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DRUG-INDUCED HEMOLYTIC ANEMIA

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The human red cell is designed to circulate in the blood stream for approximately 120 days. After this time, presumably because its metabolic machinery or stroma wears out, the cell is removed from the circulation. Certain untoward events, however, can abruptly shorten the lifespan of red cells. Prominent among these is the administration of drugs and other chemicals. Some compounds, such as phenylhydrazine, produce hemolytic anemia in all individuals who receive a sufficient dose, but such drugs are no longer employed commonly in medical practice. Other drugs produce hemolysis in only the occasional, unusually susceptible individual. This latter type of hemolytic response has proved to be a most interesting and profitable model of the pharmacogenetic diseases.

Susceptibility to drug-induced hemolysis may occur because the red blood cells of the vulnerable individual are abnormal or because abnormal plasma factors are formed in response to the administration of drugs.

I. SENSITIVITY TO DRUG-INDUCED HEMOLYTIC ANEMIA DUE TO INTRAERYTHROCYTIC DEFECTS

A. Metabolic abnormalities

1. Erythrocyte metabolism (15, 115). The erythrocyte is a living, metabolically-active cell. It must maintain a gradient of sodium and potassium across its membrane, reduce methemoglobin that has been formed by the oxidation of hemoglobin, and maintain enzymes in an active state. Glucose provides the energy source for these metabolic activities. After phosphorylation to glucose-6-phosphate in the hexokinase reaction, further metabolism may take place by one of two routes. The Embden-Meyerhoff pathway is the major way in which glucose is metabolized anaerobically to pyruvate or lactic acid. This pathway provides energy in the form of ATP and can reduce NAD to NADH for methemoglobin reduction. Although the main glycolytic pathway is of primary importance in providing energy for the cell, lesions affecting it do not appear to be involved in drug-sensitivity.

The other route of red cell metabolism is the hexose monophosphate shunt. In this pathway no ATP is formed, but NADP is reduced to NADPH. Normally, only 5 to 10% of glucose-6-phosphate is metabolized by this pathway, its rate being limited by the availability of NADP. The main function of this route of metabolism appears to be to provide reduced NADP for the reduction of oxidized glutathione. Glutathione appears to play a role in maintaining sulfhydryl

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enzymes within the red cells in active form, and in detoxifying small quantities of hydrogen peroxide. The levels of hydrogen peroxide that may appear when certain types of drugs react with oxyhemoglobin may be so low that catalase represents a relatively inefficient means for their disposal (56, 57). Another enzyme system, the glutathione peroxidase system, appears to be effective in detoxifying such small amounts of peroxide (56, 129). The glutathione (GSH) that is oxidized to oxidized glutathione (GSSG) in the glutathione peroxidase reaction, that which may be oxidized in the process of reducing mixed disulfides, and possibly mixed disulfides themselves (164), can be reduced to GSH through mediation of the enzyme glutathione reductase with NADPH as substrate. Thus, the complete pathway requires three groups of enzymes, the glucose-6-phosphate dehydrogenase (G-6-PD)-phosphogluconic dehydrogenase (PGD) system to reduce NADP to NADPH, the glutathione reductase system to oxidize NADPH to reduce GSSG to GSH, and finally, the glutathione peroxidase system that oxidizes GSH for the reduction of peroxide. The cell appears to become excessively susceptible to drug-induced hemolysis when any of these three enzyme systems is not functioning properly.

2. Glucose-6-phosphate dehydrogenase deficiency. A. HISTORICAL. Hemolytic anemia associated with the administration of 8-aminoquinoline antimalarial compounds has been recognized since shortly after the introduction of these drugs into medical practice. In 1926 Cordes (58, 59) noted manifestations of acute hemolysis in subject given pamaquine naphthoate [Plasmoquine, Pamaquine, Pamoate, Alminoquin naphthoate, Plasmochin; methylene-bis-β-hydroxynaphthoate of 6-methoxy-8-(1-methyl-4-diethylamino) butylaminoquinoline]. Many other reports of this type of hemolytic anemia appeared subsequently. Such investigations have been reviewed in detail elsewhere (12).

After the introduction of pamaquine, a large series of related 8-aminoquinoline compounds were synthesized, and a number of these were tested in man. Several of these, pentaquine phosphate [8-(5-isopropylaminoamylamino)-6-methoxyquinoline phosphate], isopentaquine [8-(4-isopropylamino-1-methylbutylamino)-6-methoxyquinoline] and primaquine [8-(4-amino-1-methylbutylamino)-6-methoxyquinoline diphosphate], were also found to be hemolytic in certain sensitive subjects (86). Primaquine was a very effective antimalarial compound, but produced hemolytic anemia in susceptible persons. The importance of this new drug made it possible, for the first time, to study a hemolytic drug reaction under well-controlled conditions.

Red cells from persons known to be sensitive to primaquine were labelled with chromium-51 and were transfused into nonsensitive recipients. Administration of primaquine to these primaquine-nonsensitive subjects resulted in rapid destruction of the labelled cells in their circulation. The fact that sensitivity to primaquine was due to an intrinsic defect of the erythrocyte was confirmed by infusing labelled cells from a nonsensitive individual into a subject known to be sensitive to primaquine. The administration of primaquine to such a recipient resulted in rapid destruction of his own red cells, but no loss of labelled erythrocytes (67).

Further investigations with the ^{51}Cr-labelling technique established that primaquine was only one of the many compounds to which these abnormal red cells were uniquely sensitive. Not only do other 8-aminoquinoline derivatives produce hemolysis of primaquine-sensitive cells *in vivo*, but a variety of other compounds such as sulfanilamide (Prontosil album, Prontalbin, Deseptyl, Septoplix, Prontylin; *p*-aminobenzensulfonamide), acetanilid (Antifebrin; *N*-phenylacetamide), phenacetin, and small doses of phenylhydrazine (65) do so as well.

It was also shown that there was selective destruction of older red blood cells by these drugs; young cells appeared to have a capacity to protect themselves against destruction (25). Thus, in Negro subjects, at least, the hemolytic anemia was self-limited; once the older cells had been destroyed the hemoglobin values returned to normal even if administration of the drug was continued (64). Later studies showed that in the more severe Mediterranean type of deficiency (see below) even quite young cells are destroyed, and hemolysis was therefore not self-limited (151).

