; there are osmotic factors which may be involved, as well as a lot of things which make it pretty obscure just what ATP actually does.

LOOS, AMSTERDAM: I should like to ask Dr. Tanaka two questions: the first question is, do you agree with me that the PK estimation in hemolysate frequently gives tricky results and that it is advisable to do more estimations of the erythrocyte 2,3-DPG-content in PK-deficient red cells or those suspected of being deficient? As you mentioned, we found already that during pregnancy one of our PK-deficient patients had erythrocytic PK activity increased to more than two times the level of normal erythrocytes, while the 2,3-DPG level remained very high. So we could have missed the PK deficiency in this patient at that time if we had not done the 2,3-DPG estimation. My second question is: you told us that in the PK-deficient erythrocytes, the steady state concentration of 3-phosphoglycerate is increased. It may be that the flow of traffic through the 2,3-DPG-cycle is promoted by an increased 3-phosphoglycerate content of the cell, while the total glucose consumption is hardly influenced. An increased formation of lactate from 1,3-DPG will decrease the phosphorylation of ADP to ATP in the phosphoglycerate kinase reaction, and so the net gain of energy-rich phosphate bonds per molecule of lactate produced is diminished. Do you think that the biochemical features of pyruvate kinase-deficient red cells may be explained on the basis of a too active Rapaport-Luebering-cycle, or in other words, on the basis of an in-

TABLE 1. *Biochemical and hematologic changes in a homozygous PK-deficient patient during pregnancy*

Date	PK activity	G-6-PD activity	ATP	2,3-DPG	Hb (g%)	Reticulocytes (%)
6-9-64	4	19.3	0.88	7.5	8.3	200
± 4° month of pregnancy = October '65						
10-29-65	19.2	27.6	—	—	7.0	760
12-22-65	24.1	28.2	0.86	6.2	7.7	830
1-25-66	28.2	27.8	1.14	9.1	7.0	465
transfusion of 500 ml of blood at 1-26-66, at 1,3, and 2-4-66						
transfusions of 200 ml of packed erythrocytes were given						
5-2-66	—	—	—	—	10—	90
transfusions of 200 ml packed cells were given at 7, and 2-8-66						
2-9-66	—	—	—	—	10.8	—
3-21-66	35.6	22.2	1.22	6.0	9.8	89
5-20-66 delivery of a healthy child (female)						
4-25-66	28.0	20.5	0.94	6.3	8.9	145
6-6-66	9.3	21.4	0.84	9.7	9.6	423
normal values	16.7	13.7	1.13	3.6	13.7	<20
s.d.±	4.2	2.7	0.13	0.7	0.8	

The enzyme activities are expressed in Mark's units. ATP and 2,3-DPG are given in μmoles/ml of cells.

efficient glycolysis in these cells (Figure 2)? The data summarized in Table 1 may be of some use in discussing this matter.

TANAKA: I certainly agree that this shunt probably does play an important role in pyruvate kinase deficiency as well as in other situations. If glucose goes primarily through the pathway, glucose would be utilized but would not yield an equivalent amount of ATP. As to what this really means in the PK-deficient cell, I am not able to say. Measuring one particular compound like 2,3-DPG is certainly important, but I think being able to demonstrate a specific defect would be a better means of detecting the patient's problem in the sense of diagnosing the disease. I certainly recognize that if the PK activity goes up during pregnancy, one might miss the situation, but fortunately this would be a rare occurrence. In overall studies of patients, the PK assay would be better, although I certainly agree that studying intermediates is very important in this kind of a problem.

Biochemical Properties of Human Erythrocyte and Leukocyte Pyruvate Kinase

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SINCE SIR ARCHIBALD GARROD first pointed the way in his classic studies on alkaptonuria,[6] rare human maladies have served to illuminate important genetic and metabolic mechanisms. Our interest in human pyruvate kinase (PK) began because of problems in the clinical care of members of several families who have hereditary nonspherocytic hemolytic anemia. When Valentine, Tanaka and Miwa in 1961[19] reported their findings in similar families of a specific deficiency of erythrocyte PK in homozygous-affected patients and intermediate levels of the enzyme in unaffected carriers, we applied their assay method and obtained comparable results in six of our families.[12] One difficulty with the assay is the need to separate erythrocytes from leukocytes since the level of PK in leukocytes from these patients is normal. This difference in the genetic control of the same enzyme in different tissues has continued to interest us, and the studies reviewed in this report have been directed toward gaining some understanding of the genetic control and the physiologic role of these two isozymic forms of PK.

EVIDENCE FOR DIFFERENT ISOZYMIC FORMS OF PK

Some biochemical properties of the erythrocyte and leukocyte forms of PK are clearly different, but there are also interesting points of similarity. The use of the term isozymes is in keeping with the original definition by Markert and Moller[14] but also carries some more specific connotations.[8]

Chromatographic Differences

An anion exchange column using DEAE cellulose and gradient elution with KCl (Fig. 1) resulted in two fractions.[12] Leukocyte PK is not adsorbed and comes off at the column front. Erythrocyte PK is eluted at .04-.12 M KCl. More recent studies in which a cation exchange column of CM Sephadex and a pH gradient[17] were used also suggest chromatographic differences.

Electrophoretic Differences

Our initial observation[12] of separation of the two forms of PK by starch block electrophoresis at pH 7.5 has since been confirmed with starch gel electrophoresis and histochemical demonstration of PK activity.[2] In both systems the

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erythrocyte form moves further toward the anode than does the leukocyte form.

Solubility in Ammonium Sulfate

The leukocyte form is precipitated at a concentration of 50 to 70 per cent $(NH_4)_2SO_4$ and the erythrocyte form at a concentration of 25 to 35 per cent from crude homogenate or from later stages of purification, following chromatography. Even on repeated precipitation, the preparations obtained have not yet yielded crystalline enzyme, but have been stable during storage at —15° C for up to 18 months.

Antigenic Differences

An antiserum to partially purified erythrocyte PK was prepared by giving New Zealand white rabbits (weighing 2.5 to 3.0 kg) three injections of 4 ml of antigen at one-week intervals. The antigen was prepared from hemolysate of leukocyte-free red cells by DEAE chromatography and precipitation of the .04-.12 M KCl eluate between 20 and 40 per cent $(NH_4)_2SO_4$. Each 4 ml injected contained 2 ml of Freund's adjuvant plus 2 ml of an enzyme preparation, which had 9 units of PK and 156 mg of protein per ml. The animals were bled on the 35th day, and serum was harvested. Although no specific precipitin reaction was detected, the antiserum caused inactivation of erythrocyte but not of leukocyte PK. The conditions used were incubation at 4° C for two hours in mixtures of 0.2 ml of antiserum with 0.1 to 0.5 ml. of test material containing 0.01-0.04 units of PK. In the case of erythrocyte PK, from 81 to 99 per cent of the activity of the control sample, without antiserum, was lost) whereas less than 8 per cent of the activity of leukocyte PK disappeared under the same conditions.

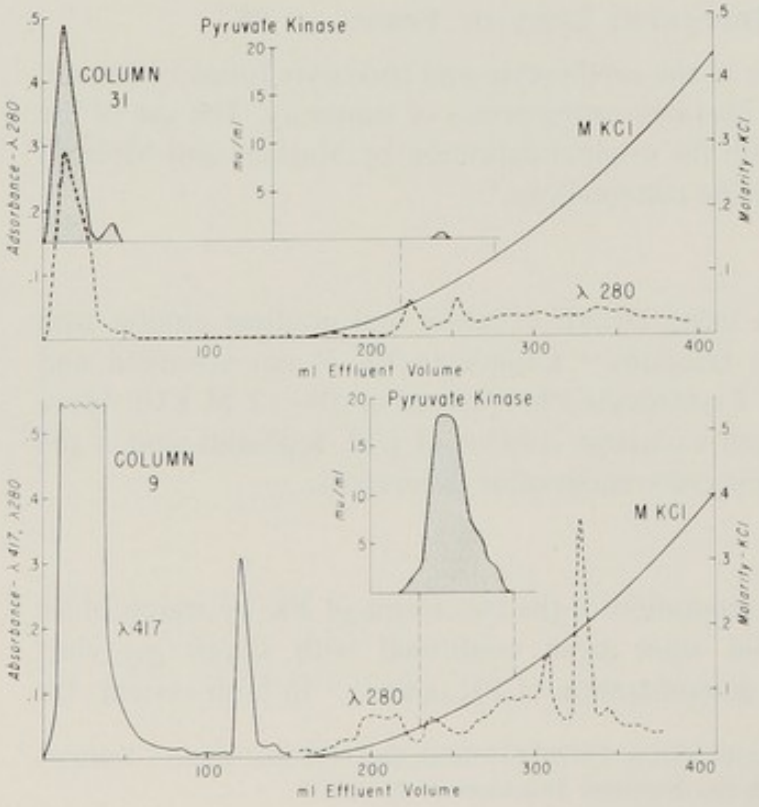


FIG. 1. DEAE chromatograms showing recovery of leukocyte PK (Column 31) and erythrocyte PK (Column 9). (See reference 12 for method.)

Kinetic Differences

Preparations of human erythrocyte and leukocyte PK purified by DEAE chromatography[12] have been compared in their kinetic properties.[3,4] Leukocyte PK (Fig. 2) has a K_m for phosphoenolpyruvate (PEP) of 1×10^{-4}M and for adenosine diphosphate (ADP) of 1.78×10^{-4}M. The results are compatible with a random addition of substrates in which the combination of the enzyme with one substrate does not influence its affinity for the second substrate Erythrocyte PK under the same conditions (Fig. 3) has a ten-fold higher requirement for PEP and a dependence of K_m for one substrate on the concentration of the second, suggesting that there is an ordered binding of ADP and PEP. A competitive inhibition between adenosine triphosphate (ATP) and PEP (Fig. 4) occurs with a K_i for ATP of 3.5×10^{-4}M. Rose and Warms[16] confirmed these results for human erythrocyte PK and made the observation that lower concentrations of magnesium (5 mM vs 15 mM) and the substitution of imidazol for triethylammonium buffer results in appreciably lower estimates for K_m PEP. They also confirmed the competitive inhibition of ATP for PEP.

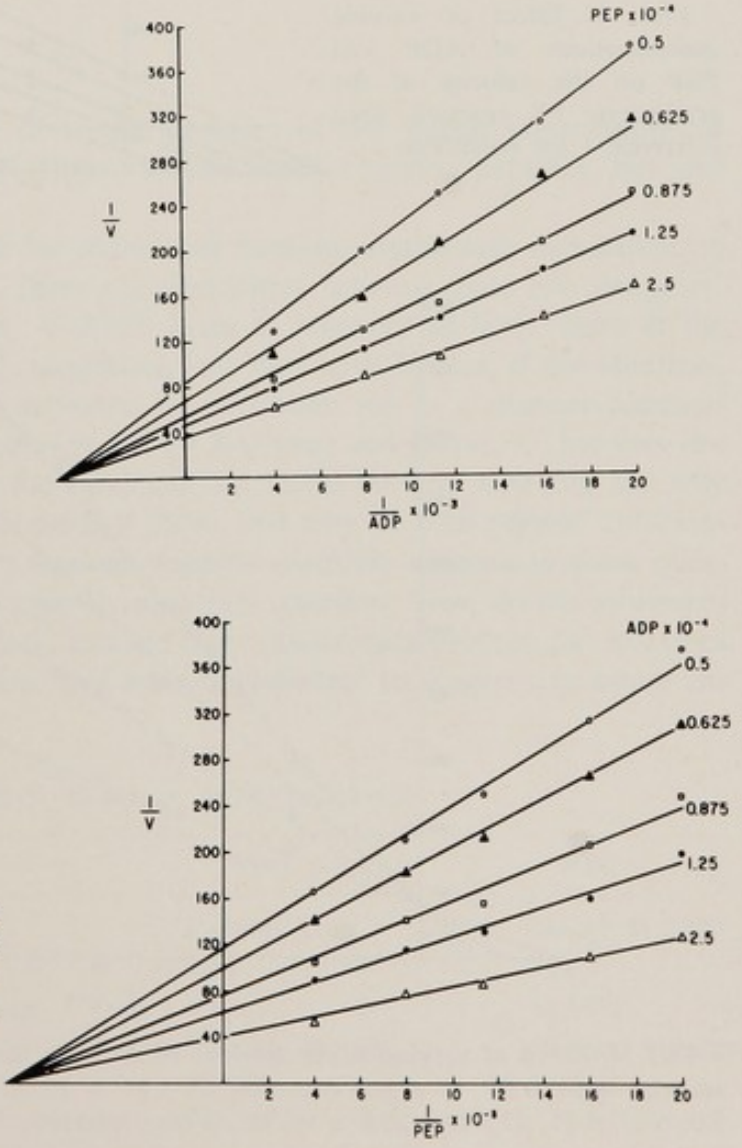
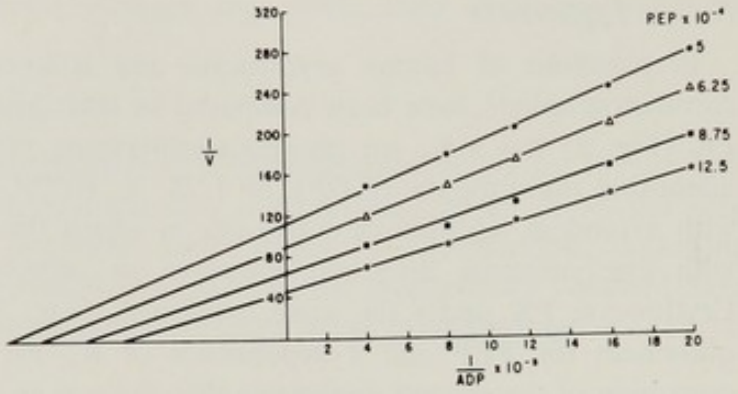


FIG. 2. Effect of varying concentrations of ADP and PEP on the velocity of leukocyte PK reaction. (See reference 4 for conditions.)



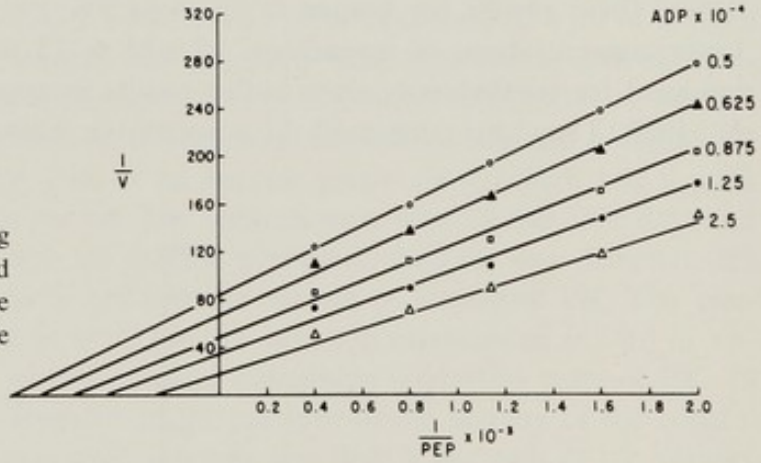


FIG. 3. Effect of varying concentrations of ADP and PEP on the velocity of the erythrocyte PK reaction. (See reference 4 for conditions.)

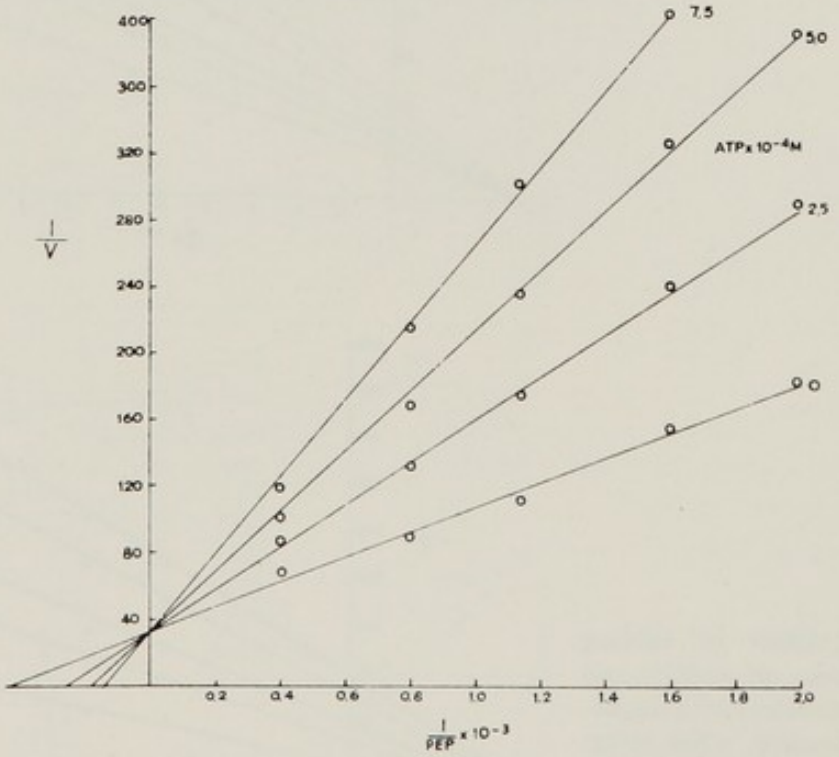


FIG. 4. Effect of varying concentrations of ATP on the velocity of the erythrocyte PK reaction with ADP at a concentration of 1.25 x 10^{-4}M and PEP at concentrations of 5.0, 6.25, 8.75, 12.5 and 25.0 x 10^{-4}M. (From reference 3.)

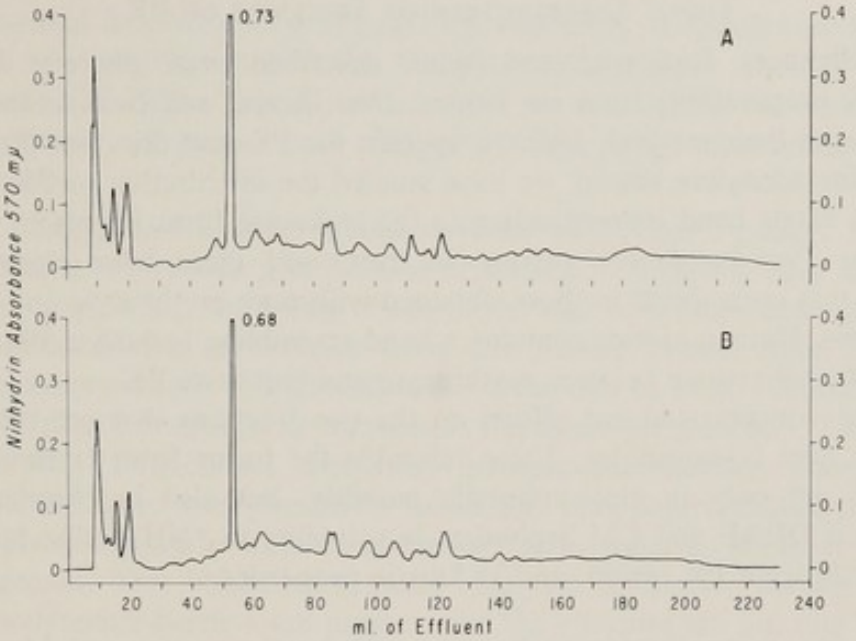


FIG. 5. Tryptic peptide patterns from automatic column chromatography of partially purified leukocyte PK (A) and partially purified erythrocyte PK (B). (See reference 12 for method.)

Structural Differences

Chromatographic patterns of tryptic peptides of our purest preparations of leukocyte and erythrocyte PK (Fig. 5) show many common peptides, but also definite differences.[12]

In contrast to the evidence for differences between erythrocyte and leukocyte PK, summarized in Table 1, there are similarities between these two isozymes. Gel filtration using Sephadex G-200[12] leads to recovery of both forms at the same region ($V/V_o = 1.74$), suggesting that they have similar, if not identical, molecular weights. Both are activated by potassium ion in a manner identical with that reported for rabbit muscle PK by Kachmar and Boyer.[10] That they are different molecular forms of the same enzyme seems certain. One can ask why the two forms have evolved in the first place, and why do both persist? Information reviewed in the next two sections suggests plausible answers to these questions. Also under study is an equally important question: How do the structures of the two forms of PK compare, and are there homologies between the isozymes like those found in hemoglobins[9] and immunoglobulins[7] to suggest a common ancestral gene?

TABLE 1. *Summary of PK properties*

	WBC	RBC
DEAE	Column front	.04-.12 M KCl
Electrophoresis	Slow	Fast
Inactivation by anti-RBC PK serum	0-8%	81-99%
Kinetics with ADP and PEP	Random binding	Ordered binding
Solubility in $(NH_4)_2 SO_4$	50-70%	25-35%

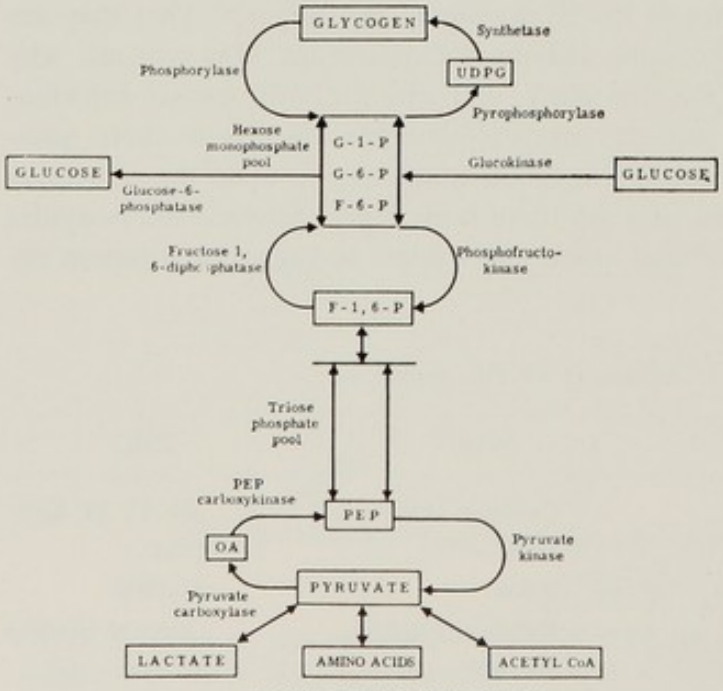
Tissue Distribution of Isozymes of PK

Von Fellenberg, Richterich and Aebi[20] described electrophoretic differences among PK preparations from rat kidney, liver, brain, and cardiac and skeletal muscle. Using their method, which is specific for PK and does not give positive reactions for adenylate kinase, we have studied the distribution of PK in human tissues.[2] A single band corresponding to the leukocyte form is present in muscle and brain. Two bands are present in liver, and these have electrophoretic mobilities that correspond to those obtained with pure erythrocyte and leukocyte preparations. Human kidney contains a band resembling leukocyte PK and a second band intermediate between erythrocyte and leukocyte PK.

We have concentrated our efforts on the two fractions that are reproducibly present in liver homogenates. These resemble the forms from erythrocytes and leukocytes not only in electrophoretic mobility, but also in chromatographic behavior on DEAE and CM Sephadex, in solubility in $(NH_4)_2SO_4$, inactivation by antierythrocyte PK serum, and in kinetic properties.[2]

Role of Pyruvate Kinase in Regulation of Carbohydrate Metabolism

Glucose metabolism in the erythrocyte is almost exclusively unidirectional from glucose to lactate through the Embden-Meyerhof pathway. Keitt[11] has recently reviewed the energy metabolism of the red cell and the importance of the 2,3-diphosphoglycerate phosphatase shunt as an "energy clutch" in uncoupling glycolysis from ATP generation. The effect of inorganic phosphorous (P_i) in stimulating erythrocyte glycolysis has prompted a number of studies reviewed by Rose and Warms.[16] Calculation of free energy changes from steady-state levels of glycolytic intermediates and nucleotides found in normal erythrocytes led to the identification of three rate-limiting steps. These involve the reactions catalysed by hexokinase, phosphofructokinase (PFK), and PK.[15] Rose and Warms[16] cite evidence that the stimulatory effect of P_i on erythrocyte glycolysis



Location of rate-limiting enzymes.

FIG. 6. Location of rate-limiting enzymes. (From reference 24.)

is the result of its ability to counteract the inhibition of hexokinase by glucose-6-P and of PFK by ATP. The increase in glycolysis results in a high concentration of PEP. This, in turn, causes an increase in the activity of PK, which normally functions in a region of first order dependence on PEP concentration.

These findings would argue against anything more than a passive role for PK in determining the rate of glycolysis in erythrocytes. That PK may be the most important enzyme for controlling the direction and, to a lesser extent, the rate of glucose metabolism in mammalian liver, has become evident from the work of Weber and coworkers.[21-26] Figure 6, taken from one of their publications,[24] is a diagram of the three groups of enzymes in the anaerobic pathway. These include the following bifunctional enzymes: phosphoglucomutase, phosphoglucose isomerase, aldolase, triosephosphate isomerase, phophoglyceraldehyde dehydrogenase, phosphoglyceric kinase, phosphoglyceric mutase, enolase, and lactic dehydrogenase. This bifunctional group catalyses reversible reactions in which no thermodynamic barriers are present, and the enzymes are not rate limiting.

The remaining enzymes fall into two groups that are unidirectional and rate-limiting. Weber has applied the term "key gluconeogenic enzymes" to glucose-6-phosphatase, fructose 1,6-diphosphatase, phosphoenolpyruvate carboxykinase, and pyruvate carboxylase. The importance of these four in regulating gluconeogenesis is apparent since their activities are the lowest in the gluconeogenic sequence, they are involved in circumventing thermodynamic barriers, and they are limited to tissues in which gluconeogenesis is known to occur, e.g., liver and kidney. More importantly, Weber has shown that the DNA-directed synthesis of these four key gluconeogenic enzymes is coordinately induced by glucocorticoids and repressed by insulin.