Examination of primaquine-sensitive cells by the then standard hematologic and immunologic techniques revealed no difference between normal cells and primaquine-sensitive cells (24). However, incubation of the red cells with acetylphenylhydrazine under standardized conditions revealed a unique pattern of Heinz body formation *in vitro* (26). Biochemical studies showed that the level of reduced glutathione was diminished significantly in primaquine-sensitive cells (27). Although earlier investigations failed to show any increase in the quantity of oxidized glutathione (GSSG), more recent studies have shown that the earlier methods of determining GSSG levels were unreliable (162). It is now possible to show that the concentration of GSSG in primaquine-sensitive cells is, indeed, increased (163). The GSH of primaquine-sensitive red cells was not only diminished in quantity but also unable to resist the stress of incubation with acetylphenylhydrazine or other similar compounds (11). When the pathways that maintain GSH in the reduced state were investigated, it was found that there was a severe deficiency of one of the enzymes in this metabolic pathway, glucose-6-phosphate dehydrogenase (G-6-PD) (51). By one of the strange coincidences that frequently characterize progress in science, Waller *et al.* (176) independently discovered a deficiency of G-6-PD activity in the red cells of an Iranian physician who suffered from nonspherocytic congenital hemolytic anemia.

It soon became apparent that glucose-6-phosphate dehydrogenase deficiency was in reality a heterogeneous group of disorders. Many types of abnormality have been defined (102), and most recently an important beginning has been made in characterizing the defect on a molecular level (182).

B. CLINICAL EFFECTS OF G-6-PD DEFICIENCY. When glucose-6-phosphate dehydrogenase deficiency is very mild there may be no noticeable clinical effects. In patients with somewhat more severe types of glucose-6-phosphate dehydrogenase deficiency, the lifespan of the red cell may be normal, or nearly so, until the cells are exposed to an abnormal stress. Such a stress may be provided by administration of one or a combination of certain drugs, infection, acidosis,

exposure to fava beans, or the neonatal state. Under such circumstances there is marked shortening of red cell lifespan with all the clinical signs of hemolytic anemia.

It must be emphasized that, in spite of extensive clinical and experimental observations, the spectrum of drugs that will produce hemolysis in glucose-6-phosphate dehydrogenase deficient red cells remains ill-defined. The data that are available have been obtained by two methods. Probably the most reliable observations are those based upon the use of ^{51}Cr-labelled erythrocytes in normal volunteers. Such studies can be well controlled and are relatively precise. However, data are available regarding only a limited number of drugs (12, 62, 65, 98, 186), and have been obtained almost entirely from subjects with the A type of G-6-PD deficiency, since this was the only type that was recognized originally.

The second body of data bearing on the spectrum of drugs that may cause hemolysis is derived from clinical observation of patients with hemolytic anemia that develops after drug is administered in the course of treatment of an illness. When such a patient proves to be G-6-PD deficient, it has generally been assumed that hemolysis was induced by the drug. This inference is not always correct, for indeed, many illnesses for which such drugs are prescribed can themselves induce hemolytic anemia in G-6-PD deficient subjects. On the other hand, it must be admitted that there are also some limitations to the validity of inferences drawn from observing the responses of experimental subjects to administration of drugs. Thus, the number of individuals tested is always of necessity small in such investigations. Therefore, if a drug produces hemolysis only in an occasional G-6-PD deficient individual, it might be considered to be innocuous on the basis of limited experimental population studies. Yet it is entirely possible that individual differences in drug metabolism and in the activity of enzymes other than G-6-PD may influence the response to drug administration. Indeed, in the case of thiazolsulfone (2-amino-5-sulfanilylthiazole; Promizole, 4-aminophenyl-2′-aminothiazol-5′ sulfone), it has been shown clearly that some recipients hemolyze a high percentage of G-6-PD deficient red cells, while others do not destroy infused red cells from the same donor. It was observed that those persons who hemolyzed a large proportion of transfused G-6-PD deficient cells were the same persons who themselves developed toxic symptoms such as methemoglobinemia from the drug (66). It is also important to recognize the possibility that drugs that may not produce hemolytic anemia in the relatively mild types of G-6-PD deficiency, such as the A type, may produce hemolysis in persons with the much more severe Mediterranean type of deficiency. A summary of some of the available data regarding drugs that may produce hemolysis of G-6-PD deficient cells is given in table 1.

Individuals with G-6-PD deficient erythrocytes may also be susceptible to the hemolytic effect of the fava bean. Favism is an extremely serious disorder that may end fatally if it is not recognized promptly and treated by blood transfusion. The etiology of favism is not well understood. It is clearly established that all persons who are susceptible to the hemolytic effect of the fava bean are G-6-PD deficient (153, 168). Most of the subjects who have been reported to suffer from

TABLE 1

The effect of drugs on G-6-PD-deficient red cells

Drug (with daily dosage)	Experimental Evidence Based on Drug Administration to G-6-PD Deficient Volunteers		Clinical Evidence Based on Case Reports	
	Negro subjects	Caucasian subjects	Negro patients	Caucasian patients
Drugs producing clinically significant hemolysis				
Acetanilid: 3.6 g Trade name or common name: Antifebrin	+++ (65)			
Diaphenylsulfon: 200 mg 25 mg Trade name or common name: DDS, Dapsone, Disulone, Sulphadione, Avlosulfon, Eporal Chemical name: 4,4′-diaminodiphenylsulfone	++ (62) +− (62)	+++ (54)	++ (78)	
Furazolidone: 400 mg Trade name or common name: Furoxone, Nifulidone, Tricofuron	++ (98) −			
Furmethonol: 1.0 g Trade name or common name: Altafur, Furaltadone	++ (43)			
Naphthalene			+++ (23, 80) 188	+++ (61, 126, 153, 173)
Neoarsphenamine: 600 mg Trade name or common name: Neosalvarsan			++ (187)	
Fava beans				+++ (110, 128)
Pentaquine phosphate: 30 mg Chemical name: 8-(5-Isopropylaminoamylamino)-6-methoxyquinoline phosphate	+++ (12)			
Pamaquine naphthoate: 30 mg Trade name or common name: Plasmoquine, Pamaquine, Pamoate, Alminoquin naphthoate, Plasmochin Chemical name: Methylene-bis-β-hydroxynaphthoate of 6-methoxy-8-(1-methyl-4-diethylamino) butylaminoquinoline	++++ (86)			
Primaquine: 30 mg Chemical name: 8-(4-Amino-1-methylbutylamino)-6-methoxyquinoline diphosphate	+++ (25, 65, 86)			
Nitrofurazone: 1.5 g Trade name or common name: Furacin, Chemofuran, Furesol, Nifuzon, Nefco, Vabrocid			++++ (149)	