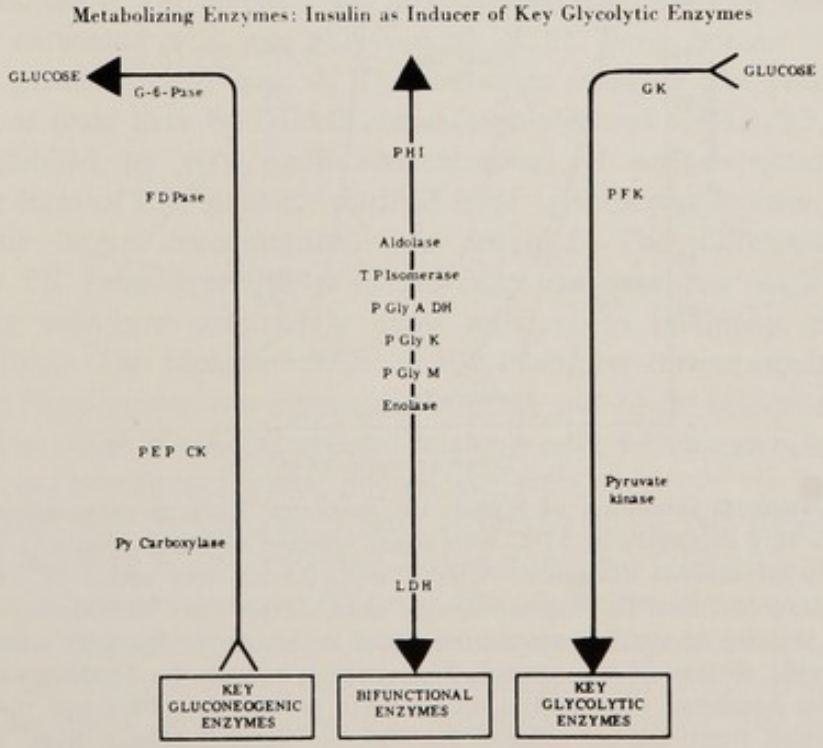


FIG. 7. The three functional genome units. (From reference 25.)

The oppositely directed "key glycolytic enzymes" in liver consist of glucokinase, PFK, and PK. They are involved in the transfer of high-energy phophoryl groups and are the antagonists of the key gluconeogenic enzymes. Weber has presented evidence that the DNA-directed synthesis of all three is unaffected by glucocorticoids and induced by insulin. He has proposed two "functional genic units" (Fig. 7), one for each group of key enzymes, and has suggested that these respond in opposite ways to insulin and to glucocorticoid hormones.

Our studies of tissue distribution[2] indicate that the majority, or some 80 per cent, of PK in human liver is of the erythrocyte type, and that the remaining 20 per cent is of the leukocyte type. Tanaka, Harano, Morimura, and Mori[18] have found two major forms of PK in rat liver. These are similar in physicochemical properties to the two forms we have described in human tissues. They have termed the form that resembles human erythrocyte PK, "L Type," and that which resembles human leukocyte PK, "M Type," for liver and muscle, respectively. No studies of rat erythrocyte enzyme were presented. Changes in the amount of the two isozymic forms of rat liver PK in response to fasting, alloxan-induced diabetes, and diabetes after treatment with insulin led them to conclude that the L-Type is directly related to the metabolic control of gluconeogenesis. The level of M-Type PK, which corresponds to that described in human leukocytes, was not affected by external conditions.

Although more work needs to be done to confirm the identity of erythrocyte

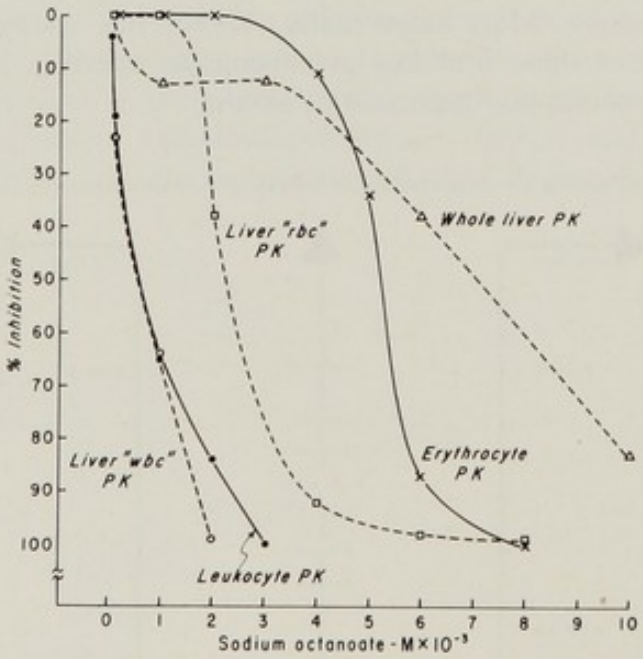


FIG. 8. Octanoate inhibition of human PK isozymes. Enzyme preparations containing 0.4 u PK/ml were incubated at 37°C with equal volumes of water (control) or increasing concentrations of sodium octanoate. After 5 min, 0.1 ml was added to 2.9 ml of the reaction mixture described by Valentine et al.[19] and the per cent inhibition was plotted by relating the velocity at varying concentrations of octanoate in the final reaction mixture (0.1 to 10 mM) to that of the control. The samples include the 28000 x G supernatant of an aqueous homogenate of normal human liver (whole liver PK) and the ammonium sulfate precipate from DEAE column fractions of normal human liver, erythrocytes, and leukocytes (see text).

PK, as we have characterized it, and L-Type PK as described by Tanaka et al.,[18] this isozyme would appear to possess the important responsiveness to hormonal control of its biosynthesis attributed by Weber to liver PK. As noted above, erythrocyte PK probably plays no significant role in the control of glycolysis in the red cell. Any reason for its existence in evolutionary terms must then relate to its function in glucose-producing tissues such as liver.

Weber[21] has extended his investigations from the hormonal induction and repression of key gluconeogenic and glycolytic enzymes, which he calls "chronic adaptation," to the problem of rapid changes in the direction of overall metabolic pathways in liver, which he calls "acute adaptation." The capacity to switch from glycolysis to gluconeogenesis is known to occur much more rapidly than could be accounted for by hormonally-induced biosynthesis of the key gluconeogenic enzymes. In comparing the activities in rat liver of the opposing enzymes at the three key steps, Weber[21] noted ratios that favor gluconeogenesis at the glucose-6-P to glucose and the fructose 1, 6-diphosphate to fructose-6-P steps, but, at the pyruvate to PEP step, glycolytic activity, represented by PK, is more than 30 times as great as the activity of the gluconeogenic enzymes, phosphoenolpyruvic carboxykinase, and pyruvate carboxylase. This large barrier to PEP formation requires a mechanism for acutely-inhibiting the activity of PK in order for gluconeogenesis to occur. Among the candidates for such a role in the rapid adjustments from glycolytic to gluconeogenic metabolism, are free fatty acids.[5,13] Weber deduced that specific inibition of the key glycolytic enzymes by free fatty acids could be the "metabolic directional switch," and demonstrated such an inhibitory effect of sodium octanoate.[21] Both the degree of inhibition and the specificity for the key glycolytic enzymes, but not for the gluconeogenic or bifunctional enzymes, are consistent with the hypothesis.

In collaboration with Weber, we have studied the effect of preincubation of sodium octanoate with our preparations of PK from human tissues. The results are presented in Figure 8. The leukocyte form of PK, prepared from leukocytes or from liver by DEAE chromatography and $(NH_4)_2SO_4$ precipitation, is inhibited by very small concentrations of sodium octanoate. The erythrocyte form of PK, similarly purified from erythrocytes or liver, is also inhibited, but larger concentrations are required. The difference between erythrocyte PK from liver and from red cells has been less marked in later studies, but both are consistently more resistant to inhibition than is the leukocyte form. The biphasic curve of PK inhibition by octanoate in whole supernatant from human liver agrees well with the sum of the behavior of the two isozymes. The range of concentration of free fatty acid, which causes inhibition of both forms, is probably well within physiologic levels.

It is possible that the different kinetic properties of the two forms of PK, as reflected by differing K_m for PEP and inhibition by free fatty acids, account for their evolution because such versatility confers a selective advantage. The results of Tanaka et al.[18] might be used to argue that a more important difference is the response of the erythrocyte but not of the leukocyte form of PK to hormonal induction. If so, and if Weber's idea of a "functional genic unit" for the three key glycolytic enzymes is considered, one might predict that structural genes for a

subunit of at least one isozymic form of all three will be found to be shared or closely linked.

If the erythrocyte form of PK has the important properties of serving as the site of action of a directional switch for gluconeogenesis in the liver, it will be necessary to explain the normal glucose metabolism of patients homozygous for deficiency of this isozyme. We have recently had the opportunity to assay the two isozymes of PK present in a liver biopsy from one of our patients.[1] At the time of an elective cholecystectomy, a sample of liver was obtained from subject C. J. (vid. 3). The comparison of the total PK activity in whole supernatants from her liver with that from the liver of two normal subjects indicates that she has about half of the normal activity (7.13 units of PK/g vs. 14.45 and 15.0 units of PKg). The amount of leukocyte PK based on DEAE chromatography of the whole supernatant was comparable in all three (2.82 unit/g vs. 2.35 and 2.16 units/g). The major difference, therefore, is due to a reduction in the erythrocyte form, and when this is expressed as per cent of normal for liver (26 per cent), it is quite similar to the per cent of normal for erythrocytes (22 per cent). One possible conclusion is that a reduction of this amount in the erythrocyte isozyme is far more deleterious to erythrocytes than to liver cells. Another is that until structural studies and more detailed information about the biosynthesis, physiocochemical, and functional properties of purified isozymes of PK are available, the observations reviewed here serve only to raise more questions than can be answered.

Summary

Two isozymic forms of PK, which were first studied because only one is present in human erythrocytes and the other in human leukocytes, are described. They have different genetic control, physicochemical, antigenic, and kinetic characteristics. Studies that suggest different functional properties, including a role in the control of carbohydrate metabolism by the erythrocyte form, are reviewed.

Acknowledgments

We thank Mrs. Irene Pierovich, Marjorie Bumm, Marie Duerst and Carolynne Dean for technical assistance in these studies. Dr. Richard Jones has given invaluable advice, and Dr. George Weber has kept us informed about his work on rat liver and has pointed out the importance of studying the inhibitors and activators of PK. Drs. Jack Fellman and Marvin Hines have advised us in the kinetic studies.

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Discussion

LÖHR, CHAIRMAN, MARBURGH: Dr. Koler's paper is open for discussion.

TOWNES, ROCHESTER: I have just a few comments to make with regard to Dr. Koler's very beautiful and extremely extensive study of the enzyme, pyruvate kinase. I should like to refer to some work that Dr. Harris and I have done very recently. We have examined the starch gel electrophoretic behavior of human pyruvate kinase, and, in general, our observations are in accord with Dr. Koler's. We examined red cell lysates by horizontal starch gel electrophoresis in

various buffer systems over a rather wide range of pH. The two systems that we found to be most informative were at pH 8.6 and 5.0, triethanolamine and succinate, respectively. For visualization we used the technique that was first used by von Fellenburg, Richterich and Aebi (ref. 20 of paper), employing coupling with a lactic dehydrogenase system and examination under ultraviolet light for the oxidation of DPNH to DPN. Red cell lysates subjected to these conditions, and electrophoresed for approximately 16 hours at pH 8.6 in triethanolamine buffer, revealed patterns that are schematically shown in Figure 1.

There is an anodal migration of enzyme, and examination of washed red cells reveals a slow band (S) close to the origin and a faster band (F), which has a mobility that is very close to that of hemoglobin A_2. White cell preparations have only a slow band and give no evidence of a fast band. The liver has a pattern that is indistinguishable from that of red cells. This is in keeping with what Dr. Koler already presented.

Other tissues—e.g., kidney, brain, heart, and muscle—and cultures of fibroblasts have only the slow band that appears to be relatively immobile under these conditions of electrophoresis. I might point out that the pattern observed in liver is probably not due to red cell contamination because there is very little hemoglobin present in the preparations. Comparable hemoglobin dilutions in red cell sonicates are far below the level at which PK is detected in this system. The liver is much more active with regard to this enzyme.

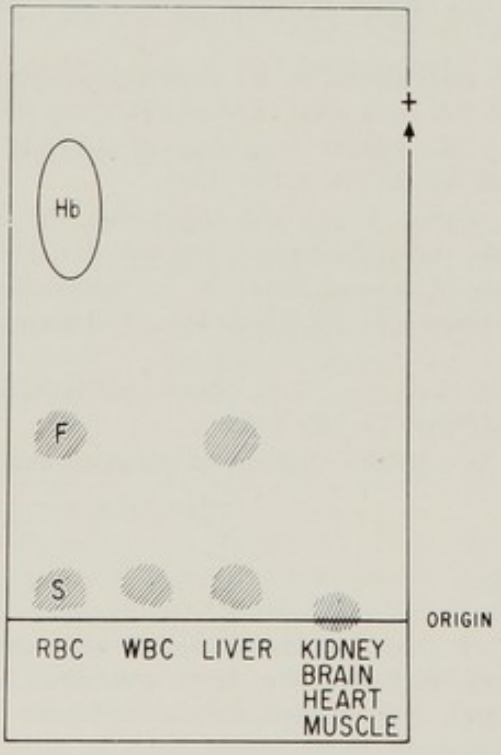


FIG. 1. Pyruvate kinase localization following electrophoresis at pH 8.6, triethanolamine buffer 0.015 M.

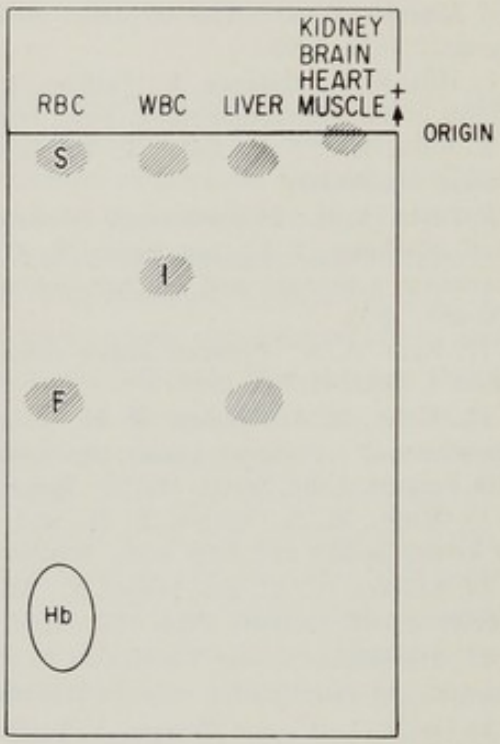


FIG. 2. Pyruvate kinase localization following electrophoresis at pH 5.0, succinate buffer 0.005 M.

Similar preparations in the succinate system at pH 5 follow the patterns that are illustrated in Figure 2. This shows a cathodal migration, and the patterns are very similar to those obtained at the higher pH. The one difference that appears to be consistent is that the white cell band now splits into two components, one which has a mobility roughly intermediate to the fast and slow bands of the red cell and the liver. This provides a rather convenient means of detecting white cell contamination, because this intermediate band does not appear in our washed red cells. As a further check for white cell contamination, we have used ACD-aged red cells after repeated wash-

ings. The kidney, brain, heart, and muscle enzyme are relatively immobile at this pH and so we have not succeeded, even at the two extremes of pH, to move significantly these enzymes.

We have examined these same components in a two-dimensional system, where in one, first electrophoresis is at pH 8.6 and then at pH 5. It appears that the slow band is slow at both pH 5 and 8.6 and that the fast is fast at both pH's. This suggests to us that the relative electrophoretic mobilities may possibly be related to molecular size in view of the well-known sieving properties of gel, and for this reason we examined red cell lysates on BioGel 200. The results of such an analysis are shown in Figure 3.

The BioGel 200 gel filtration curve (Figure 3) shows the distribution of hemoglobin, intrinsic LDH, and the blue dextran marker. When one pools fractions from such a column, concentrates them by pressure ultrafiltration, and then takes the concentrates and puts them on gel at pH 5, one sees the distribution in terms of electrophoretic behavior as it is shown in the upper left insert. This suggests that larger molecular size is represented by the slow band and that the white cell intermediate band represents molecules of intermediate size. The red cell fast band represents molecules of a relatively smaller size.

We have examined bloods of several PK-deficient individuals. We find that both slow and fast bands are absent in PK-deficient individuals. If this observation should prove to be valid, then one would assume that the slow band and the fast band are products of a single gene and there the difference in the molecular size probably reflects relative states of aggregation, something perhaps of the order of a monomer-dimer relationship.

BIGLEY, PORTLAND: First of all, I think that the apparently greater heterogeneity that Dr. Townes is seeing in red cell preparations had best be examined by trading techniques; we are most anxious to do this. I think I should point out that the amount of pyruvate kinase per leucocyte is about 50 times that per red cell. Given a contamination of, say, 500 white cells per cu mm of red cells, the amount of pyruvate kinase contributed by the leucocytes might be as much as 1/50 of that contributed by red cells.

TOWNES: I realize that the total PK activity of whole blood is actually more than that of the RBC's. The WBC activity is much greater per cell so that 50 per cent of the total activity may be due

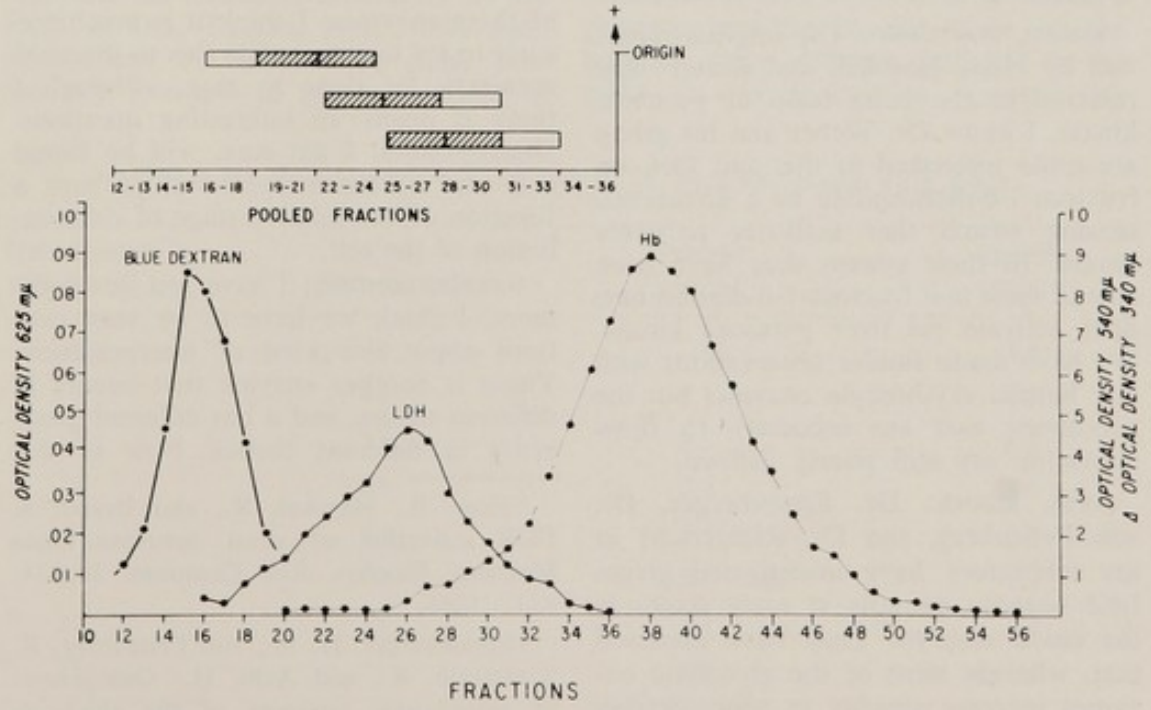


FIG. 3. Gel filtration elution pattern of white cell and red cell sonicate on BioGel P-200. In upper left insert is shown the electrophoretic localization of pooled eluate fractions.

to white cells. If we dilute our whole blood sonicates six-fold, however, we are below the level of detection for both bands. Therefore, one would require fairly gross white cell contamination to have detectable white cell activity. I do not think that the cells aged a week or more in ACD would have a significant number of white cells present after washing. I do think that the slight differences that you have observed are quite compatible with what we have found and what Dr. Koler has mentioned. Electrophoreses carried out at pH 7.4 and 7.5 do not give adequate separation. We have had similar patterns. It isn't until you get extremes of pH that you get the bands well separated.

JAFFÉ, NEW YORK: If I remember correctly, a few months ago there was a report of stimulation of pyruvic kinase activity by the addition of fructose diphosphate. This is perhaps another difference between the hepatic and erythrocytic enzyme. We had one opportunity to try the effect of the addition of fructose diphosphate in doing a pyruvic kinase determination in both a normal person and a deficient patient. In neither case did fructose diphosphate stimulate pyruvic kinase activity in the hemolysates. I wonder if anybody else has had an opportunity to look at this observation.

KOLER, PORTLAND: The original report was by Hess, Haeckel, and Brand* and referred to the yeast form of pyruvate kinase. I know Dr. Weber and his group are quite interested in this and look on fructose-1-6-diphosphate as a directional sensing switch that activates pyruvate kinase. In their system they have been able to show that fructose-1-6-diphosphate does activate rat liver pyruvate kinase. We have made similar observations with our human erythrocyte enzyme, but the conditions that are necessary to show activation are still poorly defined.

AEBI, BERNE: Dr. Eppenberger, Dr. von Fellenberg, and Dr. Richterich† in my laboratory have investigated glycolytic enzyme patterns at early stages in the chick embryo. They have observed that, whereas most of the glycolytic enzymes increase steadily in concentration at about the same rate, pyruvate kinase behaves differently, in the sense that at a very early stage pyruvate kinase activity is relatively high. It is tempting to speculate that this may be taken as evidence for an atypical pattern of glycolysis at early stages. Therefore the data on the key enzymes of glycolysis and of gluconeogenesis, as shown in Dr. Koler's illustration, might be subject to some modification. I would like to ask Dr. Koler to comment on this.

KOLER: I apologize for going over this so quickly. Figure 7 in my paper is from one of the publications of Dr. Weber's group and is a diagram showing what they call "functional-genic units." On the left are the key gluconeogenic enzymes, pyruvate, carboxylase, phosphoenal-pyruvate carboxykinase, fructose-1-6-diphosphatase, and glucose-6-phosphatase. This set of enzymes is coordinately induced in their system with glucocorticoids and inhibited by insulin. The bifunctional enzymes in the middle are apparently not affected by the hormonal systems they worked with. The key glycolytic enzymes on the right are glucokinase, phosphofructokinase, and pyruvate kinase. Again, these are coordinately induced by insulin and repressed by glucocorticoids. That is, their biosynthesis is coordinately induced or repressed by these hormones, respectively. This is Dr. Weber's work and refers to the rat levels of these enzymes. I think it is much too early to try to extrapolate this to the total mammal, let alone to man, although I think it opens up interesting questions. Discrepancies, I am sure, will be found and may be tissue-specific or perhaps a function of the relative stage of differentiation of the cell.

HARRIS, LONDON: I have just one comment. I think we have to be very cautious about this kind of interpretation. There is another enzyme that occurs in different tissues, and it has different properties in different tissues. Now in this

*Hess, B., Haeckel, R., and Brand, K FDP activation of yeast pyruvate kinas Biochem. Biophys. Res. Commun. 24:824-831, 1966.

†Eppenberger, H. M., von Fellenberg, R. Richterich, R., and Aebi, H.: Ontogenesis of cytoplasmic enzymes of the chick en bryo. Enzymol. Biol. Clin. 2:139-174, 1962-63.

TABLE 1. *Purification of erythrocyte pyruvate kinase (Exp. 11)*

	Step	Total units	Overall recovery (per cent)	Specific activity (μmoles/min/mg protein at 27° C)	Overall purification
	Hemolysate	152	—	0.002	—
1st	35-40% $(NH_4)_2SO_4$	60	39.5	0.104	52
2nd	35-45% $(NH_4)_2SO_4$	44	29.0	0.71	355
	Heat 4.5 min. 60°C.	50	32.9	1.75	875
	45% $(NH_4)_2SO_4$	34	22.3	2.6	1,300
	Alumina gel	25.7	16.9	12.9	6,460

case we know of a specific deficiency of liver aldolase. Liver aldolase is different from muscle aldolase, and I believe there is a brain aldolase, too. Liver aldolase deficiency occurs in hereditary fructose intolerance, and yet muscle aldolase is quite normal and so is brain aldolase. The interesting point in this particular context is that apparently there is some aldolase in red cells that is different from liver aldolase and resembles, as far as I gather, the muscle aldolase.

IBSEN, LOS ANGELES: Stimulated by the work of Drs. Tanaka, Koler, and their co-workers, we have made some preliminary attempts to purify erythrocyte pyruvate kinase. We tried initially to follow the procedure of Koler et al. but were discouraged by the 90 per cent loss of activity after DEAE cellulose treatment. A similar loss was also reported by Dr. Koler. We thought perhaps the enzyme remained bound to the DEAE cellulose. However, more activity could not be recovered by increasing the ionic strength or washing at different pH's. It was assumed, therefore, that for some reason DEAE cellulose caused a loss of activity. Since a primary problem in the study of the enzyme from deficient patients is the yield, it was decided to abandon this

TABLE 2. *Heat purification of erythrocyte pyruvate kinase (pH 6.03)*

Time heated min. 60° C.	Activity μM/min/ml	Protein mg/ml	Specific activity
0	48	10	4.8
3	44	9.3	4.7
6	57	8.9	6.4
8	55	8.2	6.7
9	56	8.6 (8.2)	6.5 (6.8)
10	60	8.2	7.3
12	55	8.1	6.7
13	53	8.1	6.5

Heated in a solution containing 0.1 M K phosphate buffer pH 6.03, 0.002 M mercaptoethanol, 0.002 M Mg acetate.

approach. Some preliminary observations on a somewhat different approach to the purification of red cell pyruvate kinase are shown in Table 1. In this particular instance, a washed preparation of outdated ACD blood was subjected to two sequential 35-45 per cent ammonium sulfate fractionation steps. The dissolved pellet was then heated, reprecipitated with ammonium sulfate, dialyzed, and treated with alumina gel. The overall purification was over 6000-fold and the overall yield was 16.9 per cent. The specific activity was 12.9 at room temperature. Further purification could be achieved by making 5 per cent ammonium sulfate cuts on the alumina gel eluate. The best preparations had a specific activity of 16.7 at room temperature. Protein was estimated by absorption at 280 and 260 $m\mu$. Partially-purified preparations seemed to be quite unstable. For instance, this particular preparation, left in the refrigerator, had a specific activity of 7.9 the next morning. The enzyme could be partially reactivated by incubation with mercaptoethanol and all subsequent work has been done with added mercaptoethanol. In the presence of mercaptoethanol the enzyme could be heated for 10 minutes at 60° C with no loss of activity. In fact, activity is increased from 48 to 60 units (Table 2.)

KOLER: Dr. Peter Stenzel in Dr. Jones's laboratory has made essentially the same kind of observation, and in the current efforts to purify the enzyme he is incorporating mercaptoethanol and a heat-denaturation step, so these findings are quite consistent.

Triosephosphate Isomerase Deficiency

A. A Multi-System Inherited Enzyme Disorder. Clinical and Genetic Aspects

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TRIOSEPHOSPHATE ISOMERASE DEFICIENCY is unique among the presently known inherited enzyme-deficient hemolytic anemias in that the deficiency of this glycolytic enzyme is accompanied by clinical evidence of marked neuromuscular disease and by evidence strongly suggestive of clinical involvement of other systems as well. The chronic hemolytic anemia present in this disorder is in fact only a minor component in this multifaceted clinical syndrome. Enzyme studies have revealed marked deficiency of triosephosphate isomerase in erythrocytes, leucocytes, skeletal muscle, and in spinal fluid, and a moderate reduction of activity in blood serum. There may, indeed, be a whole-body deficiency of this enzyme.

We observed our first patient[1] with triosephosphate isomerase (TPI) deficiency in late May of 1964, when we were presented with the opportunity to study the blood cells of a 13-month-old girl of French and Negro ancestry. A chronic hemolytic state had been known since she was five weeks of age. A sibling with hemolytic disease had died shortly after birth. The child had apparently developed normally until she was seven months of age, when some tightness of the Achilles tendons with inability to dorsiflex the feet was noted. Motor development was otherwise normal at that time; however, by the time she was 13 months old she had developed generalized spasticity with increased deep tendon reflexes. She had never walked, and she had lost the ability to stand or sit or to hold her head erect. Her course had been interspersed with numerous severe infections, including several bouts of pyelonephritis and pneumonitis. Such episodes were usually associated with a marked increase in the degree of anemia. Routine studies had established that she had a chronic hemolytic state with a hemoglobin concentration varying from 7.5 to 9.0 g per 100 ml between bouts of infection. No immunohematologic abnormalities had ever been demonstrated, nor were there any abnormalities of hemoglobin.

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TABLE 1. *Hematologic studies of patients with triosephosphate isomerase deficiency*

Patient	Hb (g/100 ml)	Hct (%)	RBC (10^6/mm^3)	WBC (10^3/mm)	Hb type	Reticulocytes (%)
D.D.	10.2	34	2.73	19.1	AA	13.0
S.J.	10.7	37	3.24	3.7	AA	17.0
E.JE.	9.0	32.5	—	8.2	AA	14.5

Morphologic changes in erythrocytes were not striking. There was slight macrocytosis, small numbers of target cells, and scattered small, densely contracted red cells with irregular margins.

Routine hematologic studies of this patient (Table 1) revealed a hemoglobin concentration somewhat higher than her usual level. She also had an elevated white blood cell count coincident with a urinary tract infection. Concurrent studies of her immediate family members, including her parents and two brothers, gave essentially normal results. Autohemolysis (Table 2) on sterile incubation was pronounced after 48 hours with the patient's erythrocytes. No abnormalities were noted by this technique with the erythrocytes from either parent or from the siblings. Autohemolysis was averted, however, if sterile incubation was performed with the addition of glucose, adenosine, or ATP. These findings were similar to those commonly noted in hereditary spherocytosis and were quite dissimilar to those associated with pyruvate kinase deficiency hemolytic anemia. Erythrocyte osmotic fragility was normal in family members and the patient when fresh blood was studied. After 24 hours of incubation there was a flattening of the curve observed with the patient's red cells.

Assays of several enzymes of the glycolytic and pentose phosphate pathways and of glutathione reductase (Table 3) were generally elevated in the patient and were generally normal in family members. We have frequently observed such elevations with young cell populations. It is noteworthy that most of the elevations were relatively minor, approximating no more than a two-fold increase in activity. Hexokinase activity exhibited a greater than five-fold increase. This marked selective elevation of hexokinase has been noted previously.[3]

The activity of erythrocyte triosephosphate isomerase (TPI) (Table 3) was markedly reduced to a level of approximately one-seventh of normal. A similar reduction in leucocyte enzyme activity was noted. Activity could be restored to the assay mixture by the addition of crystalline TPI. Repeated attempts to

TABLE 2. *Erythrocyte autohemolysis* (%)

Patient	No additives	Glucose	Adenosine	ATP
D.D.	8.8	1.3	1.0	0.5
S.J.	25.3	1.5	0.6	0.8
E.JE.	—	—	—	—
Normal mean	1.7	0.2	0.2	0.2

TABLE 3. *Activities of erythrocyte enzymes* in patients and their parents*

Enzymes	Normal range	D.D. Patient	D.D. Parents M	D.D. Parents F	S.J. Patient	S.J. Parents M	S.J. Parents F	E.JE. Patient	E.JE. Parents M	Parents Family R. M	Parents Family R. F†
Hexokinase	0.20-0.35	1.63	0.17	0.58	1.21	0.35	0.21	1.05	0.25		
Phosphohexose isomerase	6.9-21.0	21.1	11.6	18.5	20.5	14.1	18.9	19.3	14.4		
Phosphofructokinase	5.5-11.0	14.4	6.4	10.1	9.5	5.5	5.7	3.6	5.1		
Aldolase	1.0-1.40	2.61	0.85	1.10	1.86	0.89	0.77	2.9	1.6		
Glyceraldehyde-3-phosphate dehydrogenase	24.0-32.0	40.0	22.5	32.9	36.4	29.6	21.8	40.9	30.5		
Triosephosphate Isomerase											
Erythrocyte	128-227	12.7	90.0	125.0	13.7	80.7	92.3	71.7	102.2	92.2	105.0
Leucocyte	2,380-3,376	563	2,610	2,130	254	1,782	1,553	663	2,422	1,540	
Phosphoglycerate kinase	17.0-24.0	21.0	18.9	25.3	29.7	23.9	21.5	31.5	26.3		
Phosphoglycerate mutase	5.00-9.00	8.76	7.59	9.32	9.50	8.15	5.62	9.57	6.82		
Enolase	2.80-5.30	5.94	3.43	6.27	7.75	5.54	4.64	8.20	3.89		
Pyruvate kinase	2.00-3.40	5.83	2.69	3.24	8.12	2.79	1.75	6.86	2.84	3.21	2.52
Lactic dehydrogenase	29.0-35.0	42.2	19.5	34.9	49.8	40.0	33.0	64.1	42.1		
Glucose-6-phosphate dehydrogenase	2.60-3.40	7.63	2.53	2.47	5.92	2.38	2.50	6.09	2.50	2.23	2.40
6-Phosphogluconic dehydrogenase	1.30-2.30	3.04	2.09	1.94	3.65	2.12	1.85	2.34	1.74		
Glutathione reductase	1.20-2.20	2.52	1.70	1.29	3.17	1.73	1.86	4.03	1.82		

*All enzyme activities are expressed in International Units per 10^{10} cells.
†The symbols M and F designate mother and father, respectively.

demonstrate inhibitor substances were unsuccessful, and it appears likely that the decreased enzyme activity is due to a primary deficit in the enzyme protein itself. However, whether this deficit is qualitative as well as quantitative remains to be demonstrated

Studies in family members proved to be of great interest. Both the mother and father of the patient, as well as one of the two brothers and several other relatives, had a reduction in erythrocyte TPI activity to a level intermediate between normal and the marked deficiency noted in the patient's erythrocytes (Table 3). A similar but less marked reduction of activity was noted in the leucocytes as well. This decrease of triosephosphate isomerase activity to levels approximating one-half of the normal mean was strongly suggestive that these intermediate levels characterized the heterozygous state. The results of erythrocyte TPI assays of this patient, a second patient, and their family members are shown graphically in Figure 1. It seems apparent that these persons were biochemically separated into three groups: a clearly definable, severely enzyme-deficient group consisting solely of affected patients, an intermediate group including all the parents of affected children as well as several other close relatives, and a third group in no way distinguishable from the normal population. These data strongly support the assumption that heterozygotes were biochemically defined by intermediate enzyme deficiency. However, there was a certain degree of overlap between the heterozygous and normal groups.

The family of the first patient was part of a very highly inbred, almost entirely genetically-isolated subpopulation of French Louisiana and partially Negro origin. The family members appeared predominantly Caucasian and denied any association with their Negro neighbors. Interrelations with the white Louisiana group also were limited, resulting in the selection of marriage partners almost entirely from this very cohesive group. Although no direct evidence of consanguinity could be established between the maternal and paternal sides of the patient's family, both parents considered it quite certain that they were related in some way.

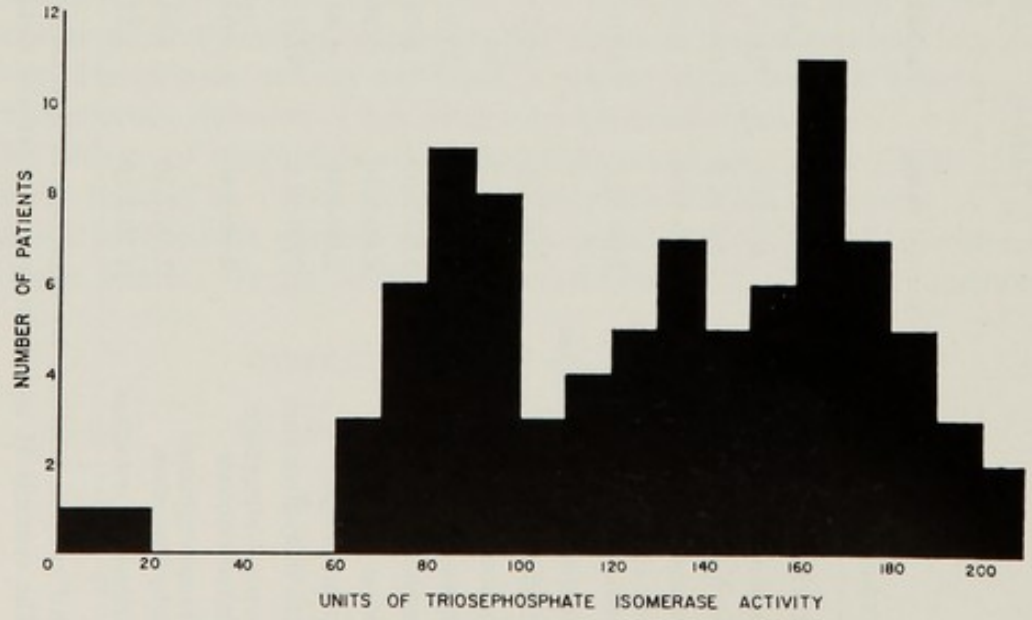


FIG. 1. TPI assays of patients and family members.

The occurrence and recurrence of the French family names throughout both maternal and paternal kindred was very striking. Shortly after family studies were instituted, it was noted by Miss Wilson in our laboratory that we had performed studies approximately one year earlier on a patient with a family name that also occurred in the ancestry of our current patient. Investigation revealed that this patient was of French Louisiana origin, and the history was strikingly analogous to that of our first patient.[2]

Pallor had been apparent shortly after birth. At one month of age, the hemoglobin concentration was 5.5 g per 100 ml, and the reticulocyte count varied from 10 to 15 per cent. There had been several episodes of increasing anemia during various febrile illnesses. However, there did not seem to be an inordinate susceptibility to infection. Neurologic abnormalities had become evident at approximately ten months of age and were characterized by generalized flaccidity. Spasticity had never been noted. Hematologic studies (Table I) were quite similar to those of the first patient. Hematologic studies of the patients

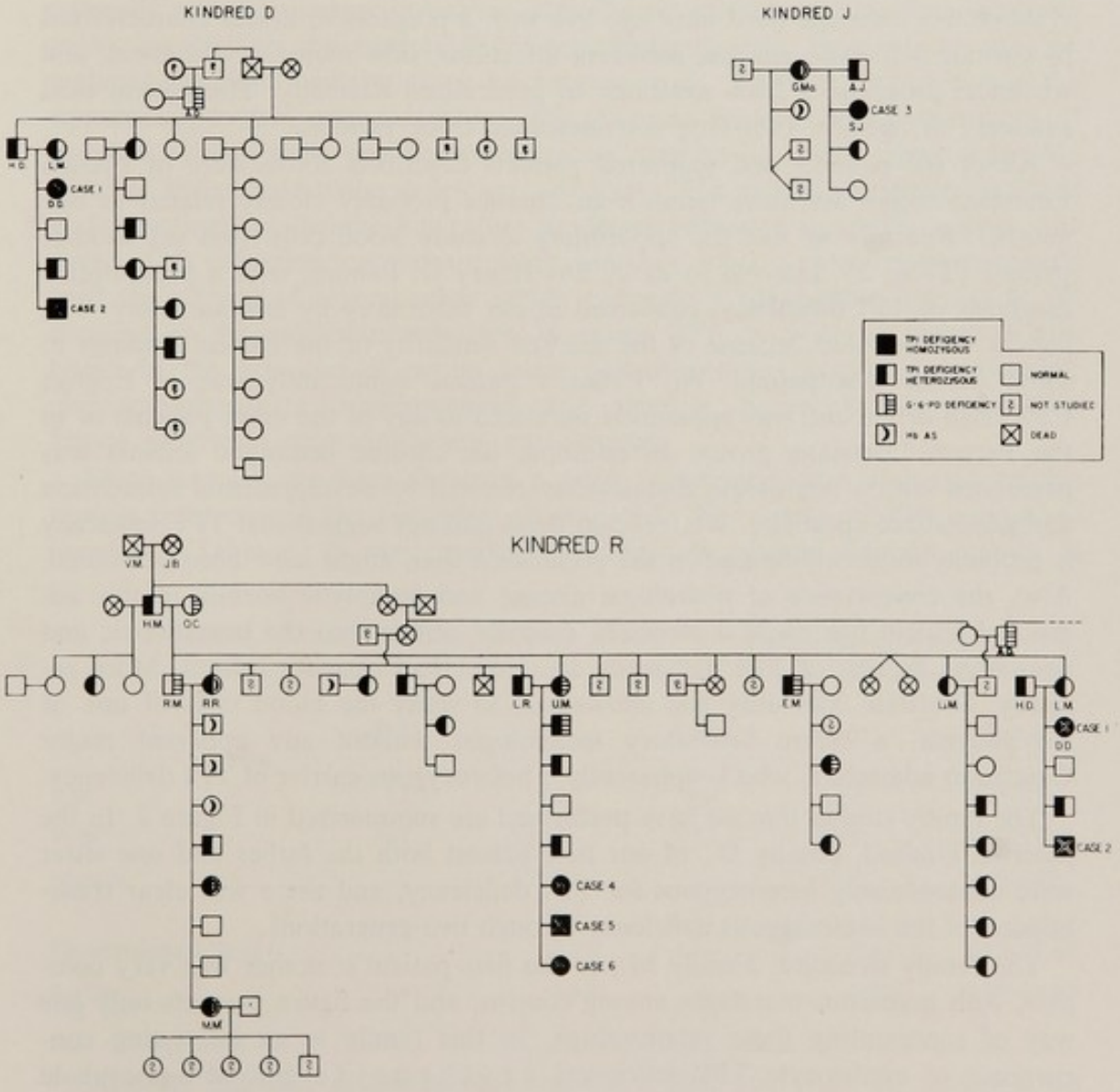


FIG. 2. Family studies of patients with TPI deficiency.

gave essentially normal results, with the exception of the presence of sickle cell trait in the patient's mother.

Again, as we fully expected by this time, a marked deficiency of erythrocyte TPI was noted in both the leucocytes and erythrocytes (Table 3). Again, both parents exhibited intermediate enzyme deficiency consistent with the heterozygous state.

The family history of our original patient, D.D., revealed that three deceased maternal first cousins (Family R.), all siblings, had had hemolytic disease. The mother, a sister of the first patient's mother, was married to a second cousin (Fig. 2). Enzyme studies of these patients' parents (Family R.) revealed that both were heterozygotes.[4]

The first of the children of Family R. had been hospitalized in Louisiana for hemolytic anemia of unknown type at age ten months and had died at one year of age of respiratory disease. A sibling had died several hours after birth of apparent hemolytic disease of the newborn. However, there was no evident ABO or Rh incompatibility and the Coomb's test was negative. The third child of this marriage lived until age five with a progressive illness characterized by chronic hemolytic anemia, recurrent infections, slow motor development, and weakness progressing from spasticity to generalized flaccidity. The patient died suddenly at age five following convalescence from varicella.

All of the patients and suspected patients described above were of French Louisiana origin and have come from families probably closely related to one another. Recently we had the opportunity to study blood cells from yet another patient (Table 2), referred to us by Dr. Henry G. Palmer, with a presumptive diagnosis of TPI deficiency, confirmed in our laboratory by enzyme assay. The patient was referred because of the marked similarity of the clinical findings to those of our first patient. Dr. Palmer's patient significantly was of English Caucasian origin and was apparently unrelated to any of the other patients or to the French Louisiana group. In addition, his chronic hemolytic anemia was associated with a neurologic disease characterized by developmental retardation and generalized spasticity. We feel that these findings suggest that TPI deficiency is probably more widespread in the population than might have been presumed. Also, the concurrence of neurologic disease and hemolytic anemia in this additional patient has made it clinically virtually certain that the hematologic and neurologic aspects of TPI deficiency are more than casually related. More recently, Dr. Paul McCurdy has allowed us to study the blood cells of one of his patients, a Negro laboratory technologist without any apparent major Caucasian admixture, who is apparently a heterozygous carrier of TPI deficiency.

The family studies that we have performed are summarized in Figure 2. In the paternal kindred, Family D., of our first patient both the father and one sister were demonstrably heterozygous for TPI deficiency, and there was clear transmission of the heterozygous deficiency through two generations.

The family structure, Family M., of the first patient's mother was very complex, with numerous marriages among cousins, and the figure presents only one way of representing these relationships. In this family is an interesting concurrence of erythrocyte TPI deficiency, erythrocyte glucose-6-phosphate dehydrogenase deficiency, and sickle cell trait. In several instances, individuals

were found to have two of these traits, and in two instances, a concurrence of all three traits was noted. One of these individuals was hematologically normal, and the second had anemia, probably secondary to menorrhagia. The limited genealogy we have been able to uncover in the second patient's family is designated Family J. in Figure 2. The child was the product of a second marriage of a heterozygous mother, this time to a man who unfortunately was heterozygous for TPI deficiency.

The patients and probable patients of whom we are now aware number seven. All but two of these individuals have died before the age of five, and one individual is now living at age five, another at age 4½. All have had severe generalized disease. There has also been at least suggestive evidence of functional impairment of the leucocytes in that some of the patients appeared to have an increased susceptibility to infection. There has also been a strong clinical impression that these patients have had a disorder of the muscles as well as of the central nervous system. Also, the rather sudden exodus of at least two of the patients has raised the question of a cardiac death. The generalized nature of the disorder has naturally raised important questions as to the extent that multiple tissues are actually involved in the enzyme deficiency. Unfortunately, none of the autopsy material has been available to us for enzyme study. We have, however, had the opportunity to gain some insight into this question. Assay of TPI in the serum of our first two patients was performed in an attempt to assess rather indirectly whether widespread tissue deficiency was likely. The results are shown in Table 4. Interestingly, a definite but rather moderate reduction in serum activity was noted. This reduction was more striking, however, when a comparison was made with the activity of several other serum enzymes in these same individuals. In contrast to the moderate reduction in serum TPI, a clear-cut elevation was noted in the activities of serum lactic dehydrogenase, glutathione reductase, glucose-6-phosphate dehydrogenase, and aldolase. Serum creatine phosphokinase somewhat surprisingly was not elevated.

More recently, we have obtained a small biopsy specimen of gastrocnemius muscle from one of the surviving patients, S.J. The results of TPI assays are shown in Table 4. A very marked deficiency in muscle enzyme activity is

TABLE 4. *Triosephosphate isomerase activity* in tissues and body fluids*

	Patients			Controls	
	D.D.	S.J.	E.JE.	Mean	Range
RBC	12.7	13.7	69	195	128-227
WBC	563	254	633	2749	2380-3376
Muscle	—	33.1 26.1	—	623	456-682
Cerebrospinal fluid	—	0.23	—	6.92	3.12-18.67
Serum	3.19 5.32	3.66	—	8.97	5.08-10.93

*Activities expressed as International Units. For RBC and WBC the activity is per 10^{10} cells; activity is per gram of muscle, and per 100 ml of serum or cerebrospinal fluid.

apparent, with a reduction to about 5 per cent of normal activity. The activities of several other muscle enzymes, including aldolase and creatine phosphokinase, were found to be normal in this sample of skeletal muscle. We have not had an opportunity to perform assays on other tissues directly, however, we have indirectly tried to assess enzyme activity in the central nervous system by performing assays on cerebrospinal fluid. Again, quite notably, a marked reduction in spinal fluid TPI has been observed in the one patient studied thus far. It thus seems quite likely that central nervous tissue is also deficient in this enzyme.

Summary

Triosephosphate isomerase (TPI) deficiency is a rare, inherited disorder characterized by progressive, severe, neuromuscular dysfunction and hemolytic anemia. The enzyme deficiency has been demonstrated directly in erythrocytes, leukocytes, and skeletal muscle. Decreased TPI activity in serum and in spinal fluid suggests that the deficiency is widespread, and may indeed involve all tissues.

The disorder has been observed thus far in three families, and genetic studies reveal that inheritance is by an autosomal recessive mode of transmission. Heterozygotes are clinically well, but biochemically detectable. The trait has been found in individuals of French-Negro ancestry, in individuals of English ancestry, and in one Negro individual without known Caucasian ancestry.

Acknowledgment

We gratefully acknowledge the technical assistance of Mrs. M. Brown, Mrs. Thea Gabor, Mr. K. Kurschner, Miss M. Mattson, Miss M. L. Wilson, and Mrs. J. Wittenberg.

The discussion of this presentation is combined with the discussion of the following paper, page 278.

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Triosephosphate Isomerase Deficiency

B. Inherited Triosephosphate Isomerase Deficiency. Erythrocyte Carbohydrate Metabolism and Preliminary Studies of the Erythrocyte Enzyme

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DEFICIENCY OF THE GLYCOLYTIC ENZYME, triosephosphate isomerase (TPI) is a disorder that has been described recently,[3,4] The enzyme deficiency has been demonstrated in erythrocytes, leucocytes, and skeletal muscle. It is associated with a severe neuromuscular disorder and a chronic hemolytic anemia and has been fatal in all but two known cases.[2]

METABOLIC STUDIES

Mature erythrocytes lack any known mechanism for the metabolism of dihydroxyacetone phosphate and are not able to further metabolize pyruvate via the tricarboxylic acid cycle. These cells depend entirely upon anaerobic glycolysis for the production of high-energy phosphate compounds. A block at the TPI level should result in certain predictable metabolic consequences unless significant compensatory mechanisms are present. The TPI step represents the only known means whereby dihydroxyacetone phosphate formed from six carbon compounds may be further metabolized via the glycolytic pathway. Therefore, a block at this level might be expected to result directly in the metabolic loss of half of the carbon compounds formed in the metabolism of glucose. Also, since two moles of ATP are consumed in the initial phosphorylation of six carbon compounds, it would be anticipated that TPI deficiency would be associated with rather marked deficiencies in erythrocyte ATP synthesis. Thus, one could quite logically anticipate a decrease in the glycolytic rate analogous to that which occurs in pyruvate kinase deficiency, a decline in the rate of ATP synthesis, an accumulation of dihydroxyacetone phosphate and perhaps other intermediate compounds such as fructose diphosphate, and a marked reduction by almost half in the amount of three carbon compounds that are eventually converted to lactic acid.

In order to evaluate the metabolic effect of triosephosphate isomerase defi-

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ciency, we performed studies on the erythrocytes of two of the patients described elsewhere.[1,3,4] Leucocyte-free erythrocytes suspended in native plasma with added glucose were incubated for 150 minutes at 37°. Aliquots of the incubation mixture were taken initially and at intervals throughout the incubation, and the concentration of several compounds of interest was determined. The data, expressed in terms of micromoles of each compound per 1.0 ml of packed erythrocytes, are shown in Table 1. Data obtained from a single, somewhat typical, young red cell population are also presented to show the general trend of changes when young cells are present, rather than for direct quantitative comparison.

Instead of the anticipated decrease in glucose utilization, there was a rather surprising marked increase to over 15 micromoles of glucose per ml of packed red cells during the 150-minute period of incubation. This increase above normal mean glucose utilization of 5.2 micromoles was very similar in both patients studied, and was rather clearly greater than that generally observed in patients with similar elevations of the reticulocyte count. Again, somewhat surprisingly, there was no deficit in the amount of lactate formed from glucose. The ratio of lactate formation to glucose utilization closely approximated normal in both patients studied.

It was thus obvious that major compensatory mechanisms must have been utilized by these erythrocytes, and it seemed most likely that such compensation was by way of the pentose phosphate pathway. In an effort to assess this mechanism of compensation, similar studies were performed with the addition of methylene blue to the incubation system. In the presence of methylene blue there was an increase in glucose utilization to 17.7 micromoles per ml of packed red cells in one patient, and to 19.9 micromoles of glucose in the second patient. These increases were considerably less marked than in the controls and in the young cell control. Such a sluggish response to methylene blue stimulation could be caused by inability of the shunt to function, as is noted in glucose-6-phosphate dehydrogenase deficiency, or, alternately, it can be due to a shunt already functioning at near-maximal levels. We feel that the latter explanation is probably the correct one in these patients and that the increased glucose utilization and the almost proportionate production of lactate occurs by way of increased activity of the pentose phosphate pathway. However, direct measurements of the activity of the pentose phosphate pathway have not been performed.

The initial concentration of ATP did not differ significantly from normal in the first patient. However, there was a considerable decrease of the initial ATP concentration in the second patient. In both instances changes in ATP concentration during the period of incubation were not appreciable. It is certain that the ATP concentration was reduced in the erythrocytes of the second patient, and it is possible that there is a relative decrease in ATP levels in relation to the cell age in the second patient. We have been less impressed with the changes in ATP concentration with cell age than have others, however. A very moderate increase in the initial concentration of fructose diphosphate was noted, much as might be anticipated. This increase was scarcely more than that noted in the young cell control, however.