TABLE 1—*Continued*

Drug (with daily dosage)	Experimental Evidence Based on Drug Administration to G-6-PD Deficient Volunteers		Clinical Evidence Based on Case Reports	
	Negro subjects	Caucasian subjects	Negro patients	Caucasian patients
Nitrofurantoin: 400 mg Trade name or common name: Furadantin, Furadonine, Chemicofuran	++ (98, 100)			
Phenylhydrazine hydrochloride: 30 mg, 90 mg	++ (65) ++++ (98)			
Quinocide: 30 mg Trade name or common name: Win 10488, Chinocide Chemical name: 6-Methoxy-8-(4-amino-4-methyl-butylamino(quinoline	+++ (3, 98)			
quinodine: 0.8 g Trade name or common name: Conquinine, pitayine				++ (110)
Sulfanilamide: 3.6 g, 5.0 g Trade name or common name: Prontosil album, Prontalbin, Deseptyl, Septoplix, Prontylin	+++ (65) ++ (12)			
N-Acetylsulfanilamide Trade name or common name: Sulfacetamide, N-Sulfanilylacetamide, Acetocid, Albucid, Sulamyd, Sulfacyl	++ (12)			
Sulfapyridine: 4.0 g Trade name or common name: Dagenan, Eubasin, Pyriamid	+++ (186)			
Sulfamethoxypyridazine: 2.0 g Trade name or common name: Kynex, Midicel	++ (99)			
Salicylazosulfapyridine: 6.9 g Trade name or common name: Azulfidine, Azopyrine, Salazopyrin	+++ (99)			
2-Amino-5-sulfanilylthiazole Trade name or common name: Promizole, Thiazolsulfone	++ (65)			
Naldixic acid	? (7)			
SN 3883: 90–180 mg [8-(4-aminobutylamino)-6 methoxyquinoline]	+++ (12)			
CN 1110: 90–180 mg [8-(2-amino-1-methylethylamino)-6-methoxyquinoline]	+++ (12)			
SN 15,324 [8-(5-isopropylaminoamylamino)-6 hydroxyquinoline]	++ (12)			

TABLE 1—*Continued*

Drug (with daily dosage)	Experimental Evidence Based on Drug Administration to G-6-PD Deficient Volunteers		Clinical Evidence Based on Case Reports	
	Negro subjects	Caucasian subjects	Negro patients	Caucasian patients
Drugs usually not producing clinically significant hemolysis under normal conditions (e.g., in the absence of infection)				
Acetophenetidin: 3.6 g Trade name or common name: Phenacetin	+ (65)			
Acetylsalicylic acid: 3.6 g, 4–12 g Trade name or common name: Aspirin	0 (12) +− (98)			Undefined (168)
Aniline: 30 mg	0 (12)			
Antazolene: 400 mg Trade name or common name: Antistine	0 (12)			
Ascorbic acid: 1,500 mg Trade name or common name: Cantaxin, Cebione, Cevalin, vitamin C	+ (43)		+ (10)	
Chloramphenicol: 1.0 g, 2.0 g Trade name or common name: Chloromycetin	0 (12)			++ (110) ++ (53)
Chloroquine phosphate: 300 mg Trade name or common name: Chloroquin diphosphate, Aralen phosphate, SN-7618 Chemical name: 7-Chloro-[4-(4-diethylamino-1-methylbutylamino)] quinoline diphosphate	+− (98)			
Dimercaprol Trade name or common name: BAL, Dimercaprol, Dicaptol	+ (153)			
Diphenhydramine hydrochloride: 300 mg Trade name or common name: Benadryl, Amidryl, Dibendrin, Dihidral, Allergen, Syntedril	0 (12)			
Menadione sodium bisulfate: 10 mg, >2.5 mg Trade name or common name: Hykinone, Klotogen, Kavitan, Ido-K	0 (186) 0 (187, newborn infants)			
Menadione sodium diphosphate: >2.5 mg Trade name or common name: Menadiol tetrasodium diphosphate	0 (187; newborn infants)			
Methylene blue: 390 mg Trade name or common name: Swiss blue, methylthionine chloride	+ (43, 98)			
p-Hydroxyacetanilide: 3.6 g Trade name or common name: Nobedon, APAP, Tabalgin, Tempra, Amadil, Eneril, Tralgon	0 (65)			
Para-aminophenol: 100 mg	0 (12)			

TABLE 1 —*Continued*

Drug (with daily dosage)	Experimental Evidence Based on Drug Administration to G-6-PD Deficient Volunteers		Clinical Evidence Based on Case Reports	
	Negro subjects	Caucasian subjects	Negro patients	Caucasian patients
p-Aminobenzoic acid: 8.0 g Trade name or common name: PABA, Amben, Paraminol, vitamin H, vitamin B_x	0 (65)			
Procaine amide hydrochloride: 3.0 g Trade name or common name: Pronestyl hydrochloride, procain amide hydrochloride	0 (12)			
Probenecid: 1.0 g Trade name or common name: Benemid	0 (186)			
Sulfadiazine Trade name or common name: Pyrimal, Debenal, Diazyl, Sterazine, Adizaine		0 (168)		
Sulfamerazine: 5 g Trade name or common name: Methylpyrimal, Primal M, Percoccide, Veta-Merazine	0 (12)			
Sulfisoxazole: 5 g, 6.0 g, 8.0 g, Trade name or common name: Gantrisin, Gantrosan, Soxisol	0 (186) 0 (186) ++ (98)			
Sulfathiazole: 5 g Trade name or common name: Thiazamide, Cibazol, Eleudron, Thiacoccine	0 (12)			
Sodium sulfoxone: 300 mg Trade name or common name: Diasone, Novotrone, Diazon	+ (65)			
Tripelennamine: 300 mg Trade name or common name: Dehistin, Azaron, Pyribenzamine, PBZ, Tonaril	0 (65)			
Pyrimethamine Trade name or common name: Daraprim, Chloridin, Darapram, Malocide	0 (65)			
Quinine: 2 g	0 (186)			
Quinacrine hydrochloride: 100 mg Trade name or common name: Mepacrine hydrochloride, Atabrine hydrochloride, Chinacrin hydrochloride	+− (98)			
Sodium nitrite: Trade name or common name: Erinitrit	0 (44)			
SN 15,305: 500 mg [8-(5-isopropylaminoamylamino)-7-methylquinoline]	0 (12)			
SN 3294: [4-(4-diethylamino-1-methylbutylamino)-6-methoxyquinoline]	0 (12)			