The most striking deviation from normal was a rather marked increase in the

TABLE 1. *The pattern of glycolytic intermediates in triosephosphate-isomerase-deficient erythrocytes**

	Normal			Patient I			Patient II			High reticulocyte control		
	0 min ± SE‡	△ 150 min ± SE	Methylene blue added △ 150 min ± SE	0 min	△ 150 min	Methylene blue added △ 150 min	0 min	△ 150 min	Methylene blue added △ 150 min	0 min	△ 150 min	Methylene blue added △ 150 min
Glucose		−5.2 ± .2	−7.6 ± .9		−15.4	−17.7		−15.8	−19.9		− 9.3	−17.8
Lactate		+8.9 ± .5	+9.3 ± .6		+26.2	+28.8		+35.3	+31.4		+17.1	+24.9
Pyruvate		− .04 ± .02	+1.9 ± .5		− .49	+ 2.4		0	+ 3.8		− .15	+ 2.2
DHAP	.09 ± .02	+ .67	0†	1.77	+ 1.04	+ .93	2.19	+ .50	+ 3.8	.30	+ .17	− .12
Gald-3-P	.03 ± .01	0	0†	.19	− .10	+ .04	.13	+ .03	+ .09	.04	+ 0.1	+ .02
F-1,6-P	.04 ± .01	+ .31	0†	.32	− .02	− .25	.93	− .36	− .33	.23	− .01	− .13
ATP	1.17 ± .09	− .03 ± .04	+ .21 ± .1	1.06	− .12	− .02	.76	+ .08	− .17	1.29	+ .09	+ .06
2,3-DPG	4.5 ± .3	+ .5 ± .2	+ .7 ± .3	4.0			6.8	+ .2	+ .6	6.3	+ 1.2	

*All values expressed as micromoles per ml erythrocytes.
†Not detectable.
‡SE is standard error of the mean.

initial concentration of dihydroxyacetone phosphate in the erythrocytes of both patients. These values of 1.77 micromoles and 2.19 micromoles of dihydroxyacetone phosphate per ml of packed red blood cells were more than twenty-fold higher than the control value of .09 micromoles per ml of packed red blood cells, and almost ten-fold greater than the concentration noted in the high reticulocyte control. The levels of the other intermediates studied, including glyceraldehyde-3-phosphate and diphosphoglycerate, did not deviate significantly from the controls. The elevation of dihydroxyacetone phosphate levels, although marked in the resting state, was not associated with an inordinate accumulation during incubation. This finding suggested that either a very great proportion of the glucose utilization occurred by way of the pentose phosphate pathway, or that alternate routes for the metabolism of the dihydroxyacetone phosphate are available. One such route would be conversion of dihydroxyacetone phosphate to alpha glycerophosphate. We have been unable to detect any activity of alpha glycerophosphate dehydrogenase in these preparations, however.

The paucity of metabolic abnormalities which seem to result from this naturally-occurring block at the triosephosphate isomerase step stands in rather marked contrast to the striking clinical abnormalities noted in these subjects. It has occurred to us that perhaps the accumulation of dihydroxyacetone itself or of some derivative product may be injurious to cells. However, we have no direct evidence regarding this possibility. It is also conceivable, particularly in tissues not lacking in alpha glycerophosphate dehydrogenase, that a lipid abnormality may result as a secondary phenomenon. Again, we have no evidence for this.

Studies of the Erythrocyte Enzyme

We have recently embarked on studies of the enzyme protein. As a preliminary step to further purification, we performed chromatography of stroma-free hemolysate on DEAE-Sephadex A-25. The blood was first collected in heparin and washed twice in saline, after which the plasma and the buffy coat were removed. The packed cells were brought to two times the original volume with distilled water and then frozen and thawed two times. Following centrifugation at 13000 x G for thirty minutes, 1.0 to 3.0 ml of the supernatant was applied to a column which consisted of DEAE A-25 in the bead form, equilibrated in 0.003 M K_2HPO_4 at pH 7. Elution was conducted with 200 ml of the same buffer at 20 ml per hour in a Buchler refrigerated fraction collector, followed by linear

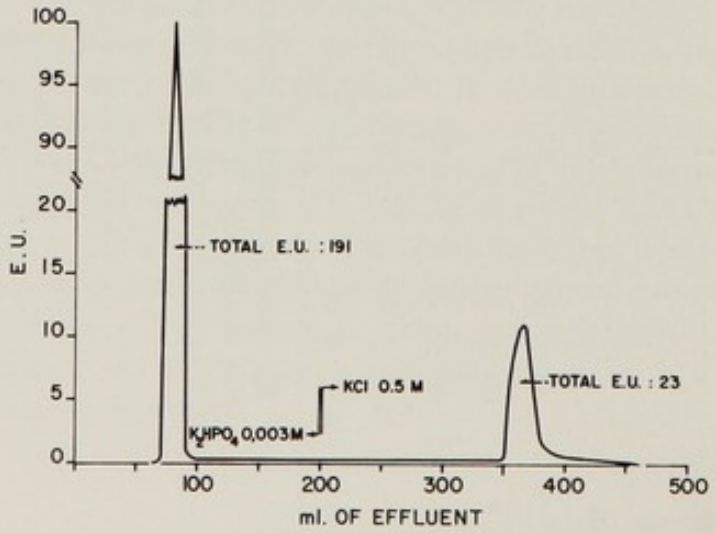


FIG. 1. Separation of TPI from normal erythrocytes on DEAE Sephadex A-25.

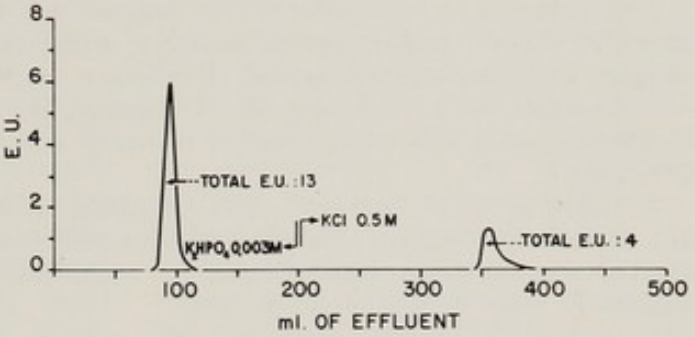


FIG. 2. Separation of TPI from enzyme-deficient erythrocytes on DEAE Sephadex A-25.

gradient elution beginning with a 50 per cent mixture of 0.003 M K_2HPO_4 and 0.5 M KCL and ending with 0.5 M KCl.

Triosephosphate isomerase activity was found in these preparations in two distinct peaks (Fig. 1). The predominant peak (A) was eluted with phosphate and appeared just after, and generally not entirely separated from, the majority of the hemoglobin. Dissociation from hemoglobin could be accomplished by treatment with 2 M urea, however. The second, lesser peak (B) was entirely separate from hemoglobin and was eluted by the KCl gradient. Chromatography of rabbit muscle enzyme obtained from commercial sources revealed only peak A. Michaelis constants for peaks A and B were the same. Similar studies of the erythrocyte enzyme from a homozygous deficient subject are shown in Figure 2. Again there was separation of activity into two distinct peaks. There was, however, a marked reduction in total enzyme activity, and this reduction seemed to involve somewhat disproportionately the major peak, A. Further experiments have suggested that these two fractions are derivatives of one another in a monomer-polymer relation, with one fraction in equilibrium with the other. Rechromatography of fraction A results almost entirely in the reappearance of the enzyme in fraction A. This can be almost entirely transformed to fraction B, however, by treatment with 2 M urea, prior to rechromatography. On the other hand, rechromatography of fraction B results in a redistribution between the two fractions. It seems likely that fraction A is the polymerized form which is predominant, except after treatment with urea which results in depolymerization. We are presently performing estimates of molecular size with gel filtration to further evaluate this hypothesis.

Summary

Metabolic studies on triosephosphate isomerase-deficient erythrocytes revealed a rather startling paucity of knowledge of the metabolic consequences of this lesion, with the exception of marked elevation of dihydroxyacetone phosphate concentration and a moderate reduction of ATP concentration observed in one of the two patients studied. Preliminary chromatographic studies suggest that the enzyme may exist in more than one molecular form.

Acknowledgment

We gratefully acknowledge the technical assistance of Mrs. V. Reagh and Mr. I. Stuart.

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DISCUSSION

LÖHR, MARBURGH, CHAIRMAN: The discussion is now open.

HUENNEKENS, LA JOLLA: Several people this morning have brought up the possibility that the decreased levels of enzyme in the glycolytic sequence may give rise to the observed physiological end-effects, not particularly because of the derangement in glycolysis per se (i.e., by changing steady state levels of intermediates), but by some other mechanism. In a way, this makes good sense because glycolysis is a central pathway in the red cell, and it ought to have the capacity to absorb a certain amount of stress and strain without the cell falling apart. There is another possibility that might be considered which has to do with some work that has been carried out recently in Green's laboratory on the mitochondrial membrane and its relation to phosphorylated compounds. In oxidative phosphorylation, the primary event appears to be the creation of some sort of an energy-rich linkage; this occurs at three points in the electron transfer chain. By an isoenergetic transfer of this energy-rich linkage, a series of intermediates are made, terminating in ATP. There is some suggestion that the penultimate compound in this sequence, or perhaps one even further back, may be a high-energy substance that is responsible for keeping together the subunits of the mitochondrial membrane. Now in the red cell, since oxidative phosphorylation doesn't occur, one might ask whether ATP or some other intermediate created by glycolysis, could give rise to an energy-rich intermediate of this type. Even existing in a low concentration, such a compound might have the same function in the red cell—that is, holding membranes together. It is conceivable that the studies reported here have illustrated that key glycolytic enzymes, when deficient, do not impair glycolysis as much as they depress the synthesis of some type of energy-rich intermediate which is involved in maintenance of the red cell membrane.

LÖHR: I think we must differ between the metabolic consequences of mature red blood cells and the other tissues, because red blood cells do not have mitochondria as Dr. Huennekens said. But in addition, mature red blood cells haven't measurable amount of glycerol-phosphate-dehydrogenase. I think in the other tissues the accumulation of dihydroxyacetone phosphate is not so great because these tissues contain this enzyme and can oxidize dihydroxyacetone phosphate to glycerophosphate. But the red blood cell does not have this opportunity. Glycerophosphate is an important substance for the lipids; it is the glycerol compound of neutral fatty acids and for some phospholipids.

SCHNEIDER, LOS ANGELES: We have considered several alternative mechanisms by which triosephosphate isomerase deficiency might produce cellular damage. First, enzyme-deficient cells are unable to convert glyceraldehyde phosphate to dihydroxyacetone phosphate. In tissues that contain alpha glycerophosphate dehydrogenase, it may well be that this may result in impairment of lipid synthesis. A second possible mechanism is that the accumulation of dihydroxyacetone phosphate in the red cell may, in itself, be toxic to these cells by some unknown mechanism. Also, it is possible that a condensation product between dihydroxyacetone phosphate and other intermediate substances may be toxic. The most logical basis for cellular dam-

age is, of course, decreased ATP synthesis. This decrease is not striking, however.

KIRKMAN, CHAPEL HILL: Would you comment on the time of onset of the neuromuscular symptoms and the speed with which they develop? Some of us are intrigued by the possibility that a congenital enzymic defect, particularly of a very active metabolic pathway, could give rise to a slowly progressive neuromuscular disease?

SCHNEIDER: These children have been apparently normal at birth; they have progressed normally through some of the initial stages of motor development, such as raising the head and sitting and this sort of thing, and then they have seemed to gradually and progressively lose their functions, finally progressing to severe flaccid paralysis. The findings in each case have been strikingly similar. At about 6 to 7 months, tightening of the Achilles tendons has been noted, progressing in almost all of the instances through a period of spasticity and finally to flaccidity. In one instance there has been a progressive flaccid paralysis from the beginning.

WAYS, SEATTLE: I would just like to suggest that if you do study the red cell lipids, you don't disregard the hemolysate—that you study the stroma and hemolysate lipids separately. It is conceivable, at least, that the stroma might be normal but that you might find something in the hemolysate which would give you a clue as to what is going on.

KOSOWER, NEW YORK: What is the specificity of this enzyme for the substrates? Could it be that, for example, in the nervous system, another substrate exists? The clinical description of neuromuscular disease starting at six months after birth with progressive deterioration is in a way analogous to some hereditary lipid storage diseases. Accumulation of such substrate could lead to progressive degeneration of the affected cells.

SCHNEIDER: This is an area that we really haven't explored at all in our own studies. So far as I am aware, triosephosphate isomerase is specific for the two substrates, dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. However, we have done no work on this at all.

LOOS, AMSTERDAM: I am puzzled by your explanation that the production of energy-rich phosphate bonds is favored by using the HMP-shunt as a bypass reaction of triose isomerase. Looking at the stoichiometry of the HMP-shunt, you may expect that three molecules of CO_2 and one molecule of lactate are formed from one molecule of glucose; this will reduce the production of energy-rich phosphate bonds from four to two; you will get the same production if you do not use dihydroxyacetone phosphate during glycolysis. So I don't see the value of the HMP-shunt.

SCHNEIDER: We have conceived of this in a somewhat different way. If you assume that the inability to utilize dihydroxyacetone backs up as far as fructose monophosphate and thence into the shunt, one could conceive that virtually no recycling would occur. Thus you lose only one carbon as CO_2. Also there would be a saving of one mole of ATP, since the phosphofructokinase step would be bypassed. Theoretically, five-sixths of the carbon would be available for ATP synthesis in the glycolytic steps below triosephosphate, resulting in a possible net gain of 2⅓ moles of ATP.

LOOS: I don't agree with you there; if there is no recycling, then fructose-6-phosphate has to pass the triose isomerase reaction. In a TPI-deficient red cell, this shunt product can't be used for improvement of energy-rich phosphate bond production.*

SCHNEIDER: We are not so certain that the products of the hexose monophosphate shunt may not enter directly and entirely at the glyceraldehyde phosphate level.

LOOS: Looking at the shunt reactions, you will notice that glyceraldehyde-3-phosphate, as well as fructose-6-phosphate, is formed and no other products for glycolysis.

*After recalculation, I agree with Dr. Schneider that the use of the HMP-shunt in TPI-deficient red cells increases the net gain of energy-rich phosphate bonds slightly (from 0 to ⅓ to 1). However, this net gain is still low in comparison with the value obtainable with normal red cells. To me it still remains puzzling how the normal ATP-level in TPI-deficient red cells may be explained on the basis of shunt stimulation.

Elevated ATP Levels in Human Erythrocytes

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THREE DIFFERENT TYPES of abnormalities characterized by a hereditary increase of red cell ATP levels have been described in the literature. In 1964 Brewer[2] reported the occurrence of a hereditary abnormality in a Negro family that was characterized by a significantly increased ATP level in otherwise normal erythrocytes; in the same family, sickle cell trait and G-6-PD-deficiency were found. More details were presented in 1965,[3] and we now know that (a) inheritance was of autosomal mode and not linked with the other abnormalities; (b) ATP-levels in erythrocytes of the affected persons varied from 128 to 198 per cent of the normal level; (c) pyruvate kinase activity of the red cells was normal; and (d) after prolonged storage at 4° C,[4] the viability of erythrocytes with high ATP levels was greater than that of stored normal red cells. The primary biochemical abnormality in these erythrocytes has not been elucidated.

In the same year, Jacobasch and co-workers[9] reported the occurrence of high ATP levels in the erythrocytes of two unrelated babies; in addition to this abnormality cerebral dysgenesis, decreased development, muscle hypotonia, achromatrichia, and hyperlipemia were found. Nothing is known about the inheritance of this abnormality. The erythrocytes of those babies were characterized by (a) spherocytosis, (b) 40 and 60 per cent increased ATP level, (c) normal 2,3 DPG content, (d) normal glucose consumption, and (e) absence of 2,3 DPG phosphatase activity.

Jacobasch and associates suppose that there is a causative relationship between this phosphatase deficiency and the high ATP levels. The 2,3 DPG phosphatase deficiency reduces the formation of 3 phosphoglycerate from 1,3 DPG via 2,3 DPG to zero, thereby promoting the phosphorylation of ADP to ATP in the bypass reaction of phosphoglycerate kinase. A peculiar phenomenon is that the blocked 2,3 DPG phosphatase reaction does not result in an accumulation of 2,3 DPG. No explanation was given for the increased amount of adenine in the nucleotide pool of the abnormal erythrocytes.

In 1964 we[15] also reported the occurrence of high ATP levels in the erythrocytes of the members of a large family in which β-thalassemia minor was found. It was observed:

a. that the inheritance of the high erythrocytic ATP levels was of a dominant autosomal mode, which was not linked with inheritance of β-thalassemia.

Dr. Loos's attendance at the symposium was made possible by the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) and the Dr. Karl Landsteiner Foundation.

b. that the ATP levels varied from 180 to 200 per cent of the normal value. (Base analysis proved that the ATP fraction, previously separated by column chromatography, contained only adenine as purine base.)
c. that of all the enzymes estimated (glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, glutathione reductase, phosphogluconic acid dehydrogenase, triosephosphate isomerase, pyruvate kinase, and lactic dehydrogenase), only the pyruvate kinase activity was abnormal, always approximately twice the normal level.
d. that glucose consumption and the lactate production with different substrates was normal.
e. that the 2,3 DPG content of the abnormal cells was only 30 per cent of the normal level.

It may be worthwhile to review the different attempts we have made to localize the primary abnormality in the erythrocytes with a doubled ATP content. The level of ATP in red cells is the result of a continuous formation and breakdown. We, therefore, thought that since glucose consumption was normal, the con-

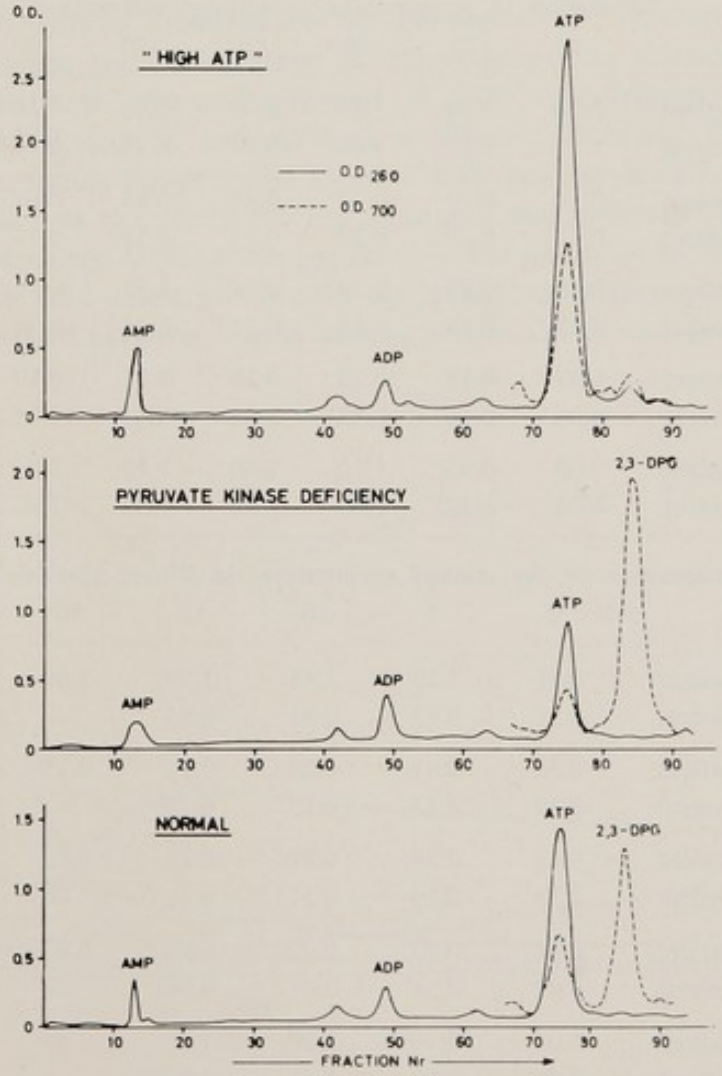


FIG. 1. Dowex-I chromatography of extracts from erythrocytes with doubled pyruvate kinase activity, pyruvate kinase deficient erythrocytes, and normal erythrocytes. Methods as described by Prins et al.[13] and by Loos.[11]

sumption of ATP might be impaired. This concept appeared particularly attractive, since Harvald and co-workers[7] reported the occurrence of hereditary ATP-ase deficiency in human erythrocytes. However, we found that the ATP-ase activity in the red cells of 7 affected members of the family was increased instead of impaired (0.070 ± 0.019; normal value, 0.040 ± 0.08 μM P_i/ml/min).

The formation of a high ATP level in red cells requires, in addition to extra adenine and pentose phosphate, a high production of energy-rich phosphate bonds. As was already mentioned, glucose consumption as well as lactate production with different substrates was normal. Chromatographic analysis of the organic phosphate compounds of the abnormal erythrocytes revealed only two abnormalities: a doubled ATP content and a very low 2,3 DPG content (Fig. 1).

TABLE 1. *Biochemical changes in erythrocytes with an increased pyruvate kinase activity and in normal erythrocytes during incubation in Ringer-solution without substrate and after washing and resuspension in Ringer-adenosine-solution*

		Incubation in Ringer-solution without substrate							
Minutes		0	30	60	120	180	240	300	345
ATP	patient	2.36	2.34	2.36	1.99	1.56	1.26	1.04	0.90
	control	1.11	1.07	1.06	0.94	0.83	0.71	0.62	0.52
ADP	patient	0.18	0.20	0.24	0.45	0.64	0.61	0.65	0.62
	control	0.18	0.20	0.20	0.23	0.33	0.33	0.43	0.38
AMP	patient	0.16	0.15	0.14	0.19	0.32	0.37	0.41	0.42
	control	0.17	0.16	0.15	0.21	0.26	0.30	0.33	0.35
Pyruvate	patient	0.07	0.17	0.23	0.29	0.31	0.37	0.20	0.44
	control	0.06	0.14	0.28	0.52	0.82	1.25	1.31	1.29
Lactate	patient	0.00	0.80	1.60	2.40	2.80	3.00	3.60	3.80
production	control	0.00	0.20	1.00	1.90	1.50	1.80	1.80	2.10

		After resuspension of the washed erythrocytes in Ringer-adenosine-solution						
Minutes		0	5	10	15	30	40	60
ATP	patient	0.89	1.19	1.48	1.59	1.68	1.73	1.75
	control	0.51	0.69	0.91	0.95	—	1.00	0.98
ADP	patient	0.56	0.41	0.21	0.18	0.15	0.13	0.18
	control	0.37	0.31	0.15	0.16	—	0.17	0.16
AMP	patient	0.44	0.29	0.21	0.21	0.20	0.20	0.23
	control	0.38	0.29	0.21	0.21	—	0.21	0.20
Pyruvate	patient	0.00	0.02	0.02	0.01	0.03	0.02	0.03
	control	0.02	0.03	0.01	0.00	—	0.02	0.01
Lactate	patient	0.00	0.10	1.00	1.30	1.80	2.30	3.10
production	control	0.00	0.30	0.60	1.00	—	2.20	2.50

The concentrations are expressed in μmole/ml cells. The methods are described by J. A. Loos[11] and by Prins et al.[13]

Mindful of the occurrence of hereditary 2,3 DPG mutase deficiency,[14] as well as the causative relationship between a high ATP level and an impaired 2,3 DPG cycle, suggested by Jacobash and co-workers,[9] it was necessary to exclude the occurrence of 2,3 DPG mutase deficiency in the abnormal cells. Incubation of red cells from two different patients with a doubled ATP content and a low 2,3 DPG level in isotonic sodium fluoride solution, with pyruvate and adenosine as additives, revealed a 2,3 DPG production, respectively 2.2 and 3.0 μmoles per ml of erythrocytes in 80 minutes. This rate of formation of 2,3 DPG is of the same magnitude as the glycolylic activity of these erythrocytes.

A comparative study of the biochemical features of red cells with pyruvate kinase deficiency[6] and of red cells with a doubled ATP content and a doubled pyruvate kinase activity led us to hypothesize that the activity of pyruvate kinase and presumably other enzymes late in the Embden-Meyerhof pathway governs the flow of 1,3 DPG to 3 PG via 2,3 DPG, thus regulating the net gain of energy-rich phosphate bonds.[10,11]

The results of an experiment that we performed directly support this hypothesis (Table 1). Red cells with high ATP content and normal erythrocytes were incubated without substrate for 345 minutes at 37° C; then the cells were washed with saline and resuspended in Ringer-adenosine solution. During this experiment the breakdown and synthesis of ATP were followed by measuring ATP, ADP, AMP, lactate, and pyruvate at different intervals. The net gain of energy-rich phosphate bonds in the form of ATP per μmole of lactate produced from adenosine was calculated for the abnormal and normal red cells; the abnormal cells produced 0.60 μmole per ml cells per μmole of lactate formed, and the normal cells 0.36 μmole per ml cells per μmole lactate formed. Thus, red cells with double-normal pyruvate kinase activity produce more energy-rich phosphate

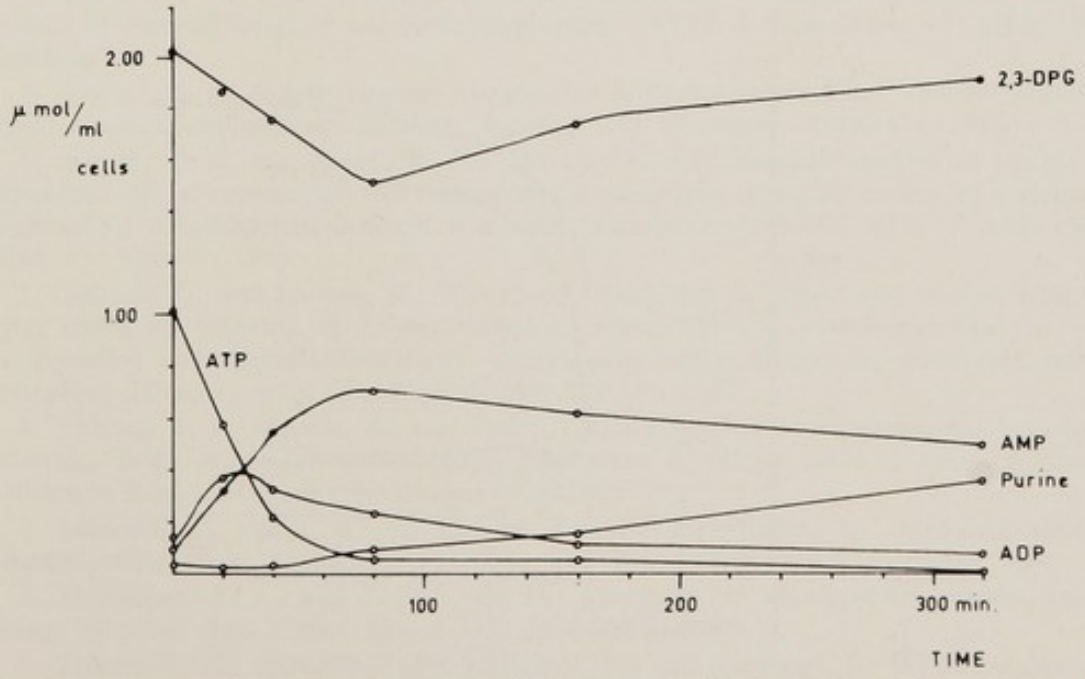


FIG. 2. Metabolic changes in normal erythrocytes during incubation with Ringer-glucose-NaF solution; analysis of the different compounds was done by Dowex-1 chromatography.

bonds during glycolysis. These results become even more pronounced as one takes into account that ATP consumption is greater in the red cells with high ATP content.

The question which immediately rises is, what is the origin of the extra adenine content of erythrocytes with elevated ATP-levels? There are two possible explanations: Reticulocytes do have a much higher ATP-content than mature red cells.[5] During maturation, the capacity of de novo synthesis of purine is lost,[12] and the production of energy-rich phosphate bonds is diminished by the loss of oxidative phosphorylation.[5] This causes a loss of adenylic acid as ATP is broken down to IMP or purine and pentose phosphate. IMP cannot be used for resynthesis of ATP,[1] while purine may diffuse out of the cell. The amount of adenylic acid that is retained after maturation will depend strictly upon the energy-rich phosphate bonds' production capacity that is left. We may say that erythrocytes with a double-normal pyruvate kinase activity will have a greater retention of adenylic acid than normal erythrocytes because the energy-rich phosphate bond production is greater in these cells.

Henderson and Le Page[8] demonstrated that in vivo mature mouse erythrocytes take up adenine originating from different tissues. The adenine is utilized for

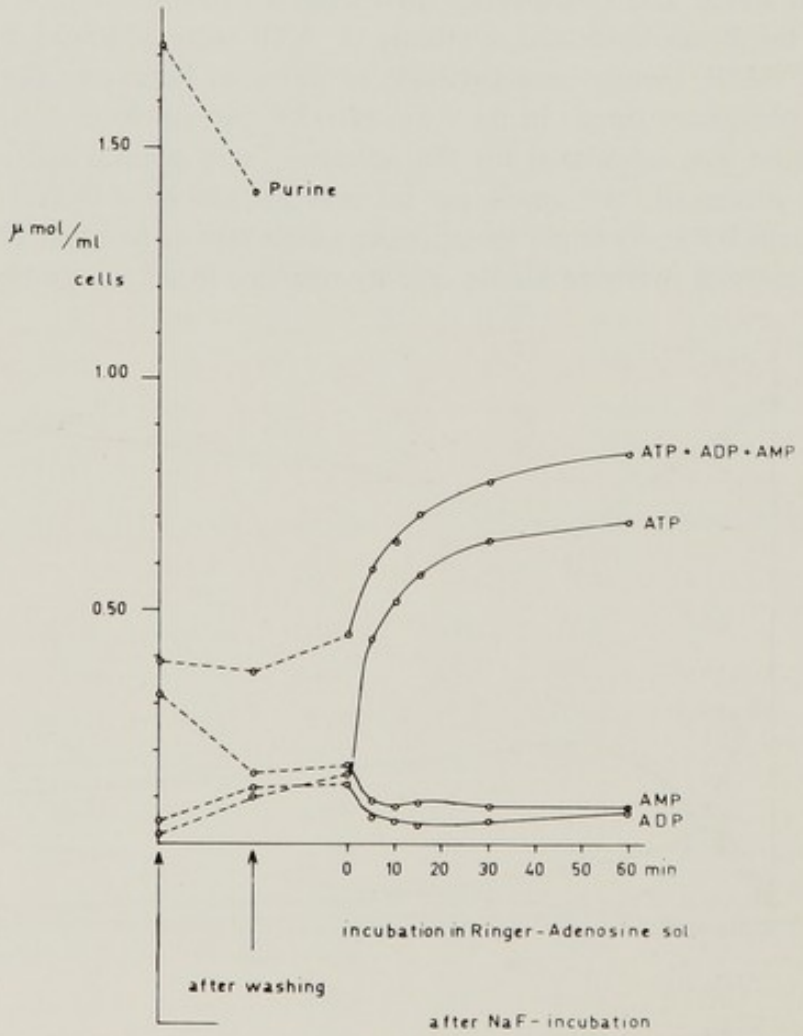


FIG. 3. Metabolic changes in normal erythrocytes after 300-min. incubation with fluoride, after washing and resuspension in Ringer-adenosine-solution. The analysis of the different compounds was done by enzymatic methods, described in detail by J. A. Loos.[11]

ATP-synthesis. The reverse of this process also appears to occur. In man, erythrocytes may perhaps use adenine from extra-erythrocytic origin for the synthesis of adenylic acid, the amount of adenylic acid formed being strictly dependent on the production capacity of energy-rich phosphate bonds in the red cells.

We have shown that normal erythrocytes can rapidly synthesize ATP from adenosine (Fig. 2). Red cells were incubated in isotonic sodium fluoride solution, and ATP was rapidly broken down to AMP via ADP, and purine was formed gradually from ATP. If the fluoride is eliminated by washing the cells and the cells are resuspended in Ringer-adenosine solution, ATP is formed rapidly by rephosphorylation of AMP and ADP.

On prolonged preincubation with sodium fluoride (300 min.), purine diffuses out of the red cells, and then the adenylic acid content is greatly diminished (Fig. 3). After washing these cells and resuspending them in Ringer-adenosine solution, ATP is synthethized from adenosine. The same phenomenon was found to occur with red cells in which the adenylic acid content was depleted by storage at 4° C.

Summary

Three different types of abnormal erythrocytes with high ATP content occur. Evidence is presented that in the Dutch type the origin of the high-energy phosphate content, as well as the extra adenine content, is caused by increased pyruvate kinase activity.

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DISCUSSION

GIBLETT, SEATTLE, CHAIRMAN: Let us begin the discussion.

JAFFÉ, NEW YORK: Do these patients in your Dutch families have any hematologic abnormalities associated with the high ATP levels?

LOOS, AMSTERDAM: No real hematologic abnormalities. In the whole blood we find a small but significantly higher hemoglobin content, and in two of our patients the apparent half-life of the erythrocytes, as determined with chromium-51, is in the lower limit of the normal range. Perhaps we can explain the latter phenomenon on the basis of the very low 2,3-DPG content, which makes the ATP unstable in the erythrocytes with high ATP levels. We found that on incubation of these cells in a medium without glucose, the ATP decreased much more rapidly than in normal red cells, and it may be that this also occurs in vivo under certain conditions, e.g., in the spleen.

AZEVEDO, HONOLULU: We measured ATP levels in a sample of approximately 400 Negroes and Caucasians. From these, a subsample of 100 was also assayed for pyruvate kinase. We confirmed the lower ATP levels in Negroes compared with Caucasians. However, we did not find any correlation between ATP and PK levels.

LOOS: That is about the same as was reported by Brewer. His patients had high ATP levels but the pyruvate kinase activity was normal.

KEITT, BOSTON: Have you measured the levels of 3-phosphoglyceric acid in the high ATP families, and has anyone ever shown enough activity of 2,3-diphosphoglycerate phosphatase to support the amount of lactic acid production from glucose that would be required for your hypothesis to be verified? Is there enough activity of that enzyme to support a normal glycolytic rate if it went by the pathway that you postulate in pyruvate kinase deficiency?

LOOS: Beginning with your second question, it is claimed in the literature that the 2,3-DPG cycle is quite active, as was estimated, e.g., by Rapaport with P^{32}-incorporation. As you know, the interpretation is rather tricky. Actually, I still believe that the real activity of the 2,3-DPG cycle is unknown, so I agree with you that this is a weak point in my hypothesis. The 3-phosphoglycerate content of red cells with high ATP was not measured.

KEITT: Would you not require that the 3-phosphoglyceric acid be low for your hypothesis?

LOOS: We would expect that the 3-phosphoglycerate content would be low in these cells; however, we have no data.

LÖHR, MARBURGH: Your interesting cases are similar in some respects and different in others from our cases with diphosphoglycerate mutase deficiency. We have investigated 6 cases with deficiency in diphosphoglyceromutase. In our cases, the diphosphoglycerate level was low, but the ATP level was also diminished. We were astonished to find that it is the monophosphoglycerate mutase reaction that needs catalytic quantities of 2,3-diphosphatase for its action—that it is not stimulated enough. Because of this, low glycolytic lactic formation and low ATP is evident in our case. In this respect these cases do differ from yours.

LOOS: I see. How did you measure the 2,3-DPG mutase activity?

LÖHR: The mutase reaction was measured following the method of Dr. Schröter.* We have used fructose 1-6-phosphate as a substrate, and it is important to eliminate from the hemolysate all traces of ADP by dialyzing the hemolysate, because in the presence of ADP one part of the fructose 1-6-diphosphate allows the direct glycolytic pathway to lactate. We have eliminated the ADP and used fructose 1-6-diphosphate as substrate. We have measured the formation of 2,3-diphosphoglycerate by enzymatic assay of monophosphoglycerate mutase.

LOOS: Yes, and you know that Dr. Keitt has remarked that in this system a deficiency of trioseisomerase might give the same effect, but I am sure you have put trioseisomerase in your reaction mixture.

LÖHR: Yes, we have measured TPI in all our cases and never have we seen a defect in this enzyme.

HARRIS, LONDON: I may have missed this in your paper: have you examined the pyruvate kinase levels in white cells or any other tissue cells in this condition?

LOOS: Up to now we have only looked at the red cells.

PRINS, AMSTERDAM: May I make a comment on the question of pyruvate kinase activity and ATP levels? Elevated pyruvate kinase activities are not always accompanied by high ATP levels. In our family the situation was such that of all the enzymes we estimated, only pyruvate kinase was elevated. However, when every enzyme is increased in the same fashion as pyruvate kinase, we would not expect a high ATP level.

*Schröter, W., and Heyden, H.: Kinetik des 2,3-Diphosphoglyceratumsatzes in menschlichen Erythrocyten. Biochem. Z. 341-387-398, 1965.

Erythrocyte Hexokinase and Hereditary Hemolytic Anemia

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THE METABOLICALLY IMPOVERISHED, non-nucleated, mature human erythrocyte is indeed unique among the cells of the human body. It lacks DNA, RNA, mitochondria, the capacity to perform oxidative phosphorylation, and an intact Krebs cycle or cytochrome system. Unlike the platelet and the leucocyte, which may be destroyed in the process of performing their physiological functions, the red cell is normally a neutral nomad, exchanging its wares of oxygen in the tissues and carbon dioxide in the lungs. It has learned the lessons of austerity. On a simple diet largely consisting of sugar, vitamins, and phosphorus it conserves its energy to meet its own simple needs: to resist the accumulation of methemoglobin; to actively transport cations against electrochemical gradients; to preserve its hemoglobin undenatured in the face of oxidative stresses. It husbands its restricted resources, and like the organism as a whole—if given a reasonable chance—lives out its allotted span. Diminishing in vigor, gradually, by a process of attrition and senility, it approaches destruction. It is not surprising that, if born with genetically-imposed handicaps or subjected to abnormally great environmental stresses, it may succumb prematurely in the body with resultant hemolytic anemia.

The adult human erythrocyte is a glycolytically-dependent entity. Under normal circumstances some 90 per cent of glucose metabolized and some 75 per cent of energy produced is derived from anaerobic glycolysis and the production of lactate via the well-known Embden-Meyerhof pathway. Net generation of ATP results and the pyridine nucleotide NAD is cycled to NADH and back again. A side reaction of great interest and possibly major importance permits the accumulation of uniquely large amounts of 2,3-DPG (which may comprise 50 per cent of the total inorganic phosphorus of the cell) through the conversion enzymatically and essentially irreversibly of 1,3-DPG to 2,3-DPG. The latter in

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turn may, under appropriate conditions, be dephosphorylated to 3-PGA. 3-PGA when produced via 2,3-DPG (the Rapaport-Luebering shunt) has been achieved while bypassing the ATP-generating PGK reaction which competes with 2,3 diphosphoglycerate mutase for the common substrate 1,3-DPG. It is apparent that variations in the amount of glucose traversing the Rapaport-Luebering shunt may have important implications in the maintenance of intracellular ATP-ADP ratios, the level of intracellular inorganic phosphate, and the delicate regulation of glycolysis. While anaerobic glycolysis per se results in the net generation of two moles of high energy ATP for each mole of glucose converted to lactate, there is no net synthesis or loss of ATP if glucose is metabolized through the Rapaport-Luebering shunt. The operation of both pathways simultaneously results in adjustable and variable net synthesis of somewhere between 0 and 2 moles of ATP per mole of glucose metabolized. Presumably this may be varied under different conditions of supply and demand.

Normally, approximately 10 per cent of glucose metabolized and some 25 per cent of energy generated occurs via the hexose monophosphate shunt pathway. Oxygen is required, carbon dioxide is generated from carbon-1 of glucose, and pentose is formed. The latter is converted by the catalysis of transketolase and transaldolase back to intermediates of the Embden-Meyerhof pathway, and glucose, metabolized either anaerobically or by way of the oxidative shunt, ultimately traverses a common pathway to lactate. The pyridine nucleotide co-factor of the oxidative shunt is NADP and not NAD. The NADPH generated plays an important role—particularly in the face of drug-induced and other oxidative stresses—in the maintenance of GSH and as a backup contributor to the reduction of methemoglobin. In the performance of these functions it is recycled to NADP and is again available for shunt pathway metabolism.

In so metabolically restricted a cell inborn errors or deficiencies in the limited metabolic pathways available can be expected to and, indeed, do result in clinically apparent and symptomatic hemolytic anemia. A deficiency of G-6-PD of the oxidative shunt pathway is clearly related to certain forms of drug-induced hemolysis,[5] and in certain deficient subjects chronic hemolysis in the absence of drug challenge may be present.[15,27] Hereditary deficiencies of erythrocyte pyruvate kinase,[29,31] triosephosphate isomerase,[24,30] 2,3-DPGM,[2,13,25] and more recently G-3-P dehydrogenase[5] have been documented. Other hemolytic processes have been defined in terms of association with ATPase deficiency,[7] with GSSG-reductase deficiency,[4,12] and with inability to synthesize the tripeptide GSH itself.[17,21,22] Such disorders have been characterized as intracorpuscular in type, have not been associated with spherocytosis, abnormal hemoglobins or a positive antiglobulin test, and have usually (though there are exceptions) exhibited a moderate macrocytosis and nondescript red cell morphology, with variable but usually small numbers of irregularly contracted erythrocytes on stained blood films. Splenomegaly has been present, and splenectomy, either partial or complete, is of little benefit. We have recently reported hereditary hemolytic anemia, having in general the characteristics noted just above, associated with a profound deficiency of hexokinase, the first catalyst of glycolysis.[29] The findings in this syndrome and the role of hexokinase in erythrocyte survival are the subject of this discussion.

KINDRED N.

The proposita (S.N.-C.H.S., #55-14-60) has had hemolytic anemia since birth and, despite lack of any evidence of maternal-fetal Rh or ABO incompatibility, required exchange transfusion in the neonatal period. The history of this female child, 11 years of age at the time of the present study, has been reported in greater detail elsewhere.[29] Jaundice, progressive anemia, reticulocytosis, and hepatosplenomegaly continued to be evident despite transient improvement following the initial transfusion. Splenectomy was performed at age five months and, because hemolytic anemia, while ameliorated, clearly persisted, accessory splenic tissue was sought but not found at laparotomy performed at age 18 months. Moderate chronic anemia, mild icterus, and reticulocytosis have characterized the patient's condition from that time until the present. The hemoglobin has usually ranged between 7.5 and 10.5 g per 100 ml of blood, and the reticulocyte count was between 7 and 24 per cent. Incubated osmotic fragility of erythrocytes is now essentially normal, though before splenectomy it was substantially increased as was red cell mechanical fragility. Figure 1 shows a representative Wright's stained blood film. Moderate anisocytosis, polychromasia, poikilocytosis, and a number of small, densely-staining cells with spickle-like surface projections are evident. Some increase in basophilic stippling, occasional cells resembling spherocytes, and occasional Howell-Jolly bodies are also seen. The hemograms of the father and mother of the proposita and those of

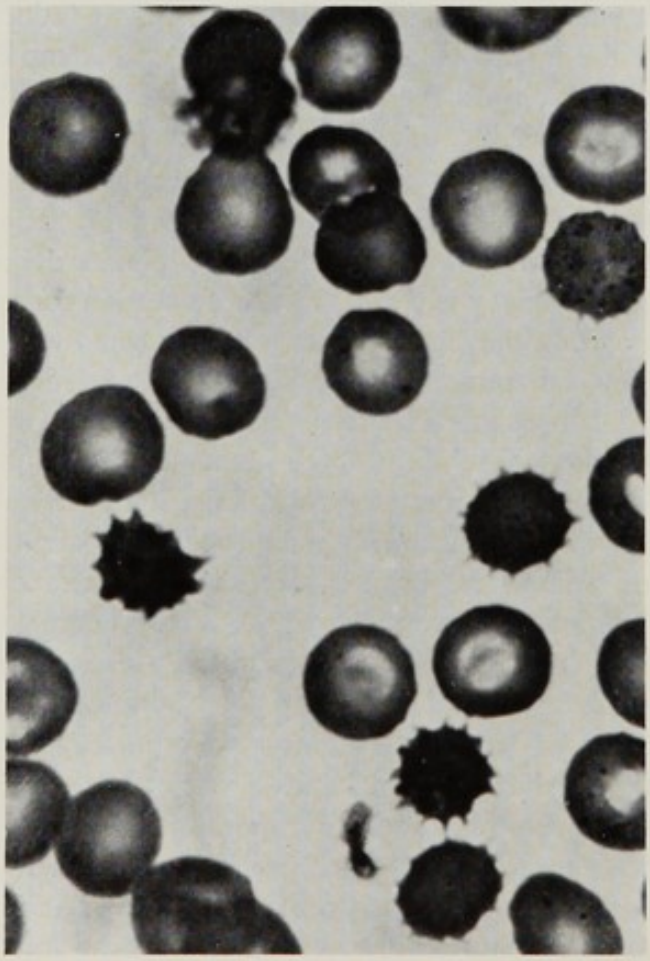


FIG. 1. Wright's stained blood film of proposita (S.N.).

four of her siblings are normal. Parents and siblings are clinically asymptomatic, and splenomegaly and jaundice are absent. The family is of English and Polish extraction. One of four siblings and the father are said to have had transient neonatal jaundice.

Materials and Methods

Hexokinase activity of both erythrocytes and leucocytes was assayed in a system containing dilute hemolysate. The components and conditions of assay are shown in Table 1. G-6-P generated by the reaction is quantitatively converted to 6-PGA by added excess of crystalline G-6-PD, and the reaction was followed spectrophotometrically at 340 mμ as NADP is converted to NADPH. The following enzymatic activities of erythrocytes were measured by assay procedures described previously:[11] glucose phosphate isomerase, fructosediphosphate aldolase, glyceraldehyde phosphate dehydrogenase, triosephosphate isomerase, phosphoglycerate kinase, monophosphoglyceromutase, phosphopyruvate hydratase, pyruvate kinase, lactate dehydrogenase, glucose-6-phosphate dehydrogenase, phosphogluconic dehydrogenase, and glutathione reductase. Adenosine triphosphatase was assayed in a system linking the conversion of ATP to ADP to the pyruvate kinase reaction. The assay conditions as well as those employed to measure phosphofructokinase activity are detailed elsewhere.[29] In certain studies the phosphorylation of fructose and the conversion of fructose, fructose-6-phosphate, mannose, and glucose-6-phosphate to other glycolytic intermediates by hemolysates were also measured according to methods outlined in our initial report.[29] Determination of erythrocyte autohemolysis,[20] glucose consumption,[19] glutathione stability,[1] ATP concentration,[19] and of $C^{14}O_2$ recovery from U-C^{14} glucose and 1-C^{14} glucose[18] have been documented elsewhere. Studies on upper and lower layers of centrifuged erythrocytes were performed on the upper and lower thirds, respectively, of a column of erythrocytes centrifuged at 3000 x G for 30 minutes. Electrophoretic studies on erythrocyte hexokinase were performed by the method of Katzen and Schimke[10] and are reported separately elsewhere.[9]

TABLE 1. *Hexokinase assay system**

	Final Quantity per Cuvette
Glycylglycine buffer, 0.05 M, pH 8.1	25 μmoles
$MgCl_2$	5 "
Glucose or fructose	5 "
ATP	5 "
NADP	0.5 "
Glucose-6-phosphate dehydrogenase	1.0 enzyme units
Hemolysate equivalent to 3.0 x 10^7 erythrocytes	
Final volume	3.1 ml

*Incubation time = 1 hour; temperature = 37.0°, change in absorbence (A) recorded continuously employing automatic Gilford Recorder. Results are expressed as micromoles of NADP converted to NADPH per minute per 10^{10} erythrocytes.

Hematologic Studies

Hematologic studies performed on the patient and her family are summarized in Table 2. The patient exhibits anemia, substantial reticulocytosis, moderate macrocytosis, a slightly elevated leucocyte count with an unremarkable differential formula, and a normal platelet count. Erythrocyte morphology has been described earlier in this report. Parents and siblings have essentially normal hematologic parameters, including lack of reticulocytosis, although the erythrocyte count per cu mm and packed cell volume in the case of the father are slightly lower than normally expected. The osmotic fragility of incubated cells of the patient is currently normal and this is likewise true for the cells of both parents. Hemoglobin electrophoresis reveals no evidence of a hemoglobinopathy. Hemoglobin A_2 constitutes 2.9 per cent and hemoglobin F, 2.2 per cent of the total hemoglobin of the proposita. The erythrocyte half-survival time of the patient's cells as measured by the Cr^{51} method is 6.0 days, while that of parents and siblings is normal. Autohemolysis of the erythrocytes of the proposita conforms to the Type I pattern as defined by Selwyn and Dacie.[26] Authohemolysis is slightly increased in the absence of additives and is partially but not entirely corrected by glucose and more so by adenosine. Adenosine does not require hexokinase for its metabolism. The data are summarized in Table 3.

Glutathione Stability

Glutathione is present in normal amounts in the cells of the proposita and is normally stable on incubation with acetylphenylhydrazine in the test devised by Beutler.[1]

Enzyme Studies

When the erythrocyte enzymatic activities of the Embden-Meyerhof and oxidative shunt pathways were measured in the patient's erythrocytes, hexokinase alone was found to be deficient. Moderately to substantially increased activities

TABLE 2. *Kindred N. — Hematologic data*

	Patient	Father	Mother	Brother (T.)	Brother (B.)	Sister (D.)
Hb (g%)	9.4	14.1	13.6	12.5	11.8	12.5
RBC (x 10^6/cu mm)	3.16	4.66	4.69	4.35	4.0	4.09
PCV (%)	34.0	41.0	38.0	37.0	33.0	36.0
Reticulocytes (%)	13.4	1.4	0.7	1.0	0.9	0.4
MCV (μ^3)	107.0	88.0	81.1	85.0	82.5	88.1
Hb type	AA	AA	AA	—	—	—
Fetal Hb (%)	2.2	—	—	—	—	—
Hb A_2 (%)	2.9	—	—	—	—	—
Antiglobulin test	Negative	Negative	Negative	—	—	—
Incubated fragility test	Normal	Normal	Normal			
RBC survival time ($T\frac{1}{2}$ Cr^{51})	6 days					

TABLE 3. *Autohemolysis studies—RBC of proposita (S. N.)*

Autohemolysis (%)	No Additive	Glucose 0.026 M*	Adenosine 0.020*
Normal mean	0.59	0.11	0.21
Proposita (S. N.)†	2.90	0.76	0.61
	3.00	1.90	0.40

*Final concentration.
†Two separate determinations.

were observed for phosphofructokinase, fructosediphosphate aldolase, glyceraldehydephosphate dehydrogenase, phosphoglycerate kinase, phosphopyruvate hydratase, pyruvate kinase, lactate dehydrogenase, glucose-6-phosphate dehydrogenase, phosphogluconate dehydrogenase, glutathione reductase, and adenosine triphosphatase. The activities of glucosephosphate isomerase and triosephosphate isomerase were normal. This conforms essentially to our usual experience when erythrocytes of young mean cell age are assayed.

Hexokinase, in sharp contrast to all other activities measured, was observed to be uniquely and markedly deficient. Data on the proposita and her family are summarized in Table 4. These were amply and reproducibly confirmed by repeated studies. The following points deserve emphasis: (1) The patient's cells possess hexokinase activity below our lower limits of normal *despite marked reticulocytosis and a very young mean cell age*. Erythrocyte hexokinase activity must be evaluated in the context of the fact that it is characteristically and uniformly increased to a marked degree—up to several-fold—in the presence of hemolytic anemia, reticulocytosis, and young erythrocyte populations. A representative example of the order of magnitude of the aberration in the cells of the proposita may be illustrated by the fact that a simultaneously assayed control

TABLE 4. *Kindred N.—Erythrocyte hexokinase activities**

Subjects	Reticulocytes	Hexokinase*
Normal (13)	(%)	(EU/10^{10} RBC)
Mean		0.24
Range	0.5-1.5	0.17-0.31
Std. Dev.		0.043
Patient S. N.	14.50	0.15
High-retic. fraction	23.10	0.17
Low-retic. fraction	6.05	0.11
Father	0.58	0.15
Mother	0.80	0.19
Brother (T.N.)	0.20	0.075
Brother (B.N.)	0.40	0.16
Sister (D.N.)	0.30	0.17

*Expressed as indicated in Table 1.

specimen of comparably young cell age and with 20 per cent reticulocytosis exhibited activity six to eight times greater than that of the patient. (2) Leukocyte hexokinase activity was normal in the cells of the patient and her family. (3) Hexokinase was clearly deficient in specially prepared "high" and "low" reticulocyte fractions of the patient's blood. Substantial elevations are found routinely when similar fractions from cells obtained from subjects with hemolytic anemia of other etiology are assayed. (4) Erythrocyte hexokinase activity is either low or low normal in both parents and the siblings of the proposita. Strikingly diminished activity is evident in the cells of sibling T.N., and substantially subnormal activity is noted in those of the patient's father and certain siblings.