this type of hemolytic anemia presumably had the Mediterranean type of defect. However, favism has also been reported to occur in persons in West China (70), and it is possible that a different variant is involved in this area. In contrast, favism is not known to occur in Negro subjects with G-6-PD deficiency. It appears that other factors, in addition to G-6-PD deficiency, are required for persons to be sensitive to the hemolytic effect of the fava bean. Thus, it is possible for several persons in a single family group, all G-6-PD deficient, to enjoy a meal of fava beans but for only one to develop severe favism. What the other factors leading to this disorder may be is not clear in spite of extensive studies. It has been suggested that immunologic reactivity may play a role, and it has been found that favism is more common in some G-6-PD deficient families than in others (165). Thus, additional genetic predisposition to favism may be required. This represents an important and interesting area for further investigation.

G-6-PD deficient individuals may also develop hemolytic anemia during the course of an infection even when no drugs are administered. It is this phenomenon that makes evaluation of the hemolytic potential of a drug under clinical conditions particularly hazardous. Clinically, hemolysis in G-6-PD deficiency has been associated with a variety of infections (16, 38, 46, 55, 84, 127). It has been shown that under experimental conditions, influenza A virus will produce hemolysis of G-6-PD deficient cells *in vitro* (135). It is not known whether clinical hemolysis occurring in G-6-PD deficient persons represents the same type of phenomenon. Diabetic acidosis also appears to cause the development of hemolytic lesions in G-6-PD deficient persons (77). It is not known whether the lowering of blood pH exerts an effect through inhibition of the hexokinase reaction or by other means that may limit further the metabolism of glucose-6-phosphate through the monophosphate shunt.

In some populations it has also been noted that G-6-PD deficient infants have an increased incidence of hemolytic disease of the newborn. It is noteworthy that while such an increase has been observed in Greece (69) it could not be documented in Israel (169). This was true in spite of the fact that the G-6-PD variant that is most prevalent in these regions is the same, G-6-PD Mediterranean. It is possible that environmental factors may play a role in this type of hemolytic disorder. In one study (74), a dye mixture used to sterilize the umbilical cord stump was implicated as an exogenous agent producing hemolytic anemia in G-6-PD deficient infants. In other instances, it is possible that the routine administration of vitamin K derivatives may play a role (187). In most cases, however, no exogenous agent is implicated and it is likely that the association of G-6-PD deficiency with the normal enzymatic immaturity of the erythrocyte results in hemolysis spontaneously or in the presence of very minor stress.

Although the vast majority of G-6-PD deficient subjects have erythrocytes with a normal or nearly normal life span in the absence of stress, there are rare G-6-PD deficient patients with the clinical picture of nonspherocytic congenital hemolytic anemia. These patients have hemolytic anemia even in the absence of exposure to any stressful situation. Biochemical investigations of erythrocytes

demonstrate that their enzyme generally has such poor stability *in vivo* (102, 141), marked decrease in activity, or unfavorable alterations in kinetic constants (103), that virtually no metabolism of glucose-6-phosphate through the hexose monophosphate pathway is possible. Some of the variants that produce this clinical picture are listed in table 2.

C. MECHANISM OF HEMOLYSIS. The sequence of events leading to destruction of G-6-PD deficient red cells when the drugs are administered is poorly understood. Although very high concentrations of primaquine produce potassium loss (178) and eventually lysis (24) of G-6-PD deficient cells, they are no more susceptible to this type of destruction *in vitro* than are normal erythrocytes. It must be assumed that the administration of drugs inflicts a lesion on these cells that permits them to be selected for destruction by the reticuloendothelial system. The most easily measured of these changes are the loss of reduced glutathione (27, 72) and the formation of Heinz bodies (26).

The interaction of a hemolytic drug with hemoglobin *in vitro* results in the generation of low levels of hydrogen peroxide (57). It is likely therefore, that peroxides may also be generated *in vivo* when a hemolytic drug is administered. The glutathione peroxidase-glutathione reductase-glucose-6-phosphate dehydrogenase system efficiently disposes of this oxidizing agent in normal red cells. In G-6-PD deficient red cells, however, the GSH is merely oxidized to GSSG. Accumulation of this disulfide compound may in itself alter some metabolic steps within the red cell. The depletion of GSH may render susceptible to inactivation sulfhydryl-containing enzymes in the red cell such as hexokinase or glyceraldehyde phosphate dehydrogenase. The fact that no system exists for efficiently removing the H_2O_2 that may be generated, may result in peroxidative damage to hemoglobin, red cell enzymes, and soluble constituents. Mixed disulfides between GSH and hemoglobin may also be formed. The relative role of these possible mechanisms of damage has not been defined satisfactorily. There are some reasons to believe, however, that the changes induced by chemicals may not depend upon the generation of peroxide alone. Thus, we have been able to recover completely, in the form of GSSG, the glutathione that disappears from red cells when they are exposed to peroxide. In contrast, recovery of glutathione is incomplete after exposure to acetylphenylhydrazine (32) or to phenylazoformate (azoester) (164).

Considerable attention has been paid to the possible role of methemoglobin formation in drug-induced destruction of hemoglobin. It has been suggested that methemoglobin may be an intermediate in the formation of denatured hemoglobin (Heinz bodies) as the result of drug administration (81, 94). It is true that most drugs that produce hemolytic anemia in G-6-PD deficient cells do induce the production of some degree of methemoglobinemia. It has also been shown that the heme group of methemoglobin is not bound tightly to globin and moves freely from one molecule of globin to another (45). Since heme-free globin is quite susceptible to denaturation (150), the possibility that methemoglobin is an intermediary in the oxidative destruction of hemoglobin is a reasonable assumption. But the evidence supporting a causal relationship between methemoglobin on the

one hand and oxidative destruction of hemoglobin and hemolysis on the other is not strong. Indeed, a considerable body of negative evidence has led us to question whether methemoglobin is necessary as an intermediate compound in the oxidative destruction of hemoglobin (22).

1) Some Heinz body-producing drugs do not produce any measurable methemoglobinemia (123).
2) The rate of formation and disappearance of methemoglobin in cells exposed to oxidative drugs does not seem to bear a precursor relationship to the formation of denatured hemoglobin (81, 148).
3) The conversion of oxyhemoglobin to methemoglobin by nitrite does not increase susceptibility of red cells to Heinz body formation *in vitro* (22) or hemolysis *in vivo* (13, 23, 30).
4) "Stabilizing" methemoglobin by the addition of cyanide does not prevent the formation of Heinz bodies (22).
5) Methemoglobinemia, *per se*, whether induced by nitrite or caused by a deficiency of NADH-methemoglobin reductase, does not lead to hemolytic disease.

D. TYPES OF G-6-PD VARIANT. It is now recognized that many variants of G-6-PD exist. Some of these are subactive variants, *i.e.*, associated with decreased enzyme activity in the mature erythrocytes; others merely show alteration in electrophoretic mobility. Variants of G-6-PD that appear to be distinct along with criteria for their characterization are listed in table 2.

Sufficient purification of the enzyme to permit fingerprinting has been achieved in the case of the normal (type B) G-6-PD and the common A+ variant. The difference between the two enzymes has been found to consist of a single amino acid substitution, asparagine for aspartic acid (182). To fingerprint G-6-PD it was necessary to purify the enzyme from the red cells of more than 10 liters of whole blood of each type. In the case of subactive mutants vastly larger amounts of blood would be required unless new methods for enzyme purification and fingerprinting are developed. Until such methods are available, it will continue to be necessary to use functional criteria such as pH optimum curves, K_m values, and the comparison of substrate analogues, the thermal stability of the enzyme, and electrophoretic mobility as means of characterizing mutant enzymes. Naturally, it is probable that some subjects, who might be regarded as having identical variants, may actually have variants that are quite different when fingerprinting studies are performed. A possibility must also be considered that some subjects with variants that appear to be different in nature may actually have the same variant. Even with the best effort, it is difficult to achieve complete standardization between laboratories. To help to minimize this type of difficulty an International Reference Laboratory for G-6-PD has been set up under the direction of Dr. Arno Motulsky at the University of Washington, Seattle, Washington.

E. GENETICS OF G-6-PD DEFICIENCY (19). The structural gene for glucose-6-phosphate dehydrogenase is located on the X-chromosome. Studies of families reveal that transmission occurs from mother to son, but not from father to son. The degree of expressivity varies greatly in heterozygous females so that some

TABLE 2

Table of G-6-PD variants

Variant (references in parentheses)	Population	RBC Activity: % of Normal	Electrophoretic Mobility: Approx. % of Normal	K_m G-6-P: μM	K_m NADP: μM	2dG-6-P Utilization: % of G-6-P	Heat Stability	pH Optima	Population Frequency
Normal B	Various	100	100	50–78	2.9–4.4	<4	Normal	Normal	Usual
Hektoen (63) (66a)	U.S. White	400–500	100 129 (PO_4)	51	3.0	3	Normal	Normal	Rare
King County (184)	Negro	100	105	61	4	6	?	Normal	Rare
Madison (134)	Norwegian	100	70 (TEB) 90 (Tris)	?	?	?	?	?	Rare
Ijebu-Ode (119, 118a)	Negro	100	85	60	24	11 (14mM)	Decreased	Biphasic	Rare
Ita-Bale (119, 118a)	Negro	100	65	91	11	14 (14mM)	Slightly decreased	Normal	Rare
A+ (40, 101)	Negro	88	110	Normal	Normal	<4	Normal	Normal	Very common
Baltimore-Austin (118)	Negro	75	90	68	3.1	<4	Normal	Normal	Rare
Ibadan-Austin (118, 142)	Negro	72	80	62–72	3.3	<4	Normal	Normal	Rare
Minas Gerais (5)	Brazilian	<70	95	41	4	9	?	Normal	Rare
Madrona (87)	Negro	70–80	80	32	3.5	Normal	?	Normal	Rare

Barbieri (122)	Italian	40–60	135	Increased	Increased	?	Normal	?	Rare
Capetown (39)	Cape Colored Norwegian	53–80	55–65 (TEB) 76–88 (Tris) 35–48 (PO_4)	11–14	0.2–1	7–16	Normal	Biphasic	?
Kerala (4)	Asian SE Indian	50	75 (TEB) 90 (Tris)	23	1.5	7.4	Normal	Biphasic	Rare
Columbus (140)	Negro	35	100	Normal	Normal	Normal	?	?	Rare
Tel-Hashomer (147)	Sephardic (Tunisian) Jew	25–40	60–70	30–40	?	Normal	Normal	Slightly biphasic	Rare
Athens (166)	Greek	20	98	19	3	15	Slightly reduced	Slightly biphasic	
Puerto Rico (124)	Puerto Rican	19	112	18.6		2.7	Slightly decreased	Probably normal	
Washington(124)	Negro	16	95	57.4		1.6	Normal	Normal	
Markham (102)	New Guinea	15–10	105 108	4.4 6.3	?	162–222	? Reduced	Very biphasic	?
Kabyle (97)	Algerian	14–36	104 (TEB) 110 (PO_4)	68		10 (same as control)	Normal	Normal	
Freiburg* (47, 179)	German	10–20	85 (TEB) 90 (PO_4)	87–118	4			Biphasic	

* Associated with nonspherocytic congenital hemolytic disease.