Figure 2 records our observations of erythrocyte hexokinase activity in patients with hemolytic anemias over the past three years and places the proposita in perspective. Fifty-four cases of hemolytic anemia of various etiologies have been grouped arbitrarily according to the reticulocyte percentage of the populations assayed. The mean of each group is plotted against the mean hexokinase activity observed in the assays of the same erythrocyte populations. It is abundantly clear that striking increases in red cell hexokinase activity accompany increasing reticulocytosis in assayed blood. Standard deviations are shown. The erythrocytes of the proposita are uniquely deficient. The magnitude of the observed deficiency may be documented in still another way. It is well established, and our own clear experience confirms the observation, that certain other red cell enzymes, glucose-6-phosphate dehydrogenase and pyruvate kinase among them, show increased activity in young erythrocytes. There is then a computable ratio of hexokinase (HK) to G-6-PD and PK in normal and abnormal blood. Table 5 records these ratios in normal control blood, in 16 cases of miscellaneous

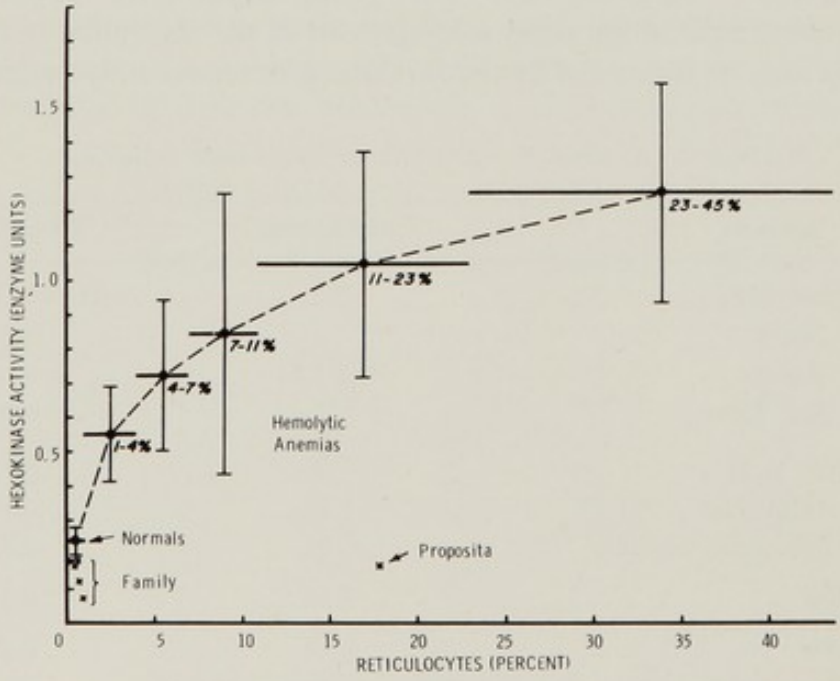


FIG. 2. Hexokinase activity observed in 54 cases of hemolytic anemia of various etiologies plotted against reticulocyte percentage in cells assayed. Cases are grouped according to reticulocyte levels. Mean hexokinase activity for each group is plotted against mean reticulocyte percentage in cells of that group. Standard deviations are shown. Values for proposita and her family are designated separately.

TABLE 5. *Ratio of hexokinase (HK) to glucose-6-phosphate dehydrogenase (G-6-PD) and pyruvate kinase (PK) activities**

	HK/G-6-PD (x 10^{-2})	HK/PK (x 10^{-2})
Normal subjects	8.37 ± 0.82	9.0 ± 1.66
Hemolytic anemias (16)	18.86 ± 7.32	21.84 ± 9.45
Proposita S. N.†	3.40	3.32

*Enzyme units in each instance are equivalent to micromoles of product produced per 10^{10} erythrocytes per min at 37° C.

†Mean of three separate determinations.

hemolytic anemias, and in three separate assays of the cells of the proposita. The HK-G-6-PD and HK-PK ratios in the case of the patient are far lower than those obtained with normal blood. This is vastly exaggerated when comparison is more appropriately made to ratios obtained in blood from cases of hemolytic anemia with erythrocytes comparable in age with those of the proposita.

In additional studies, enzyme kinetics were investigated by varying substrate concentrations of glucose and ATP independently over a wide range in the assay system. Computation of Michaelis-Menton constants for each substrate revealed no discernible difference qualitatively between the enzyme in patients and in normal control cells. Similarly, preliminary electrophoretic studies of erythrocyte hexokinase of the patient and normal controls showed no detectible qualitative abnormality.[9] Type I and Type III hexokinase bands were observed in both, and these appeared to differ only by the fact that both types were reduced in the patient's blood. No evidence of a hexokinase inhibitor could be ascertained in mixture experiments employing combinations of the patient's and normal hemolysate.

Sugar Metabolism

Table 6 summarizes data relative to the capacity of the patient's erythrocytes to utilize various sugars in vitro as compared with the capacities of normal red cell populations and young populations from subjects with other types of

TABLE 6. *Erythrocyte carbohydrate consumption*

	(μmoles/ml RBC/hr)		
Substrate	Normals	High-Retic. Blood†	Patient S. N.‡
Glucose:	1.60 (20)*	2.70-4.41 (7)	0.57-1.08
Fructose:	1.38 (5)	1.77-2.80 (3)	1.22, 1.53
Mannose:	1.10 (5)	3.28-4.11 (3)	3.00, 3.20
Galactose:	0.20 (5)	0.44-0.92 (3)	2.11, 2.65

*Number of cases in parentheses.

†Reticulocytes = 10.0-19.8% for glucose, and
= 7.8-15-1% for fructose, mannose, galactose.

‡Patients' reticulocytes = 13.0-22.1%.

hemolytic anemia. It is apparent that the cells of the patient utilize both fructose and glucose at rates below those of normal cell populations and far below those of comparably young, "high reticulocyte" blood. Mannose, on the other hand, is actively metabolized by the patient's cells with values greater than those for cells from normal subjects and equivalent to those found when erythrocytes from patients with other hemolytic anemias were studied. Galactose, for reasons not clear, appears to be much more actively utilized than it is by other young erythrocytes. Similarly, low activity is manifest when either glucose or fructose are substrates in the hexokinase assay system. If triose production from glucose or fructose is measured, it is found that this is less than that from normal blood; however, if triose production from either G-6-P or F-6-P is determined, it is comparable to the high level characteristic of young erythrocytes and "high reticulocyte" preparations. Only hexokinase is deficient. Mannose, in sharp contrast, is converted to triose much more actively by the patient's cells than by normal ones. In short, the hexokinase responsible for glucose and fructose phosphorylation appears identical and deficient for both substrates; mannose appears to be phosphorylated by a separate and unaffected kinase. It should be noted that (a) fructose is phosphorylated by erythrocytes of all types at only about one-third the rate of glucose for the same cells in our hexokinase assay, and (b) the conditions of the assay make it certain that the kinase measured is phosphorylating fructose to F-6-P, and not F-1-P. A fructokinase of liver results in the latter product which, however, would not be measured in our assay procedure.

Studies employing 1-C^{14} and U-C^{14} labeled glucose are briefly summarized in Table 7. Despite a diminished total glucose consumption, the absolute amount of glucose metabolized via the oxidative shunt was greater than that observed for normal blood and approached that found in reticulocyte-rich blood. This resulted in a marked increase in the *per cent* of total glucose metabolized via the shunt. When the shunt pathway was stimulated by methylene blue, 10^{-6} M, the per cent of glucose shunted was comparable for normal blood and that of the patient. The *increase* in glucose shunted under methylene blue stimulation was markedly less for the patient's blood than for normal blood. The increased initial shunt pathway activity was, in short, already closer to the maximal activity than is true for normal blood. The reasons for these aberrations from normal are not entirely clear. However, it must be remembered that the generally heightened enzyme activities of reticulocyte-rich blood in the case of both the anaerobic and oxidative

TABLE 7. *Glucose metabolized via pentose-phosphate pathway*

Substrate	Normals (20)* (%)	High-Retic.† Blood (7) (%)	Patient S.N.‡ (%)
C^{14}-U-0_2	2.4-5.7	3.1-5.5	22.7
C^{14}-1-0_2	2.0-2.7	1.9-2.3	10.9, 9.3

*Number of cases in parentheses.

†Reticulocytes = 10.9-19.8%

‡Patient's reticulocytes = 13.0-22.1%.

shunt pathways are in the proposita competing for a uniquely low level of their common substrate, G-6-P, occasioned by the erythrocyte hexokinase activity. The role of levels of co-factors such as NADP in young cells in the face, again, of diminished G-6-P production may likewise conceivably have altered the relative proportion of shunted and anaerobically metabolized glucose. Other possibilities exist, but, in any event, the combination of young cells and deficient hexokinase activity appears to result in a new ratio of shunted glucose to total glucose metabolized.

Genetics

While it appears likely by analogy with similar syndromes that the patient is homozygous for a defective gene, the data do not permit any flatly categorical conclusion. The hexokinase activity of the father's cells appears definitely low and that of the mother suspiciously so. One male sibling, T.N., without evidence of a significant hemolytic process, has values substantially lower than those of the patient—though these values represent the activity of a cell population whose mean age approximates that of normal blood. The apparent possession of the defective gene by a clinically and hematologically normal father and male sibling as well as by the female patient with symptomatic hemolytic anemia points to a probable autosomal mode of transmission. Values for the mother and certain other siblings suggest, but do not definitely prove, that they, too, are heterozygous for erythrocyte hexokinase deficiency. A maternal uncle studied recently has exhibited the low value of 0.15 enzyme units and is likewise very probably a heterozygous carrier of the defective gene. It is tempting to postulate a semirecessive, autosomal mode of transmission of the deficiency, heterozygotes possessing a mild biochemical lesion and the homozygote having clinically-apparent anemia and hemolysis. Caution dictates that additional data be secured before a final decision on the mode of inheritance is made, however.

Comments

It is generally accepted that ultimate destruction of the erythrocyte is dependent upon the gradual attrition of its metabolic machinery and occurs when it is no longer able to support its own small but finite needs and meet the challenges of its environment. However, the critical factor normally conditioning senility and destruction remains undetermined with certainty. Grignani and Löhr[6] assumed a key role for hexokinase because of its strategic location at the beginning of glycolysis and the fact that, at least in vitro, human erythrocyte hexokinase exhibited the lowest activity of any glycolytic enzyme. Hinterburger et al.,[8] Rapaport and associates,[23] and Ockel and co-workers[16] came to similar conclusions. They pointed out that the erythrocytes of different species have variable rates of glycolysis and that for each species the capacity for glycolysis and the activity of hexokinase closely parallel each other. This was found to be true in individual blood samples as well as between species. They also pointed out that in the transition from reticulocyte to adult erythrocyte, the reduction in glycolytic capacity approximates the diminution in observable hexokinase activity. These workers conducted systematic investigations of anaerobic glycolysis under carefully controlled conditions of pH. Glycolysis was optimal at pH 8.5 while hexo-

kinase activity was optimal at pH 8.1. Because of the Donnan distribution of diffusible ions, an external pH of 8.5 is associated with a lower intracellular pH of about 8.0, very close to the optimal value for hexokinase. Since the optimal pH for hexokinase and glycolysis appeared to correspond, this was regarded as further suggestive proof that hexokinase is a decisive link in determining the glycolytic rate. Further support was rendered by the fact that a decrease in dietary magnesium was associated both with a decrease in glycolysis as well as in hexokinase activity.

Not all are in agreement that hexokinase is the pacemaker of glycolysis. Others, for example, are of the opinion that the capacity for anaerobic glycolysis is limited, not by enzymes, but rather by co-enzymes, metabolites, or the availability of enzymes within cells.

Brok and co-workers[3] determined the activity of a variety of enzymes in red cells of different ages separated into groups according to their specific gravity. Hexokinase exhibited the steepest decline in activity between young and old cells. We have had this same clear experience[29]: Of all the enzymes of glycolysis, hexokinase shows substantially the greatest rate of activity decay as the reticulocyte becomes an adult erythrocyte and the latter moves toward senility.

While it cannot be stated categorically that hexokinase is rate-limiting in glycolysis, its activity as the mediator of the first step cannot be substituted for by any naturally occurring pathway and the evidence documented above makes it suspect, indeed, as a possible critical step in survival. The present patient has hexokinase activity in her reticulocyte-rich blood several-fold less than expected and levels appropriate to, or even less than, those present in normal blood. On the hexokinase scale of aging, her reticulocytes are already approaching physiologic senility. They cannot survive to a much riper age, and she has hemolytic anemia.

As discussed in a previous report,[29] it is intriguing to speculate on the reasons why T.N., a sibling, has erythrocyte hexokinase activity substantially lower than that of the proposita and yet has no evidence of hemolytic anemia. His measured chromium-51 half-survival time is essentially normal. Of course, the observed hexokinase levels are those of his normally aged cells while those of the proposita are the levels of reticulocytes and very young erythrocytes. The data for T.N., nonetheless, must mean that if hexokinase is critical in bringing about ultimate erythrocyte destruction, its level in the older, moribund erythrocytes under normal circumstances must be less than that observed as mean value for T.N.,'s cells. It is possible that reticulocytes and young cells, with their greater metabolic activity, may conceivably suffer more from hexokinase deficiency than their older brethren, though this is frankly speculative. Also, it is impossible to predict from the measured mean hexokinase activity in the patient's reticulocyte-rich, young cells how fast this is decaying and what level it attained in those unmeasured cells immediately before their demise. Figure 2 illustrates the striking elevation of hexokinase as reticulocytosis increases in miscellaneous hemolytic states. Despite the slope of the curve, it should not be inferred that data are sufficiently precise to predicate continual exponential decay of hexokinase activity as the reticulocyte matures and the adult erythrocyte ages. Data to be reported elsewhere suggest that this is not quite the case.

The data relative to the consumption of various sugars are of substantial interest. In this experiment of nature, it is clearly apparent that fructose as well as glucose is phosphorylated almost certainly by the same deficient enzyme. Conversely, mannose is metabolized as actively as it is by other reticulocyte-rich bloods and must be phosphorylated by a separate kinase. The reasons for the marked increase in galactose metabolism are not clear.

In 1965 Löhr et al.[14] described diminished hexokinase in red cells, leucocytes, and platelets of three juvenile male patients in two families. These patients bear no resemblance to our subject with marked hemolytic anemia. Not only were all the blood cells hexokinase-deficient but, in addition, each subject exhibited panmyelopathy, multiple congenital abnormalities of the classical Fanconi's syndrome, marked chromosomal aberrations, abnormal enzyme kinetics, and absence of, or at most low-grade, reticulocytosis. None of these features characterized the syndrome in our case.

The nature of the ultimate deficiency in the hexokinase activity of the subject here reported remains obscure. Unlike the cases of Löhr et al.[14] no abnormality in enzyme kinetics is yet apparent. It is not known whether absolute deficiency of enzyme protein exists or whether altered enzyme protein accounts for either diminished activity or accelerated decay. Presence of an inhibitor has not been demonstrated but cannot be regarded as ruled out unequivocally. This would appear to represent another instance of definable molecular disease associated with hereditary hemolytic anemia.

Summary

Studies of a patient with hereditary hemolytic anemia associated with a severe deficiency of erythrocyte hexokinase activity are reported. The proposita has exhibited a continuous hemolytic process since birth with anemia, reticulocytosis, absence of demonstrable hemoglobinopathy, and a negative antiglobulin test. Splenectomy has resulted in only modest amelioration of the process. Despite the fact that hexokinase, of all the erythrocyte glycolytic enzymes, is uniquely elevated several-fold in reticulocytes, the patient's reticulocyte-rich blood has lower hexokinase activity than even that of blood from normal subjects. All other glycolytic enzymes, the activities of the oxidative shunt pathway catalysts, and of several nonglycolytic enzymes possess either normal or greater than normal activity. Glutathione levels are normal and GSH is stable in the acetylphenylhydrazine test, and the autohemolysis pattern conforms to that designated by Selwyn and Dacie as Type I. Fructose and glucose are phosphorylated at less than normal rates by the patient's erythrocytes; mannose, in contrast, is very actively metabolized, apparently by a separate, unaffected kinase. With regard to per cent, a greater than normal amount of glucose is metabolized via the oxidative shunt. Kindred studies indicate normal hematologic parameters in parents and siblings. Hexokinase activity of erythrocytes is low in the father, probably below normal in the mother, and strikingly reduced in a hematologically and clinically normal sibling. It is probable that the symptomatic proposita is homozygous for a semirecessively inherited autosomal gene which in heterozygotes is manifested by a mild biochemical lesion unassociated with

clinical or hematologic abnormalities. White cells do not share in the abnormality.

Acknowledgments

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DISCUSSION

GIBLETT, SEATTLE, CHAIRMAN: Dr. Löhr.

LÖHR, MARBURGH: Dr. Valentine, I also believe that your case is different from our observations about Fanconi myelopathy. In 1965 we published three cases from two different families with hereditary panmyelopathy, and we found hexokinase deficiency in all kinds of blood cells—white cells, platelets, and particularly in red blood cells (ref. 15 in paper). Up to now we have investigated 6 cases. Four of our cases of panmyelopathy had a lack of hexokinase and the fifth had no lack of this enzyme. The K_m for glucose was increased by a factor of three. The ATPase activity was about normal or high, with ATP content and lactate production, decreased. The Heinz body test was positive in one case. It was interesting that Miss Schroeder* has found multiple aberrations of chromosomes in six cases. The karyotype was normal, but the chromosomes showed aberrations like breakage of chromatids and isochromatids. Approximately 50 per cent of all metaphases in lymphocytes treated with colchicine showed these aberrations. She also found tripolar mitotic figures in vivo.

We feel that hexokinase deficiency is directly responsible for these aberrations of mitosis. In some samples we have added an inhibitor of hexokinase to normal lymphocytes and have found that,

*Schroeder, T. M.: Cytogenetsche und cytologische Befund bei enzymophenischen Panmyelopathien und Pancytopenien. Humangenetik 2: 287-316, 1966.

after incubation with it, breakage of chromatids was also present. Perhaps the difference in our cases is one of age. The clinical manifestations of the Fanconi myelopathy occur in about the second decade. Perhaps your patients will still develop a Fanconi syndrome.

SMITH, STILLWATER: I wonder if Dr. Valentine ran GSH stability tests on his patients or did the Heinz body test.

VALENTINE, LOS ANGELES: The level of reduced glutathione was normal, and the stability was likewise normal. The level of the ATP was in the normal range—not as high as seen in comparably high reticulocyte blood, however.

BEUTLER, DUARTE: I am not certain that I understood your interpretation of the brother of the proposita. Did you interpret the brother as being a homozygote or a heterozygote?

VALENTINE: I do not know if one can be absolutely certain; he has no clinical evidence of disease. We are inclined to interpret him as a heterozygote.

BEUTLER: It seems to me that when an apparently identical biochemical lesion produces markedly different effects, we are inclined to regard it as being due to a genetic polymorphism involving the locus under study. I think that very often, though, the expression of the gene may be markedly influenced by the genetic milieu in which it is found.

We have recently, for example, studied a G-6-PD-deficient patient with nonspherocytic congenital hemolytic anemia. We thought that this must be one of the rare variants of G-6-PD deficiency, but upon study of the enzymatic properties, found it to be a typical Mediterranean variant in a Jewish woman.* I wonder whether in Dr. Valentine's case, for example, it is possible that the brother is also homozygous for the same gene as the proposita, but being genetically different from the proposita, the defect is not expressed.

VALENTINE: We do not have any data that would enable us to choose between these two alternatives at this time. I think the case illustrates one of the dilemmas of measuring enzyme activity. We know what the hexokinase is in the blood of the proposita. The blood has 15 per cent reticulocytes and the remaining cells are probably only a little bit older than reticulocytes. We have no way of knowing what levels her blood would have achieved had the cells undergone a normal decay curve for 120 days. That would not answer the question either, but I just want to emphasize that you measure the cells that are there, and you cannot tell what level of enzyme the moribund cell possesses or what enzyme level a given cell will have tomorrow or the next day.

BEUTLER: The brother's enzyme activity is remarkably lower than the parents, however. Is this not correct?

VALENTINE: There is no question about that. He could be homozygous without any clinical expression. I do not know any way to choose between those points at the present time.

CARSON, CHICAGO: Is there appreciable contribution by 6-phosphogluconic dehydrogenase of the hemolysate to the quantity of TPN reduced in the assay system?

VALENTINE: I do not think we know the exact answer to your question, but I think by all odds that we are getting 2 moles of TPNH for every mole of G-6-P formed. The extra crystaline G-6-PD, I think, just gets the G-6-P into the shunt pathway. But the actual activity of 6-PGADH on a molar basis in our system would be about 10 times higher than hexokinase. I think, therefore, that we are getting 2 moles.

*Beutler, E., Mathai, C., and Smith, J.: New biochemical variants of G-6-PD giving rise to congenital nonspherocytic disease (CNHD). Clin. Res. 15: 130, 1967 (abstract).

Contributions of Hereditary Disorders of Red Cell Metabolism to Human Genetics

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THE RED BLOOD CELL is a unique object for studies in human genetics. Unlike most tissues, blood can be obtained easily. No complicated separation techniques are required, so that a variety of biochemical and serologic studies can be performed readily. Three broad areas of investigations with red cells have contributed greatly to knowledge of human genetics: (1) studies of blood groups; (2) studies of hemoglobins; and (3) studies of metabolic disorders and enzymatic variation of the red cell.

Most, if not all, principles of human genetics can be illustrated using examples from these areas. Investigations of hereditary disorders of red cell metabolism and enzymatic variation, the most recent additions to these studies, already have contributed significantly to the progress of human genetics.

GENES AND GENE PRODUCTS

Results of studies of human hemoglobin and its variants have served as models for the genetic control of protein synthesis. It is generally recognized that single polypeptide chains are the products of structural gene action. Most genetically-determined protein variants are believed to owe their origin to single amino acid substitutions, resulting from base pair mutations in the colinear DNA segment that determine the structure of the polypeptide chain under study.[121] However, other chromosomal mechanisms, such as unequal crossover yielding a fusion-gene product, have been demonstrated to result in some variant proteins, such as certain haptoglobins and Hb Lepore.[101] Even a single amino acid deletion, presumably caused by intragenic crossover, has been demonstrated recently in an abnormal human hemoglobin—Hb Freiburg.[55] Unlike hemoglobin, enzymatic proteins are present in only trace quantities in the red blood cell. Detailed characterization of normal and mutant gene products, using purified red cell enzymes, is only in its infancy. However, recent studies have demonstrated that two different red cell enzymatic variants—Type A+ G-6-PD[126] and one of the carbonic anhydrase variants[112]—are caused by single amino acid substitutions. These were the first demonstrations that mammalian enzyme variants owe their abnormality to the same mechanisms demonstrated for bacterial trytophane synthetase mutations[124] and for mammalian hemoglobin variants.[51] In the case of both the G-6-PD and carbonic anhydrase variants, the mutant protein was associated with differences in electrophoretic behavior rather than with a decrease in enzyme activity. Even so, 40 blood donors (20 for the normal

Aided by grants of the NIH (He 3091) and of the Commonwealth Fund.

enzyme and 20 for the mutant enzyme, each donating 500 ml of blood) were required to isolate a sufficiently large quantity of enzyme to demonstrate the single amino acid difference between normal and mutant A type of G-6-PD. The full structural characterization of red cell enzyme proteins exhibiting deficiencies in enzyme activity will be much more difficult, since the amounts of blood required are too large to make isolation of the mutant enzyme practical with existing methods.

Fortunately, even short of purification, mutant enzymes can be characterized in many other ways not possible with nonenzymatic proteins. A variety of measurements relating to functional enzymatic characteristics—such as pH optimum, substrate affinity (K_m), utilization of substrate analogs, serologic activity, and action of enzyme inhibitors—can be performed even if there are no electrophoretic differences. Significant and consistent abnormalities in these parameters, as compared with the normal enzyme, suggest structural differences of genetic origin. Thus it was inferred that the Mediterranean variant of G-6-PD was altered structurally because its affinity for G-6-P and TPN, as well as its utilization of a substrate analog (2-deoxyglucose), differed from normal.[60] It was demonstrated recently that chromatographic techniques could be used to separate physically the Mediterranean variant from the normal enzyme.[128] No separation has as yet been achieved with electrophoretic techniques.

In some instances, the various enzymologic measurements fail to differentiate between a normal and mutant enzyme. If there are consistent alterations in electrophoretic or chromatographic mobility, particularly if demonstrable in several members of a family, a difference in primary protein structure can usually be inferred. Theoretically, however, protein molecules with identical primary structure can be altered in charge by complexing them with small molecules, causing a change in electrophoretic mobility or column behavior. Electrophoretic, enzymologic, and serologic identity between the normal enzyme and that derived from an enzyme-deficient subject may, however, still be compatible with structural differences. Often application of multiple electrophoretic and chromatographic methods may disclose previously undetected differences between proteins.[105]

Genetic studies may sometimes give information about structure of an enzyme without enzyme purification. For instance, the existence of a "hybrid" enzyme in heterozygotes suggests at least dimeric structure of the enzyme under investigation. Thus, in our studies of cat 6-PG dehydrogenase,[114] we observed three electrophoretic patterns in different cats: a single fast (B) band, a single slow (A) band, and a triple pattern consisting of both the fast (B) and slow (A) bands, as well as a third band of intermediate mobility with approximately twice the enzyme activity of the fast and slow bands. Genetic investigation showed that cats with the fast bands and cats with the slow bands were homozygotes (respectively, i.e., Pgd^B Pgd^B and Pgd^A Pgd^A) and that animals with the triple band were the corresponding heterozygotes, i.e., Pgd^B Pgd^A. The presence of the third band suggested that the functional enzyme must at least be a dimer, since independent assortment of gene products of type A and type B would produce two AB units for every single AA and BB units. Similar results have been obtained with rat 6-PG dehydrogenase.[84]

Independent assortment of the two basic units (A and B chains) of lactic dehydrogenase into subunits of four chains produces an isozyme pattern of five bands on electrophoresis (LDH_1:B_4, LDH_2:B_3A_1, LDH_3:B_2A_2, LDH_4:BA_3, LDH_5:A_4).* Mutations will produce a complicated pattern. Since the mutational alteration either affects the A or B chain of lactic dehydrogenase, as many as 10 additional components have been seen (see Shaw[94] for references). A single gene mutation of conventional type may, therefore, produce a complicated phenotypical pattern with a total of 15 electrophoretic enzymatic components (5 + 10). Heterozygotes for both A and B chain mutations could manifest 25 different enzyme types (5 + 10 + 10) on electrophoresis.