TABLE 2—*Continued*

Variant (references in parentheses)	Population	RBC Activity: % of Normal	Electrophoretic Mobility: Approx. % of Normal	K_m G-6-P: μM	K_m NADP: μM	2dG-6-P Utilization % of G-6-P	Heat Stability	pH Optima	Population Frequency
West Bengal (4)	Asian Indian	9	90 (TEB) 94 (Tris)	31	6.6	4	Normal	Normal	Rare
Chicago* (105)	West Europe	9–26	100	58–76	3.1–3.7	<4	Very low	Normal	Rare
Seattle (107, 157)	Welsh-Scottish	8–21	90	15–25	2.4–2.8	7–11	Normal	Slightly biphasic	Rare
A− (40, 101, 103)	Negro	8–20	110	Normal	Normal	<4	Normal	Normal	Common
Duarte* (20)	U.S.	8.5	100	58	5	5.4	Markedly diminished	7.0	Rare
Hong Kong (181)	Chinese	<8	100	½Normal	Normal	Sl.↑	Normal	Normal	?
Mediterranean (29, 106, 122, 146)*	Greek Sardinian Sephardic Jews Asian NW Indian	0–7	100	19–26	1.2–1.6	23–27	Low	Biphasic	Common
Benevento (124)	Italian	7	93	4.6		245	Decreased	5.5–9.75 Double peak	
Bangkok* (171)	Thai	5	100	60	5.3	8.4	Markedly diminished	8.0–8.5	
Panay (71)	Visayas Islands, Philippines	<5	96	30	4.7	Normal	Slightly increased	Biphasic	

Canton (125)	South Chinese	4-24	105	20-36	2.0-2.4	4-15	Slightly reduced	Biphasic	Common
Oklahoma* (104)	West Europe	4-10	100	127-200	20	<4	Low	Narrow peak	Rare
Paris (36)	French	4	?	280	?	?	Very unstable	High peak at 9.5	Rare
Union (183)	Philippino	<3	Fast	8-12	3.6-5.2	180	?	Biphasic	?
Ohio* (140)	Italian	2-16	Fast 110	Slightly increased	Slightly increased	Normal	Very labile	?	Rare
Torrance* (172)	U.S.	2.4	103 (PO_4)	48 60	6	2.4	Markedly diminished	Normal	Rare
Clichy* (36)	Greek	2	100	178	?	?	?	(Abnormal plateau 9-10)	Rare
Albuquerque* (29)	U.S.	1	100	115	11	0	Markedly diminished	Peaked with optimum at 8.5	Rare
Milwaukee* (180)	Puerto Rican White	0.5	92	224		3.7		8	Rare
Tübingen* (175)	Southern USSR Holland	0.3	100	↓	↓	?	?	?	Rare
Berlin* (83)	German	0-1		?↑	?	0	?	?	Rare
Eyssen* (40)	West Europe	0	90	?	?	?	Labile (in cold)	?	Rare
Beaujon* (36)	French	0	Fast	182				Peak at 9.5	Rare

* Associated with nonspherocytic congenital hemolytic disease.

have normal enzyme activity, some have virtually no enzyme activity, and most manifest intermediate degrees. It has been shown that in each female cell only one of the two loci for G-6-PD deficiency is active. Thus, the blood of a heterozygote for G-6-PD deficiency contains a mosaic of cells, some with normal G-6-PD activity and some with a marked decrease of G-6-PD activity. The process of inactivation and mechanisms by which it may bring about greatly varying proportions of normal and deficient cells of heterozygotes have been discussed in detail elsewhere (14).

Because of the unequal distribution of glucose-6-phosphate dehydrogenase among the red cells of heterozygotes, detection of deficiency presents special problems. Techniques in which the overall enzyme activity of a red cell hemolysate is measured are less sensitive than those in which cells react as individual metabolic units. Several methods designed especially for the detection of heterozygotes have been described (21).

The locus for glucose-6-phosphate dehydrogenase is linked closely to the locus for color blindness (2, 130, 160) and for hemophilia A and B (159). No linkage with the locus for Xg^a has been found (161).

F. DETECTION OF G-6-PD DEFICIENCY. The G-6-PD activity of erythrocytes may be measured by estimating at 340 mμ the rate of reduction of NADP in the presence of buffer, hemolysate, and glucose-6-phosphate. Several quantitative assay systems have been described (18), but clinically important G-6-PD deficiency is usually sufficiently severe so that the diagnosis may be made by the use of simple screening tests. A number of such tests have been described in the last few years. All of them seem to be quite adequate. From the point of view of rapidity and ease of performance, we prefer the fluorescent spot test procedure (17, 31).

3. Glutathione reductase deficiency. A. HISTORICAL. In 1959 Desforges *et al.* (68) reported studies of a patient with sulfoxone-induced hemolytic anemia whose red cell G-6-PD activity was normal. In the course of other investigations, it was found that glutathione reductase activity was only approximately 60% of that of a normal control. Subsequently, a Caucasian subject, who was in the first series of "primaquine-sensitive" persons investigated, was found to have normal G-6-PD activity. However, his glutathione reductase activity was 57% of normal (50). In Germany, Waller and associates (174, 177) have collected data from a large number of patients with glutathione reductase deficiency. Most of our information regarding this deficiency is drawn from work of these investigators.

B. THE CLINICAL EFFECTS OF GLUTATHIONE REDUCTASE DEFICIENCY. The original cases of glutathione reductase deficiency reported by Desforges and by Carson were patients who developed hemolytic anemia after the administration of sulfoxone or primaquine. In the larger number of cases subsequently reported by Waller *et al.* (177), however, the clinical manifestations have been much more varied. Deficiency of glutathione reductase of the red cells has been associated with pancytopenia, with nonspherocytic hemolytic anemia with or without thrombocytopenia and leukopenia, with thrombocytopenia, and even

with isolated instances of leukemia and hemophilia B. In nearly one-fourth of the cases the condition was associated with nonhematologic disorders. When hematologic manifestations were evident they were often aggravated by the administration of drugs. However, the drugs that were implicated were not the "oxidant" compounds summarized in table 1. Rather, many other types of drugs and chemicals were associated with the development of the dyscrasias. These compounds are listed in a recent review (174).

C. MECHANISM OF HEMOLYSIS. Although glutathione reductase plays a vital role in the chain of reactions that detoxifies hydrogen peroxide in the red cell and maintains disulfides in the reduced form, the relationship between the deficiency of this enzyme and the hematologic symptoms that are reported is not at all clear. This is true because the level to which activity of the enzyme is reduced in "enzyme deficient" subjects is about one-half of normal. This level of activity still greatly exceeds the activity of the hexokinase, the rate-limiting enzyme in the series of reactions that reduce NADP for glutathione reduction. Thus, it has not been possible to demonstrate a metabolic deficit in glutathione-reductase deficient red cells (185). Even the steady-state level of GSSG is normal in cells that have only one-half the normal enzyme activity (164).

Some apparently normal patients have glutathione reductase activity that is as low or lower than the activity in the red cells of affected individuals. It is of particular interest that some patients with homozygous hemoglobin C disease have a marked diminution of red cell glutathione reductase activity (93, 174). Yet, such individuals are not known to be prone to drug-induced hemolysis or to exhibit the other clinical effects that have been associated with deficiency of this enzyme. It has been suggested (174) that administration of drugs may result in further inhibition of already diminished levels of glutathione reductase. Nonetheless, the possibility remains that lack of glutathione reductase does not play an etiologic role in the development of hemolytic anemia, thrombocytopenia, leukopenia, and hematologic disorders. Rather, it is possible that this lack represents a secondary manifestation of a poorly-understood basic disorder.

D. TYPES OF VARIANT. It is not possible to delineate clearly different biochemical types of glutathione-reductase deficiency. Electrophoresis of the enzyme on starch gel revealed the presence of only one band (96), which was the same in a patient with moderately reduced glutathione reductase activity as in normal individuals. However, Blume *et al.* (34) have described recently a high-voltage electrophoretic technique that resolves the enzyme into two bands. In patients with glutathione reductase deficiency, the more rapidly moving of these bands is reported to be absent. Similar alterations are found in samples from a small percentage of normal persons.

An electrophoretically detectable mutation involving glutathione reductase has also been found among American Negroes (96, 117). The activity of the electrophoretically fast enzyme appears to be normal, and although there has been a questionable association of this variant with gout, it does not have any clear-cut clinical effects.

E. GENETICS. The genetic pattern of glutathione reductase deficiency has not

been worked out completely. However, inheritance of the electrophoretic variant of the enzyme appears to be that of an autosomal gene (118). The enzyme deficiency, too, appears to be transmitted as an autosomal dominant trait (174). The possibility of multigenic inheritance cannot be ruled out though on the basis of the data that are presently available.

F. DETECTION. Glutathione reductase deficiency can be detected by means of a variety of assays that depend upon measurement of the rate of oxidation of NADPH in the presence of enzyme and its other substrate, GSSG (33). A simple screening test for the enzyme deficiency has also been devised. This test depends upon the rate of defluorescence of a buffered solution of NADPH and GSSG as compared with the rate when control cells are added (17).

4. Glutathione deficiency. A. HISTORICAL. A marked diminution of the content of both reduced and oxidized glutathione in red cells was first described by Oort *et al.* (136) in 1961. The condition seems to be very rare and, in addition to the initial Dutch family, only a few cases are known (35, 37, 116). However, knowledge of the existence of this disorder has been very helpful in providing information about the role of this tripeptide in normal red cell metabolism.

B. CLINICAL EFFECTS. Glutathione deficiency is associated with a mild nonspherocytic hemolytic anemia (144). Ashby survival studies showed that the mean lifespan of glutathione-deficient red cells was only one-third to one-fourth that of normal red cells. Clear-cut clinical correlation between drug ingestion and hemolysis could not be established in the family that was studied, but 30 mg of primaquine given daily produced marked acceleration in the rate of red cell destruction in these individuals. Some evidence suggests that fava beans might induce hemolysis in glutathione-deficient patients. One deficient subject passed "cola-colored" urine and developed severe jaundice after eating fava beans.

C. MECHANISM OF HEMOLYSIS. It seems likely that glutathione deficiency is due to an incapacity of the red cell to synthesize glutathione. Incubating normal red cells with glycine or with glutamine-^{14}C resulted in incorporation of radioactivity into glutathione of normal cells. No labelled glutathione was found in deficient cells (144, 145). These studies did not rule out the possibility of an exchange reaction. However, Boivin and Galand (35, 37) have reported that while hemolysates from two glutathione-deficient subjects could incorporate radioactivity into the dipeptide glutamyl-cysteine, glycine could not be incorporated to form the complete tripeptide, GSH.

The mechanism by which glutathione deficiency renders red cells excessively sensitive to hemolysis by oxidant drugs remains unknown. Nevertheless, it may be presumed that such cells, in common with G-6-PD deficient cells, are unable to protect erythrocytes against low levels of hydrogen peroxide.

D. TYPES OF VARIANT. No distinct variants of glutathione deficiency are known.

E. GENETICS. Glutathione deficiency is inherited as an autosomal recessive disorder (144). The glutathione levels of heterozygotes appear to be normal.

F. DETECTION. Detection of glutathione deficiency depends upon the estima-

tion of red cell glutathione levels. This is best done with DTNB [diothiobis-(2-nitrobenzoic acid] (28).

5. Phosphogluconic dehydrogenase (PGD) deficiency. A. HISTORICAL. Three groups of investigators (42, 111, 138) independently reported the occurrence of partial phosphogluconic dehydrogenase deficiency in 1964. Other such cases have been described since. The complete absence of phosphogluconic dehydrogenase was described by Parr and Fitch (139).

B. CLINICAL EFFECTS OF PHOSPHOGLUCONIC DEHYDROGENASE DEFICIENCY. Sensitivity to oxidant drugs would be expected to occur in individuals with severe PGD deficiency, since such a defect would lead to impairment of shunt activity and thus of glutathione reduction. In the only severely affected subjects known, drug challenge has not been carried out (139). Dern *et al.* (66) have, however, administered 60 mg of primaquine daily to a partially PGD-deficient subject and have observed about 20% destruction of ^{51}Cr-labelled red cells. However, interpretation of these data is complicated by the fact that the subject challenged also happened to be an unexpressed heterozygote for PGD deficiency.