The association of multiple subunits—all identical, such as in G-6-PD, or different, such as in lactic dehydrogenase—appears to be a common feature of many functional enzymes. Genetic study of mutational alterations can aid in elucidating the subunit structure of enzymes. In red cell phosphoglucomutase, 8 isozyme bands are seen and designated as bands a-h. Some mutations produce only changes in isozyme bands a-d, while other mutations leave bands a-d intact and alter bands e-h. Genetic analysis of appropriate matings has shown that the enzyme is produced under the direction of at least two unlinked autosomal loci: PGM_1 and PGM_2. PGM_1 specifies bands a-d, and PGM_2 specifies the remaining bands (see Harris[47] for references).

Although starch gel electrophoresis has been helpful for understanding of the genetics of a variety of red cell enzymes, the exact interpretation of the biochemical meaning of the various bands seen on zymograms remains difficult. In many instances, a single gene appears to control several electrophoretic components because even in homozygotes for a given gene (i.e., PGM_1, acid phosphatase), several bands are seen. Possible interpretations are discussed by Harris in this symposium.[47]

Regulatory Genes in Man?

The exciting discoveries of Jacob and Monod[53] on regulatory genes have elicited much discussion. So far, no convincing proof for regulatory mutations in mammals has been presented. Some suggestive findings are of interest. Assays of red cells from affected subjects have revealed that the activities of both orotidylic pyrophosphorylase and orotidylic decarboxylase are decreased in patients with orotic aciduria.[100] Since the two enzymes catalyze adjoining reactions in biosynthesis and since enzyme activity was only depressed to 20 per cent of that of normal individuals, it has been concluded tentatively that an operator mutation may be involved in this disease. Other explanations, such as sharing of common polypeptide units by the two enzymes, however, might apply.

The finding of very small quantities of chemically and serologically indistinguishable catalase in a patient with the Swiss type of acatalasia has been claimed as an example of a regulatory type of mutation.[3] Further studies have shown, however, that electrophoretic differences exist between normal and mutant catalase, suggesting a structural mutation.[2] Furthermore, since the code is degenerate and different codons may specify the same amino

*Red cell lactic dehydrogenase may only exhibit patterns 1-3; i.e., components BA_3 and A_4 may be lacking.

acid, a mutation to a homonymous (i.e., one of different base pair composition but specifying an amino acid identical to the one replaced) codon may entail a less plentiful supply of a given amino acid-specifying sRNA. Consequently, reduced synthesis of a gene product, which is identical in primary structure to its normal counterpart, may result.

Tissue Expression of Enzyme Deficiency

The study of enzymes with identical function in different tissues is of considerable interest. Since all cells in a given individual are assumed to carry identical genetic information, it might be inferred that the same gene specifies the structure of a given enzyme that catalyzes the same reaction in different tissues. This hypothesis might be tested by determining whether enzyme deficiency can be demonstrated in the white cells or other tissue cells in patients with erythrocytic enzyme deficiency. Table 1 indicates that G-6-PD deficiency of the Negro type,[68] pyruvic kinase deficiency,[108] and hexokinase deficiency[117] are not associated with a corresponding deficiency of these enzymes in white blood cells. Before concluding that the corresponding white cell enzymes are determined by different genes and are not affected by the mutation that causes red cell enzyme deficiency, an important distinction between red cell and white cell physiology must be realized.

Mature red cells do not have a nucleus and, therefore, lack the means to synthesize proteins. White cells, being nucleated cells, apparently have this means. The mechanism of the G-6-PD-A-mutation appears to be caused by a more rapid decline of enzyme activity in aging red cells.[127] Since no new enzyme molecules can be synthesized by the red cell, enzyme deficiency results. Production of new enzyme molecules presumably prevents enzyme deficiency in white cells with the same defect. In contrast, in the Mediterranean type G-6-PD deficiency, the structural defect causing the deficiency is so severe that, even with continued synthesis of enzyme by white blood cells, enzymatic deficiency results.

Despite the presence of almost normal white cell enzyme activity in Negroes with red cell G-6-PD deficiency (A type), the characteristic electrophoretic abnormality is seen in white cells[21] and other tissue cells,[11,64,81] suggesting that

TABLE 1. *Red cell enzyme deficiencies with (or with potential) hemolysis*

Red cells alone affected
G-6-PD deficiency (Negro type)[68]
Pyruvic kinase deficiency[109]
Hexokinase deficiency[118]
Glutathione synthetase deficiency (?)[57]
Red cells and other tissue cells (i.e., WBC) affected with enzyme deficiency
G-6-PD deficiency (Chicago, Oklahoma, Mediterranean and other types). See Kirkman[59] for references.)
Triose phosphate isomerase deficiency[92]
GSH reductase deficiency. (See Waller[120] for references.)

TABLE 2. *Red cell electrophoretic variants*

G-6-P dehydrogenase*—common[55]
6-PG dehydrogenase*—common[29]
Phosphoglucomutase*—common[47]
Adenylate kinase*—common[47]
Acid phosphatase—common[47]
Carbonic anhydrase*—rare[55,113]
Lactic dehydrogenase*—rare. (See Shaw[91] for references.)

*White cell electrophoretic difference also seen.

an identical structural gene specifies both white and red cell G-6-PD. Enzymatic and serologic characteristics of normal G-6-PD have also failed to show differences between white cell and red cell G-6-PD.[128] Claimed differences of this type[19,20] could be traced to secondary alterations of white cell preparations by proteolytic enzymes.[128] These findings allow the following generalization: Any mutation that accelerates a normally-existing decline of enzyme activity in aging red cells may express only with enzymatic deficiency in erythrocytes but not in other tissues. The measurement of enzyme activity alone may be less decisive than the use of other biochemical criteria, which may also allow detection of the mutant enzyme in other tissues than red cells.

In all red cell variants, which were first detected by electrophoretic differences (Table 2), an identical electrophoretic abnormality has been noted in white cells as well as in other tissue cells. It can, therefore, be concluded that an identical gene is operating in the various tissues (the situation is less clear for acid phosphatase). (See Harris[47] for references.)

A series of enzymatic-deficiency disorders exhibit decreased enzyme activity in the red cells (Table 3), but are not associated with blood destruction, nor has hemolysis been reported on drug administration. In these instances, erythrocytes have been used as readily available sources of tissue to demonstrate enzyme deficiency which is primarily manifested in other tissues, again demonstrating that the mutant gene was operative in more than one tissue.

Occasionally, however, different enzymes may be involved in various tissues. In pyruvic kinase deficiency, separate genes appear operative in white cells and red cells.[61,62] Structural differences between purified white cell and red cell pyruvic kinase could be demonstrated on peptide analysis. Recent enzymologic studies, reported at this symposium, clearly indicate that the two enzymes are dif-

TABLE 3. *Red cell enzyme deficiencies without hemolysis*

Catalase deficiency. (See Aebi[2] and Takahara[110] for references.)
P-Gal transferase deficiency[32]
Galactokinase deficiency[43]
6-PGD deficiency[29]
Orotidylic pyrophosphorylase deficiency[100]
Orotidylic decarboxylase deficiency[100]
Diaphorase deficiency[41]

ferent but possess structural similarities and, therefore, probably share a common evolutionary origin.[61]

Heterogeneity of Various Red Cell Enzyme Abnormalities

Careful investigations of human red cell enzymes have revealed a remarkable degree of heterogeneity. The example set by the multiplicity of hemoglobin variants may be general. Each component amino acid of a polypeptide chain theoretically could be replaced mutationally by another amino acid. If there is no difference in charge, such a mutationally-altered polypeptide would be difficult to detect and might exert its normal function if the tertiary and quarternary structure of the protein were not disturbed. Many different mutations could interfere with enzymatic function, not only by amino acid replacements near the active site of the enzyme, but also by changes elsewhere in the chain that could distort the active site and cause interference with normal enzyme-substrate interaction and resultant enzyme deficiency. Some mutations would be expected to prevent the chain from assuming any functional configuration; no enzyme activity at all might be detectable. Lastly, homonymous codon mutations would not change the amino acid composition of the gene product but might be associated with differences in synthetic rates.

G-6-P Dehydrogenase

A most remarkable degree of heterogeneity has been discovered with G-6-P dehydrogenase. At least 20 different variants are known.[128] In all cases where family investigations with G-6-PD variants have been performed, X-linkage could be demonstrated or the family data were compatible with X-linkage. Most, if not all, of these variants presumably are structural mutations affecting the single G-6-PD chain (mol wt 40,000 and thus comprising about 330 amino acids),[126a] which is elaborated under control of a locus on the X-chromosome. The amino acid sequence of the G-6-PD chain is not known, and in only one variant (A+) has the exact amino acid replacement been worked out.[126] No inferences, therefore, can be made yet about the relation of genetic fine structure to the different G-6-PD mutations. It is conceivable but unlikely that the G-6-PD locus, as the hemoglobin locus, may be a mutational "hot spot" and that the relatively large number of mutations found at these loci may not be a general rule.

Investigations of G-6-PD indicate that a large variety of measurements needs to be made before concluding that two similar variants are, in point of fact, identical. Thus, G-6-PD Seattle and Kerala appear identical on enzymologic studies but differ when additional electrophoretic techniques are employed.[7a]

Pyruvic Kinase

Heterogeneity of red cell pyruvic kinase deficiency has also been found. So far, no mutations with normal enzyme activity have been described. It is likely that the discovery of a good method to visualize pyruvic kinase on starch gel electrophoresis will uncover such mutants. Busch[25] described a family in which both red cell and white cell pyruvic kinase was deficient. In all other families with pyruvic kinase deficiency where this problem was examined, only red cell deficiency was detected.[108] Patients with hemolytic anemia and levels of pyruvic kinase that were one-half of normal (usually only found in heterozygotes) have

been reported.[26,62] Conversely, low levels of pyruvic kinase have been detected in some individuals without or with only minor hemolysis.[50] The genetic and biochemical findings in one family have been interpreted to mean that patients with low pyruvic kinase levels, in the absence of hemolysis, were doubly heterozygotic for two types of pyruvic kinase deficiency, each of which leads to hemolytic anemia in the homozygotic state.[50] All these data suggest that pyruvic kinase deficiency per se is not the cause of hemolysis in patients with pyruvic kinase deficiency and that further search for the "basic lesion" is indicated.

Galactosemia

Although most patients with the clinical diagnosis of galactosemia have reduced P-gal-transferase activities, an adult patient with galactose diabetes and cataracts and red cell galactokinase deficiency has been described.[43]

It has become clear that certain "galactosemic" individuals, who have very low P-gal-transferase activities in their red cells, tolerate galactose clinically without difficulties regardless of their age.[9,93] Apparently all these individuals were Negroes. In this regard, it is of interest that 3 Negro heterozygotes for galactosemia had only red cell enzyme deficiency and not white cell enzyme deficiency, while 4 non-Negro heterozygotes had deficient enzyme activities in both red cells and white cells.[72] A tentative interpretation would be that absent erythrocytic P-gal-transferase activity in some Negroes is caused by a mutation that is *not* associated with inability of the liver to metabolize galactose. It is not quite clear, however, why the clinically-nonaffected homozygous Negro patients exhibited transferase deficiency in both red and white cells, while their heterozygote parents, unlike the usual heterozygotes, had normal white cell transferase activity. The finding of individuals with low red cell P-gal-transferase activity who can tolerate milk well, illustrates the difficulty of screening programs. Milk-free diets may not be necessary for some "affected" babies.

A further mutation—the Duarte variant[12,15]—reduces P-gal-transferase activity to one-half normal activity in homozygotes and that of heterozygotes to about 75 per cent of normal. This variant may be fairly common.[16] Its existence invalidates all frequency studies of galactosemia based on heterozygote surveys. Enzymologic characterization studies have not revealed any abnormalities of the Duarte enzyme,[14] but the findings of a difference in electrophoretic mobility suggest that this variant represents a structural mutation.[69]

Acatalasia

The classic type of acatalasia first found in Japan is associated with nearly absent catalase activity in the red cells of homozygotes and intermediate activity in heterozygotes (see Takahara,[110] for references). In one pedigree, an affected man had three sons, all with normal catalase activity, suggesting a different type of acatalasemia.[111] A third variety of this disorder was discovered by Hamilton and Neel[45] in a large Japanese kindred in which there was considerable overlap between blood catalase activites of heterozygotes and normal homozygotes, unlike the usual pattern, in which heterozygotes could clearly be differentiated from normal homozygotes. The Swiss type of acatalasia is somewhat different from the Japanese type in that residual catalase activity is demonstrable in

erythrocytes (see Aebi[2] for references). In Israel, hypocatalasia was observed in an individual who had 8 per cent of normal enzyme activity with values of 50-65 per cent of normal catalase in heterozygotes.[107]

Drug Acetylation

In an attempt to study acetylation of foreign substances in a readily available tissue, acetylation of various chemicals was studied in human red cells.[75,77] No enzymatic mechanism for acetylation of isonicotinic acid hydrazide (INH) and sulfadiazine acetylation could be detected in red cells. However, red cells acetylated para-aminosalicylic acid (PAS). INH is known to be acetylated in the liver, as is PAS.[34] Rapid and slow "acetylators" of INH can be clearly differentiated in population studies.[33] Population studies of PAS acetylation, however, indicate that variability followed a normal Gaussian rather than discontinuous distribution.[75] The data suggest that acetylation of PAS and related substances, on the one hand, and that of INH, on the other, is carried out by different enzymes. The acetylating enzyme that accepts PAS as a substrate exists presumably both in liver and red cells and exhibits no discontinuous variation in a population, while the INH acetylating enzyme does not exist in erythrocytes but exists in alternate forms in the liver.

Genetic Heterogeneity of Normal Enzyme: "Isoalleles"

The studies of Harris and his collaborators[47] have shown that electrophoretic variation of normal red cell enzymes is a common phenomenon. As has been discussed at this symposium, variants of phosphoglucomutase, acid phosphatate, adenylate kinase, and 6-PG dehydrogenase exist in fairly high frequencies in most human populations. The significance of this remarkable heterogeneity is not clear. It is unlikely that the different enzyme types simply exist by chance. More probably, selective environmental factors have brought the different enzyme types to their present frequencies. Recent studies of enzymatic polymorphisms in fruit flies have disclosed a similar degree of heterogeneity (see 1965 and 1966 issues of *Genetics*). The existence of different genetically-determined forms of enzymes appears to be a general biological phenomenon and poses new problems in population genetics to explain the present distribution of the genes determining the structure of closely similar, yet differing enzymes in various populations. If there are functional advantages, they must be extremely subtle and are unlikely to be detected by present techniques. Genetic drift alone cannot explain the remarkable variety of these proteins.

In most instances of this type of heterogeneity, enzyme deficiency does not exist. However, there are indications that different structural enzymes are associated with characteristic levels of enzyme activity within the normal range. The clearest example is furnished by three allelic genes determining three structurally-different types of red cell acid phosphatase in normal man. When enzyme activities were determined, the C enzyme had the highest activity; B enzyme had less activity than C enzyme but more activity than A enzyme (C>B>A).[48,102] The wide range of red cell acid phosphatase activity in normal individuals thus can be explained largely by the existence in various individuals of genetically- and structurally-differing enzymes, each with its own characteristic enzyme activity.

A further example of altered enzyme activity by a structural gene mutation is the common electrophoretic variant A+ type of G-6-PD, which is associated with 15-20 per cent less enzyme activity than observed in subjects with the normal B+ enzyme (Long, personal communication).[99]

However, there appears to be further heterogeneity in the absence of demonstrable electrophoretic differences. A convenient genetic technique to test the existence of genetically-controlled variation is the study of intra-and inter-family variation, using an X-linked trait such as G-6-PD enzyme activity in red cells. There is wide variation of G-6-PD activity among normal unrelated males. Measurement of G-6-PD activity indicated similar G-6-PD levels among normal brothers whose mothers were heterozygotes for G-6-PD deficiency, carrying both a "deficiency" and a "normal" allele.[30] Within sibships, the single normal G-6-PD gene in each of such male sibs must have been identical to the "normal" allele of the heterozygote mother since males carry only one G-6-PD gene. Other correlations of enzyme levels between pairs of relatives within families (i.e., father-daughter, mother-sons) also showed significant values, while husband-wife and father-son correlations were not significant (Stamatoyannopoulos et. al., unpublished data). Since fathers cannot transmit an X-linked gene to their sons, no significant correlations of enzyme levels would be expected—just as for the genetically-unrelated husbands and wives. Studies on familial correlations of enzyme levels controlled by autosomal genes are more difficult to interpret since enzyme activity in a given individual is determined by two allelic genes; while with an X-linked trait in males, the effect of only one allele can be assessed.

Since enzyme activity can be affected by many factors—such as age, sex, season, time of day, and nutritional and endocrine status, as well as by variables in the methodology of the measurement—studies relying on genetical correlations from measurements of enzyme activity alone are more difficult to interpret than studies in which the enzyme activity of a demonstrable electrophoretically-detectable structural variant can be assessed, e.g., acid phosphatase.

The results of the correlation studies are best explained by postulating the existence of several "normal" alleles (isoalleles) in the population,[106] each of which determines a certain level of enzyme activity. This level of enzyme activity is the property of the specific gene and is not regulated by feedback. The finding that heterozygotes for various red cell enzyme deficiencies usually have 50 per cent of normal enzyme activity[46] is further proof that the control of the level of enzyme activity is a function of the structural genes involved. If control of enzyme activity would be exerted by cytoplasmic control mechanisms, enzyme activities higher than 50 per cent might be expected in heterozygotes. It can, therefore, be concluded that the level of activity for most enzymes is determined directly by the gene that specifies the structure of the enzyme.

The molecular basis of alleles, whose gene products do not exhibit electrophoretic differences, remains speculative. The failure to find electrophoretic mobility changes does not rule out structural differences caused by single base pair mutations. Thus, the A^+ and A^- mutations of G-6-PD have identical electrophoretic mobility under various conditions; yet column chromatography can

separate the two enzymes and, therefore, indicates presumable differences in primary structure.[67,127] Regulatory mutations, homonymous codon mutations, or closely-linked modifiers (in the case of G-6-PD) are other interpretations compatible with the data.

The existence of isoallelic variation has an important bearing on heterozygote detection for various types of enzyme deficiency. In most instances, the mean enzyme activity of heterozygotes is approximately 50 per cent of the mean enzyme activity of normal homozygotes.[46] In practically all systems studied, however, there is overlap in enzyme activity between obligatory heterozygotes and normal homozygotes. If isoallelic variation exists, heterozygotes with one "deficiency" allele and one "high capacity" allele may have levels of enzyme activity within the same range as normal persons with two low capacity alleles. Even the best-controlled measurement of enzyme activity is unlikely to reveal all heterozygotes under these conditions. For detection of all autosomal heterozygotes, test systems based on structural differences of the mutant enzyme or on differential behavior of the mutant enzyme to various enzyme inhibitors are more likely to yield critical results. In an X-linked trait, such as G-6-PD deficiency, heterozygote detection methods that are based on the demonstration of cellular mosaicism, as expected under the Lyon hypothesis, will be more useful than enzyme assay[104] (see below).

Gene Inactivation

Females have two X-chromosomes and males possess a single X-chromosome, yet the amount of gene products of X-chromosomally-controlled proteins is identical in the two sexes. Several workers, such as Mary Lyon, suggested that only one of the two X-chromosomes is genetically active in females (for reference see Beutler[10]). These concepts have been reviewed adequately and will not be discussed in detail.

So far, incontrovertible proof for this hypothesis has come from the G-6-PD system. A few years ago, Beutler et al.[18] showed that in following the course of TPNH-coupled methemoglobin reduction, a reaction dependent on G-6-PD activity, the red cells of G-6-PD-deficient heterozygotes behaved as if composed of two cell populations, one with normal enzyme activity and the other with enzyme deficiency. Similar results were obtained with more complicated, but highly ingenious techniques.[13] Various other studies appeared to confirm these findings, particularly when it was shown by methemoglobin staining that two cell populations could be visualized in such heterozygotes.[91,115] The interpretation of these experiments became confusing when it was shown that a double population of red cells could also be demonstrated in male individuals with mild G-6-PD deficiency, such as in the mild Greek type.[83] This "pseudomosaicism" in males was shown to be the result of declining G-6-PD activity on red cell aging, and in the threshold in the histochemical technique that appeared to give rise to cells with and without methemoglobin.[17,83] Further investigations with red cells showed that mosaicism was, in fact, present in both the young and older red cell populations of female heterozygotes, vindicating the validity of the differential X-inactivation hypothesis for red cells.[103]

The existence of two genetically-differing cell populations, produced by ran-

dom inactivation in embryonic life, makes heterozygote detection more difficult. The more normal cells that are present in a given heterozygote, the more likely it will be that the enzyme values of such a person will be in the normal range. The mosaic nature of the red cell population in heterozygotes for G-6-PD deficiency explains why tests based on methemoglobin reduction are more effective in detecting heterozygotes than are enzyme assays, even though the level of enzyme activity is a more direct reflection of gene activity.[104] Methemoglobin reduction proceeds in individual cells. If there exists a population of enzyme-deficient red cells, methemoglobin reduction will be abnormal in these cells. The contribution of these cells to the total methemoglobin reduction of the entire red cell population is usually a yield of abnormal values. Comparison of methemoglobin reduction in female heterozygotes (Mediterranean type of deficiency) with male hemizygotes of the mild Greek deficiency type demonstrated this fact clearly.[103] At identical intermediate enzyme levels in both groups, males with only one enzyme-deficient population of red cells had considerably less residual methemoglobin than female heterozygotes with two genetically-different populations (one normal and one enzyme-deficient).

The final distribution of G-6-PD enzyme values in a given heterozygote will be a function of (a) the type of the isoalleles present (see above), (b) the time of inactivation in embryonic life that determines the ratio of normal to abnormal cells, and (c) possible selective factors that may favor the proliferation of either normal or abnormal cells.

Thus, the finding of significant correlation of G-6-PD values in monozygous twins, heterozygotic for G-6-PD deficiency, as compared with DZ twins[22] could be caused by identical isoalleles in the MZ twins or may be an example of selection in the identical genetic environment of MZ twins. With random inactivation, no correlation in enzyme values of MZ twins would be expected, although it is conceivable that the time of inactivation may be genetically controlled. Possibly, all three factors may be operative.

In autosomal systems, both alleles appear to be equally active, so that heterozygotes for enzyme deficiency have a single cell population with 50 per cent enzyme activity rather than two populations: one with no activity and one with 100 per cent activity.[10] As an example, when the red cells of heterozygotes for acatalasia are stained for methemoglobin under conditions in which the amount of methemoglobin is made to depend on the catalase content of the red cell, one single population of red cells is observed.[4,5] The rate of methemoglobin production in such heterozygotes appears quite different from that of artificial mixtures of normal and acatalasemic cells, where two populations can be detected.

Heterozygotes for electrophoretic variants of phosphoglucomutase and lactic dehydrogenase[31,95] also exhibit both gene products in clones of tissue culture cells, and similar results would be expected with red blood cells. All evidence with autosomal alleles, except for the antibody genes, therefore is against autosomal inactivation.

Mutational Frequency and Biochemical Enzymatic Defects

A mutation from Hb A to Hb S, or from Hb A to Hb E, requires the change of

only one among the 146 codons that specify the Hb beta chain of 146 amino acids. Estimates of mutational frequencies for hemoglobins of the order of 10^{-8} to 10^{-9} have been calculated from scanty data.[39] The mutation rates for hereditary diseases would be expected to be higher, since several mutations within a given gene may give rise to the biochemical defect responsible for that disease.[38] In point of fact, most estimates of mutation rates for such diseases showed much higher values than 10^{-8}. Studies of the mutation rates for "enzyme deficiency" would be expected to show similar high or higher values, since many different mutations affecting structural and regulating genes of a given enzyme would cause enzyme deficiency. However, if by using more refined biochemical methods, one were to study the mutation rate of, for instance, the Negro type of G-6-PD deficiency, rather than that of G-6-PD deficiency in general, a much lower mutation rate (of an order similar to that obtained for a given abnormal hemoglobin) might be expected. These considerations have important consequences. The rare codon mutation leading to a specific type of enzyme deficiency may only occur once in a generation in the whole human population existing at the present time.[38] In earlier times, with smaller populations, many generations had to pass before such a mutation could occur. With 10 million people, for example, a structural hemoglobin or specific enzyme mutation would be expected to occur approximately once in 100 generations. It is therefore suggested that in most cases the existing hemoglobin or specific G-6-PD variants originated by a single mutation and then spread by migration. As an example, the findings of the Mediterranean G-6-PD mutation in India[7a] is in keeping with historical evidence that migrations from the Near East to India occurred. The above considerations would suggest that this gene was introduced into India by migration rather than by mutation. By this reasoning, specific hemoglobin and enzyme markers probably have given more information in recent years about population affinities than has been believed possible. On the other hand, if mutational "hot spots" exist, identical codon mutations might exist that would negate any anthropologic conclusions.

Linkage

Studies on linkage of X-chromosomal genes are simpler than linkage studies with autosomal traits, since males only have one X-chromosome. Therefore, studies of crossover frequencies for X-linkage need only be concentrated on mothers and their sons. The high frequency of G-6-PD deficiency in many populations provides a useful marker that has contributed new knowledge about gene localization on the X-chromosome. Studies of both the Negro and Mediterranean types of G-6-PD deficiency indicated very close linkage with the deutan type of color blindness. The best estimates at this time give a recombination frequency of only 0.03 (for references see Siniscalco,[96] and McKusick[70a]). These studies also indicate that the Negro and Mediterranean type of G-6-PD deficiency are allelic (or closely linked, because true allelism can never be demonstrated with human material), since different recombination frequencies for each type of deficiency would be expected with nonallelism.

There is some evidence that the gene for protan color vision may be further away from the G-6-PD locus than the deutan locus (0.07 recombination fre-

quency), but the recombination values for deutan and protan color vision are not yet statistically significantly different from each other (Siniscalco, unpublished data). However, the finding that a mother heterozygous for both protan and deutan color vision had sons with normal color vision in one pedigree,[119] and the detection in another pedigree of a son with characteristics suggestive of both deutan and protan anomalies,[97] suggest that crossover between the loci for deutan and protan color vision may occur and that these loci, therefore, are not allelic. Some of these facts have led to the hypothesis that the G-6-PD locus may be located between the genes for the deutan and protan color vision anomaly.[56]

No crossovers have been detected between hemophilia A and G-6-PD type A in 17 subjects and none has been observed between hemophilia A and G-6-PD deficiency among 23 (Siniscalco, unpublished data). The G-6-PD locus and the hemophilia A locus must, therefore, be very closely linked, thus supporting previous findings of close linkage between hemophilia and color blindness. The study of hemophilia B pedigrees with G-6-PD deficiency or G-6-PD variants also should give further information regarding the problem of allelism of hemophilia A and B, which on other grounds probably are not allelic.[123]

Extensive work in Israel and Greece with the X-linked blood group Xg suggested initially that the genes for G-6-PD and Xg were measurably linked (26 recombination units). No linkage between these two genes was apparent in the Sardinian data, and it was suggested that the X-chromosome in Sardinia harbored an inversion, including the G-6-PD locus, thus making for different linkage relationships (for references see Siniscalco et al.[98]).

Collection of three-generation families allowed definite assignment of the phase (coupling or repulsion) of the two genes in doubly heterozygotic women and indicated both in Israel[1] and Greece (Stamatoyannopoulos et al., unpublished) that no measurable linkage between these two genes existed. No unusual cytologic abnormalities need, therefore, be assumed in Sardinia. Future studies on linkage between the G-6-PD variants, ocular albinism, and X-linked ichthyosis will be of great interest because measurable linkage has been demonstrated with a high degree of probability for both traits and the Xg blood group.[37,122] Such studies may establish whether the genes for these traits can be localized between the G-6-PD locus and the Xg locus.

Autosomal Linkage

Despite attempts to map autosomes, using the various red cell enzymatic variants and red cell enzyme deficiency markers, no successful results have been obtained. Thus, acid phosphatase, PGM, adenylate kinase, and 6-PGD variants are not linked measurably to any of the other common markers. No linkages have been established between any of the hereditary hemolytic anemias and common markers.

However, hereditary elliptocytosis could be shown to be linked closely to the locus for the Rh blood groups (recombination frequency of 3 per cent[63]). Soon after this discovery, it became apparent that this linkage could not be found in all kindreds.[73] It was concluded that two types of hereditary elliptocytosis exist, each one determined by a different autosomal locus. Until recently, phenotypical differentiation of these two postulated types was not possible. The studies of

Geerdink et al.,[40] however, suggest that hemolysis is more severe in patients with the type of elliptocytosis not linked to the Rh group.

It should be stressed that the classical linkage approach cannot localize autosomal genes to a given chromosome but can only establish that two genes are linked.

Chromosomal Abnormalities and Red Cell Enzymes

A completely different approach to linkage makes use of the many different types of chromosomal abnormalities discovered in recent years. If a given trait is present on a triplicated chromosome, such as on the 21st chromosome in trisomy 21, three doses of a given gene, rather than only two exist and 50 per cent greater than normal enzyme amounts might be detected if a gene specifying a given enzyme were located on the triplicated chromosome and perfect gene dosage action would apply. Similarly, with heterozygote chromosomal deletions, only one dose of a given gene would be present and only one-half of the enzyme might be formed, as already suggested by the results in the usual heterozygotes for the different enzyme deficiencies.

A considerable number of investigations have been made of trisomy 21 (see Rosner et al.[90] for references). It soon became apparent that careful distinction needs to be made between red cell and white cell enzyme measurements, since elevations of a given white cell enzyme in trisomy 21 may not necessarily be associated with elevation of its red cell counterpart.[49] Thus, P-gal uridyl transferase activity in red cells was normal while white cell P-gal uridyl transferase in Down's syndrome was elevated. However, some investigators[90] reported elevations of red cell galactose transferase and red cell galactokinase.[32] The significance of these observations for gene localization became questionable when high G-6-PD levels were found in the white cells of individuals with trisomy 21.[71] Since the gene for G-6-PD is X-linked, elevation of this enzyme must be an indirect effect. However, not all investigators have detected G-6-PD elevations in white cells[49] and in red cells.[8] The studies of Baikie et al.[8] are of particular interest. This group studied 13 red cell enzymes (hexokinase, phospho-hexose isomerase, phospho-hexokinase, aldolase, triosephosphate isomerase, G-6-P dehydrogenase, glyceraldehyde phosphate dehydrogenase, phosphoglycerate kinase, PGM, enolase, pyruvate kinase, LDH, and GS reductase). The red cell activity of all enzymes was normal except for phosphophexokinase which was 50 per cent elevated in 20 mongoloids with no overlap with normal individuals. One trisomic patient who had normal phosphohexokinase activity was found to be a normal-trisomy 21 mosaic, 10 per cent of his blood cells being trisomic. The authors suggest that unlike some of the other more inconstant enzymatic abnormalities, their findings would be compatible with localization of the "phosphohexokinase gene" to the 21st chromosome.

It would be hazardous to venture any definitive interpretations, except to point out that this type of approach to structural gene localization is hazardous. The presence of many genes on the triplicated chromosome in trisomy 21 is likely to cause profound metabolic disturbances during embryonic development and later in life. These abnormalities may be reflected by changes in enzymatic activity that are far removed from primary gene action.

The findings of Rosner et al.[90] are particularly puzzling. These authors report failure to find abnormal enzyme activity (including red cell P-gal uridyl transferase and red cell G-6-PD) in the blood of translocation mongolism patients as compared with trisomy 21 patients. Translocation mongolism patients are clinically indistinguishable from trisomy 21 patients. It would be surprising if several genes whose enzyme activity was studied would all be localized on the small bit of chromosome missing in translocation mongolism, but present in the usual trisomy 21. Further studies are required.

Deletion mapping[79] in hereditary enzyme deficiency and enzyme variation may ultimately yield results. Thus, if a patient with a "homozygous" phenotype (i.e., pyruvic kinase deficiency) carries a chromosomal deletion, localization of the pyruvic kinase gene to the deleted chromosomal fragment is suggested if both parents can be identified biochemically as pyruvic kinase hetrozygotes, while the one parent only carries the chromosomal deletion. The patient would, therefore, have one mutant and one deleted gene for pyruvic kinase. This approach would be particularly convincing with electrophoretic enzyme variants where one could be quite certain about the nature of the gene product and need not rely on enzyme activity measurements.

Formal Genetics

Following clinical, biochemical, and family studies of a given hereditary disease, more detailed genetic studies on a population of patients and their families are desirable. Frequency of the trait, biologic fitness of affected patients, mutation rates, and penetrance under specified conditions of ascertainment are valuable parameters. These studies are often quite laborious and require sophisticated techniques for data analysis. A complete study of this sort has only been reported for hereditary spherocytosis among the various genetic disorders of red cell metabolism. Morton and his group[74] showed that the segregation frequency was .51 and, therefore, compatible with autosomal dominant inheritance. The absence of consanguinity among the parents of patients with hereditary spherocytosis argued against the possibility of an autosomal recessive form of hereditary spherocytosis, such as observed in the deer mouse.[6]

Twenty-six per cent of cases were found to be sporadic. A few among these probably could be explained by illegitimacy, several by acquired hemolytic anemia simulating the hematologic findings of hereditary spherocytosis, and a significant number by mutation. In fact, if the biologic fitness of the gene were 15 per cent less than that of normal individuals and the gene for hereditary spherocytosis were in equilibrium, one would expect 15 per cent of detectable cases of that disease to have arisen by mutations. In the investigations of Morton and his co-workers,[74] the fertility of affected patients was 14 per cent less than that of normal parents, and there was no difference in prenatal and infant mortality among offspring of normal and affected parents, giving a net diminution of fitness of 14 per cent. The prevalence of the disease was 2.2 cases per 100,000 population, or roughly 1 in 50,000. The mutation rate calculated by use of these various parameters was 2.2×10^{-5}, a figure that is in reasonable agreement with estimates of other dominant mutation rates in man. The probability that a given patient could be ascertained as having this condition was 9.9 per cent.

Otherwise stated, 9 out of 10 patients would not have come to clinical attention if it had not been for the family studies.

The condition, therefore, in general is very mild and only a small proportion of patients with more severe manifestations of the disease come to the attention of physicians. This result illustrates a not infrequent finding in various hereditary diseases in which family studies disclose undetected disease as the "submerged bottom of the iceberg," thus giving a much better idea of the total clinical expression of the mutant gene than clinical studies alone can reveal.

The fundamental chemical lesion in hereditary spherocytosis has not yet been discovered. There is general agreement that abnormalities in red cell carbohydrate and electrolyte metabolism[54] are secondary to a biochemical defect in the red cell membrane.

Metabolic and Enzymatic Variation of the Red Cell in Animals

Hereditary spherocytosis in the deer mouse is pathophysiologically indistinguishable from its human counterpart.[6] There is a compensated hemolytic syndrome with reticulocytosis, erythropoetic hyperplasia, increased osmotic fragility, and splenomegaly. The life span of affected red cells is decreased in the affected animals' own circulation or in a normal circulation. Splenectomy corrects the hemolytic process. Unlike in man, the condition is inherited as an autosomal recessive trait, which is not true in man, and heterozygotes are indistinguishable from normal animals. If it can be shown that the yet unknown basic defect in man and the deer mouse is identical—an unlikely event in view of the consideration discussed above—the condition in the deer mouse may be an example of dominance modification. According to this concept, the originally dominant mutation is harmful but over generations has acquired genetic modifiers, so that ultimately the dominant trait becomes recessive and requires the double dose of the gene before becoming phenotypically manifest.[6]

No red cell enzyme deficiency associated with anemia has been discovered in animals. It is of interest, however, that the mean normal level of G-6-PD varies in different strains of inbred mice, and mating studies suggest single gene inheritance.[44]

Biochemical variation of G-6-PD in primates has been found.[27] Various primate species have a different mobility of G-6-PD in keeping with amino acid substitutions of the G-6-PD molecule in diverse species. Intraspecific variation has so far only been observed in chimpanzees and gorillas[27] (Crawford et al., unpublished observations). Of 23 male chimpanzees from different geographic sites, 4 had a fast band similar in mobility to the fast band seen in some human populations. Of 28 female chimpanzees, 2 had a double band of equal staining intensity identical to the double bands observed in human heterozygotes for A-B polymorphism. It is likely, therefore, that the mutation is X-linked in chimpanzees. In an effort to find out whether the chimpanzee "fast" mutation and the human A+ enzyme were identical, micro-complement fixation tests were done (Sarich and Wilson, unpublished data), using anti-serum that was obtained by immunizing rabbits with crystalline human normal G-6-PD. This technique in other hands has revealed differences between Hb A and S. The normal chimpanzee enzyme could be distinguished from both normal (B) and A+ human enzymes, although these

two enzymes could not be distinguished from each other.[126] It is, therefore, most likely that the human A+ and chimpanzee "fast" are not structurally identical but represent independent single mutations from their respective normal enzymes. The environmental factors causing the relatively high frequency of these variant enzymes are completely unknown. Since the enzyme deficiency A— mutation presumably owes its frequency to malarial selection (see below), it is tempting to postulate that malaria was the selective factor for the A+ polymorphism, particularly since the A+ allele is associated with 15-20 per cent less enzyme activity than normal.

The gene for G-6-PD is not only located on the X-chromosome of man and chimpanzees, but also on that of *Drosophila,*[129] *Lapidae,*[82] and *Equidae.*[70,116] Interspecific crosses have been particularly instructive in showing that G-6-PD bands in male hybrid offspring had the electrophoretic mobility of the mother's G-6-PD, rather than that of the father's, thereby proving X-linkage.

Catalase deficiency mutations have been induced by radiation in house mice.[36] One of the mutants appears similar to the Swiss type of human acatalasia in that the homozygote had 1 per cent of enzyme activity, while heterozygotes had 50 per cent enzyme activity.[35] Several other different catalase mutations were detected in over 12,000 tested offspring of irradiated parents. Depression of catalase levels in homozygotes to 18-20 per cent of normal and difficulties in distinguishing heterozygotes from normal homozygotes were encountered. Various breeding studies have established that the catalase mutants probably are allelic. This approach demonstrates that other enzymatic mutations might be found among the offspring of irradiated mice if appropriate screening studies were to be instituted.

The Interaction of Heredity and Environment

G-6-PD Deficiency and Malaria

While the high frequency of some of the red cell enzyme polymorphisms cannot yet be explained, a variety of studies has given some information about the population distribution of certain traits. The limitation of glucose-6-phosphate dehydrogenase deficiency to subtropical and tropical areas, as well as the metabolic requirements of malarial organisms within red cells for GSH and for the shunt pathway suggested that G-6-PD deficiency might owe its relatively high frequency to a selective advantage vis-a-vis falciparum malaria. This subject has been extensively reviewed previously (for references see Motulsky[76]). Although no protection by the G-6-PD trait could be demonstrated when volunteers were infected with falciparum malaria in vivo,[85] these experiments do not compare with actual field conditions since experimental infections had to be terminated at relatively low parasite levels. The distribution data of enzyme deficiency from many areas of the world, such as Greece. Thailand, Sardinia, and Africa, are all compatible with the malaria hypothesis. Recent data from Nigeria on children with very severe falciparum malarial infections indicate a deficiency of G-6-PD deficiency among such patients.[42] There is a strong correlation of sickle gene frequencies and G-6-PD gene frequency in all populations where the frequency of these traits was tested, suggesting selection of two genetically-differing red cell

traits by a common ecologic agent—falciparum malaria. This correlation holds not only in large populations, but also in the micropopulations of small Congo villages.[78] Similar correlated gene frequencies have been obtained with G-6-PD deficiency and thalassemia, as well as with G-6-PD deficiency and Hb E (see Motulsky et al.[78] for references).

Red cell acid phosphatase variants have similar distributions in Sardinia and in Rome unlike G-6-PD and thalassemia.[72a] It has, therefore been concluded that malaria does not play a role in selection for this polymorphism.

In order to get more definite data regarding the malarial hypothesis, our group is carrying out large-scale studies in Greece, where G-6-PD deficiency, Hb S, and thalassemia are common. Malaria in Greece was endemic and severe for many generations but was eradicated sharply in 1945-46. We are testing families in two areas with a high frequency for these traits, and plan to analyze the data according to the following plan. If malaria had a selective advantage during malarial times, we expect to find lower mortality and higher fertility (or both) of trait-carriers among the older generation who were exposed to the selective action of malaria. The data on the younger population should give information on deleteriousness of these traits in the absence of malaria. In addition to mortality and fertility figures, data on the segregation ratios before and after 1945 should show an excess of trait-carriers before 1945, if mortality was higher among those not protected by these traits. So far, a statistically significant increase of fertility of 10 per cent has been noted among G-6-PD hemizygotes during malarial times but not thereafter. This result is of interest; it may mean that the G-6-PD polymorphism is not in equilibrium yet and may have been on its way to fixation. Otherwise stated, over many generations, everyone in the population might have become G-6-PD deficient if malarial selection would have continued. However, the problem of evolutionary trends of the X-linked G-6-PD deficiency is a more complicated one than that for autosomal traits.[57,65] For instance, if the biological fitness of the heterozygote is higher than that of hemizygotes, equilibrium gene frequencies between 50-100 per cent can be obtained.[64a] If there is any significant disadvantage of the hemizygote, such as is seen in the G-6-PD variants associated with nonspherocytic hemolytic disease, even a great selective advantage in females (which is unlikely) would require many thousand years before a significant gene frequency would be obtained.[57] The actual findings of never having observed any polymorphic gene frequencies for G-6-PD deficiency associated with hemolytic anemia is in keeping with these observations.

Already certain general conclusions can be drawn from population studies which aim to measure biologic fitness before and after malarial times. A very large body of data is required to prove selection that has been sizable for traits such as that of the sickling gene. We know from other sources that protection vis-a-vis malaria exerted by the G-6-PD trait must have been considerably less than that exerted by the sickling trait, and a considerably greater body of data will be required to prove the hypothesis. Such approaches are, therefore, unlikely to achieve meaningful results for polymorphisms in which the selective advantage is small.

Red Cell ATP and Malaria

Red cell ATP levels are lower in Negroes than in Caucasians, with considerable overlap (Azevedo and Motulsky, unpublished data).[23,24a] The unimodal distribution of ATP levels in both populations is compatible with polygenic inheritance. ATP is a nonprotein substance and its red cell concentration is dependent upon many enzymatic reactions, including pyruvic kinase. However, no relationship was found between the levels of red cell pyruvic kinase and ATP (Azevedo and Motulsky, unpublished data). High ATP levels have occasionally been reported to be caused by a single gene inheritance (Dutch type of high ATP, American type of high ATP, and 2,3 diphosphoglycerate deficiency as cause of high ATP levels). (See Loos[66] for references.) Brewer and Powell[23] demonstrated with in vivo experiments that parasitemia in falciparum malaria increases faster in persons with relatively high levels of red cell ATP than in persons with relatively low ATP levels. These data are in keeping with in vitro findings, indicating that ATP facilitates the growth of malarial organisms. It has been suggested[86] that red cells with relatively low levels of ATP may have a better capacity to remain intact when they are parasitized. In those with higher ATP levels, hemolysis may occur before mature schizonts have formed with resultant release of immature parasites into the circulation. This hypothesis would be in keeping with the suggestion that a single type of mechanism—shortening of the life span of parasitized red cells[80]—may be responsible for malarial protection by the various red cell traits. In G-6-PD-deficient subjects, no differences in mean red cell ATP has been found in comparison with normal subjects.[24] However, Powell et al.[86] suggest that there may be different utilization or formation of red cell ATP in parasitized G-6-PD cells as compared with parasitized normal cells.

Before it is accepted that low ATP levels protect against malaria, more studies will have to be done on red cell ATP levels of children. It has been reported,[89] and we have confirmed (Azevedo and Motulsky, unpublished data), that ATP levels in children are considerably higher than in adults. In limited studies we found no racial differences in red cell ATP levels of children. Since malarial selection occurs in young children, these points need further investigation.

Hereditary Spherocytosis and Elevated Environmental Temperature

Hereditary spherocytosis is said to occur with higher frequencies among European populations than among Negro populations,[28] although no definite comparative studies of frequency are available.

Red cells from patients with hereditary spherocytosis hemolyze more rapidly than those of normal individuals when exposed to an in vitro temperature of 37° C. These findings suggest that the trait might be at a relatively greater selective disadvantage in hot climates by producing a more severe degree of hemolysis. We tested this hypothesis by subjecting deer mice with hereditary spherocytosis to elevated environmental temperature (35° C)[7]. Fifty per cent of spherocytic mice, but none of the normal animals, died within 10 days of exposure to this temperature. Marked anemia with accentuated spherocytosis, fragmentation, and hemoglobinuria was observed in the heat-exposed animals. Osmotic fragility increased further, the spleen enlarged, and the red bone marrow expanded. Initially, the number of reticulocytes decreased. This was followed by hyper-

reticulocytosis. Only minor changes of this sort and no deaths were noted in the control animals. These findings illustrate effectively the interaction of heredity and environment in the pathogenesis of disease. The mutation is benign at ordinary room temperature in temperate climates and the mice are outwardly healthy. An elevated environmental temperature acts as a specific noxious agent that transforms a harmless mutation into a lethal phenotype. This mutation would be very harmful in a hot climate. Whether these studies are relevant to the human disease will have to await more critical studies of Caucasians and Negroes.

Summary and Conclusions

The study of hereditary disorders of red cell metabolism has contributed significantly to genetics. The following findings are of general interest.

Enzymatic variants probably owe their origin to single amino acid substitutions in most cases and can be functionally characterized by a variety of methods that are not feasible for nonenzymatic proteins.

Use of multiple electrophoretic techniques may uncover otherwise undetectable genetic differences.

Genetic analysis of mutants may give information about subunit structure of enzymes.

Genetic heterogeneity of each type of enzymatic deficiency is a common phenomenon. Multiple structural alleles appear to be the most common mechanism explaining this finding. This heterogeneity also appears to explain, at least partially, the wide variation of activity for many enzymes in the "normal" population. Its molecular basis remains unknown.

In most instances studied, a given enzyme appears to be specified by an identical gene in various tissues. Exceptions, however, have been noted.

X-chromosomal inactivation in females has been unequivocally proved for the G-6-PD locus and applies to all tissues studied. No inactivation of autosomal loci of "red cell" enzymes has been demonstrated.

The mutation rate of single codon mutations, giving rise to specific enzymatic mutants, may be lower than that of human hereditary diseases in which, presumably, many codon mutations within a given gene could give rise to the identical disease. Identical enzymatic mutants in different populations, therefore, may often share a common origin.

Information on linkage of the genes for red cell enzymes and metabolic variants to those for other traits so far has been scanty. One type of hereditary elliptocytosis is linked to the Rh locus. The G-6-PD locus is located on the X-chromosome and is closely linked to hemophilia A as well as to the deutan and protan color vision loci. Attempts at obtaining linkage data by assaying enzyme activity in the red cells of trisomic patients are hazardous and allow no conclusions at this time.

The full formal genetics of only one hereditary disorder of the red cell has been worked out—hereditary spherocytosis.

Variation at the G-6-PD locus is X-linked in all mammals that have been studied. Several catalase mutations resembling the human hypocatalasias have been induced in mice by radiation. This method promises to be useful for the experimental study of enzymatic variation in mammals other than men.

Hereditary spherocytosis in deer mice has been shown to be an example of a trait with normal biologic fitness in temperate climates. Elevated environmental temperature transforms this harmless trait into a lethal phenotype.

G-6-PD deficiency of the Negro and Mediterranean type appears to have been selected for by falciparum malaria. Problems of the evolutionary dynamics of such an X-linked trait raise different problems from those posed by autosomal traits. Equilibrium gene frequencies of more than 50 per cent are possible.

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DISCUSSION

GIBLETT, SEATTLE, CHAIRMAN: Thank you, Dr. Motulsky, for providing us with this very interesting discussion.

BEUTLER, DUARTE: Dr. Motulsky, I think that you are creating certain problems for your colleague, Dr. Yoshida. It seems to me that by proposing a structural isoallele in one part of your laboratory and using 20 units of blood for "fingerprinting" in the other part of the laboratory, you create certain difficulties. The best evidence that you have regarding isoalleles is the lack of correlation in father-to-son transmission, while you do have correlation in the mother-to-son transmission. I would suggest that one way to reconcile these findings with the protein structure data of Dr. Yoshida would be to abandon the idea that isoalleles are structural in nature, and to consider the possibility of operator isoalleles. In addition, is it not possible that there are other factors on the X-chromosome that also affect G-6-PD activity? Most particularly, I would consider red cell age. I wonder whether you have used the approach that Dr. Kirkman used in some of his studies. He did not plot the G-6-PD activity alone. Instead, he determined the ratio of G-6-PD activity to transaminase or to hexokinase activity.

MOTULSKY, SEATTLE: We have not yet used Dr. Kirkman's approach. These data can be explained equally well by postulating isoalleles of identical structure, such as created by homonymous codon mutations, or by closely-linked modifiers of some sort on the X-chromosome. You are quite right; if there are isoalleles producing gene products with different structures, the pooling of 20 units of blood creates difficulties.

We cannot forget about structural isoalleles in view of Dr. Harris' acid phosphatase data. In this system, each isoallele owes its origin to a structural mutation and has been proved to have different enzyme activity.

KOSOWER, NEW YORK: Dr. Motulsky, you discarded the possibility of a gene regulating the amount of enzyme synthesized, and you put the emphasis on the structural aspect. It is quite possible that a closely-linked regulating gene also exists in higher organisms. This would mean that you could take 20 units of blood and find the same general structure for G-6-PD. One possible example in human genetics for mutations in the regulatory system might be the human porphyrias.

MOTULSKY: Dr. Kosower refers to increased delta-amino levulinic synthetase activity in the livers of patients with acute intermittent porphyria. This disease has been cited as supporting the theory of a regulatory type of genetic mechanism. At about the same time, a paper* that has not been as generally quoted described markedly elevated pseudo-cholinesterase levels in a family. Electrophoretic studies revealed, however, an additional component with higher activity than normal.

The increased enzyme level therefore was caused by a structural mutation. I am fascinated by regulatory systems so brilliantly elucidated by Jacob and Monod and, in fact, was one of the first to try to apply them to a human mutation—hereditary persistence of fetal hemoglobin; something of this sort probably exists in man. I am, however, impressed by the fact that, in several examples, a structural mutation finally explained a set of data initially fitting a regulatory hypothesis.

CARSON, CHICAGO: Dr. Motulsky suggested that I ought to say something about the malaria hypothesis. It has seemed to me that the malaria hypothesis is so attractive that it might keep us from thinking of other possibilities. Drs. Bowman and Frischer† some years ago showed that there was no incidence of G-6-PD deficiency in Zoroastrians; presumably, a population that had been exposed for a long enough time to falciparum malaria would have shown this. There was also a very low incidence in Armenians from similar endemic malarial

*Neitlich, H. W.: Increased plasma cholinesterase activity and succinylcholine resistance: a genetic variant. J. Clin. Invest. 45: 380-387, 1966.

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areas. The statistics indicating a positive correlation with G-6-PD deficiency turned out to be not significant. Another point that my colleagues, Drs. Powell and Brewer,* made was that G-6-PD deficiency in the Negro did not show any decreased morbidity with falciparum malaria. There are other parasite morbidity rate data. A report coming from Thailand and covering an extensive study indicates no correlation between G-6-PD deficiency and morbidity.

We are still left with a nice geographic correlation. Nonetheless, it seems to me that one ought to consider other possibilities. It is beginning to develop that G-6-PD-deficient individuals have something different about their overall carbohydrate metabolism, so we think of nutritional factors. It is also intriguing that *Pasturella pestis* has no G-6-PD. There is a possibility that the incidence of plague could have been different in people with G-6-PD than in those without.

MOTULSKY: In a report that just appeared in *Lancet*,† further proof for the malaria hypothesis is given. The authors took children up to the age of 4 or 5 years with severe malaria and parasite counts over 100,000 and assessed the frequency of G-6-PD deficiency by quantitative techniques. They could show that G-6-PD-deficient boys were less frequently affected.

KOSOWER: I think more studies of this kind must be done. I have also seen one or two cases in Africa of G-6-PD deficiency correlated with falciparum malaria. These patients had severe hemolytic episodes, and one might suggest that they were more susceptible. This has to be worked out further.

GIBLETT: I doubt very much if we are going to settle that problem this afternoon.

BEUTLER: I should like to thank all of the participants for their excellent contributions to this conference.

The conference stands adjourned.

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