C. MECHANISM OF HEMOLYSIS. Phosphogluconic dehydrogenase is the second enzyme in the hexose monophosphate shunt. Decrease in its activity would limit reduction of NADP to NADPH, and could also result in the accumulation of 6-phosphogluconic acid in red cells. If drug-induced hemolysis does occur in PGD deficiency its mechanism would be essentially the same as that in G-6-PD deficiency.

D. TYPES OF VARIANT. An extensive electrophoretic polymorphism involving PGD has been described (139). Severely deficient subjects have been designated as the "Whitechapel" type. Deficiency of this type of PGD deficiency is through an allele designated as PGD^{W}, which appears to produce very unstable enzyme so that homozygotes have scarcely any activity in mature red cells and reduced activity in white cells. Another allele, PGD^{O} appears to result in the production of little or no enzyme.

E. GENETICS. Partial PGD deficiency generally appears to be due to an inheritance of a single gene, such as the PGD^{O} or PGD^{W} gene, and severe deficiency is due to inheritance of both abnormal genes.

F. DETECTION. Detection of PGD deficiency depends upon performance of an NADP-linked enzyme assay.

B. The unstable hemoglobins

1. Biochemical considerations (88). Hemoglobin of normal adults is comprised of 4 polypeptide chains, 2 *alpha* chains and 2 *beta* chains. Each polypeptide chain binds a heme group, the iron of which forms a bond with the so-called proximal histidine of both chains. These histidine residues occupy position 92 of the *beta* chain and position 87 of the *alpha* chain. The sixth coordination position of the heme iron, the site of reversible oxygenation, is oriented toward the so-called distal histidine residue, which is in position 63 of the *beta* chain and position 58 of the *alpha* chain. Mutations involving amino acids at or near the

distal or proximal histidyl residues appear to give rise to abnormalities in the stability of the hemoglobin or to methemoglobinemias.

2. Historical. In 1952 Cathie (52) reported a child who had undergone splenectomy because of severe hemolytic anemia. Large Heinz bodies were present even in the absence of drug administration. Subsequently, similar cases were described by Schmid *et al.* (155) and by Lange and Akeroyd (109). The urine of the patient reported by Lange and Akeroyd contained a dark pigment that appeared to belong to the bilifuscin and mesobilifuscin group. It was not until Hitzig *et al.* (85) studied a child who developed a severe hemolysis during treatment of otitis with sulfisomidine (Elkosin), that it was recognized that this type of disease was due to a hemoglobinopathy. In this patient, most of the red cells contained inclusion bodies, but investigations of G-6-PD activity, glutathione concentration, and glutathione stability all resulted in normal values. Electrophoresis revealed that two hemoglobins were present, and the structural abnormality was elucidated subsequently at the molecular level. Soon other investigators found similar patients in whom there was either hemolytic anemia after administration of drugs, or, in some cases, evidence of hemolysis even without drug therapy.

3. Clinical effects of unstable hemoglobins. Since several different unstable hemoglobins have been described, it is not surprising that the clinical picture lacks uniformity. In patients with hemoglobin Zürich, shortening of red blood cell survival was observed even when no drugs were given, but severe hemolytic anemia ensued when certain drugs were administered. A systematic investigation of the susceptibility of ^{51}Cr-labelled transfused red blood cells to a variety of drugs was carried out (75). The results of these studies are shown in table 3. It is apparent that the spectrum of drugs that hasten the destruction of cells with hemoglobin Zürich is similar to that which causes destruction of G-6-PD deficient cells. It seems, however, that some sulfonamides such as sulfisoxazole

TABLE 3

Susceptibility of erythrocytes with hemoglobin Zürich to various drugs

Hemolysis	No Hemolysis
Sulfisomidine (Elkosin, Elkosil)	Digitoxin (Unidigin, Purodigin, Crystodigin)
Sulfamethoxypyridasine (Lederkyn)	Ethinyl estradiol
Sulfadimethoxine (Madribon)	Methandrostenolone (Dianabol)
Sulfaproxyline + Sulfamerasine (Dosulfin)	Phenobarbital (Luminal)
Primaquine	Acetylsalicylic acid (Aspirin, Empirin, Acetophen)
Causyth [8-(dimethylaminoantipyrin) oxyquinoline sulfonic acid]	3-Pyridinemethanol (Roniacol)
	Phenprocoumon (Marcoumar)
	Atropine
	Insulin
	Nicotinic acid (Niacin)
	Isoamyl nitrite (Amyl nitrite)

(Gantrisin), which do not produce hemolysis of G-6-PD deficient cells, have the capacity to destroy the hemoglobin Zürich-containing red cells. In the case of hemoglobin Köln, it was found that infection exacerbated an already existing hemolytic anemia (143). Quite surprisingly, however, the administration of drugs failed to exacerbate hemolysis (91). In the case of other unstable hemoglobins, the effect of drug administration can only be inferred.

Presplenectomy blood films are usually relatively normal in the absence of drug administration, but some hypochromia may be present, possibly because of "pitting" of denatured hemoglobin from red cells in the spleen (90, 95). When drugs are given, however, many erythrocytic inclusion bodies, staining with brilliant cresyl blue, crystal violet, or Nile blue, appear. Such bodies become especially prominent in patients who have undergone splenectomy, and may be seen in the absence of drug administration when the spleen has been removed from the circulation. Many of the patients with this type of hemolytic anemia excrete dark urine pigments of the "dipyrole" type, which have been identified tentatively as dipyrrylmethenes of the mesobilifuscin group, particularly during hemolytic crises. Since these pigments are not excreted by most patients with hemolytic anemia, it has been suggested that the route of metabolism of precipitated hemoglobin differs from the metabolism of hemoglobin derived from red cells destroyed by other hemolytic mechanisms (79).

4. Mechanism of hemolysis. Unstable hemoglobin apparently readily becomes denatured into insoluble aggregates. This process appears to be hastened by drugs that catalyze the oxidation of hemoglobin. The role of methemoglobin in this process is not entirely clear. The rate of spontaneous methemoglobin formation on autoincubation of blood is more rapid than normal in these disorders, but methemoglobin-forming substances such as amyl nitrite apparently do not have the capacity to precipitate the hemolytic reactions (see table 3). However, the precipitation of hemoglobin Köln is inhibited by cyanide (92). It is possible that a lesion near the site of the attachment of heme to globin results in weakness of the heme-globin bond, with splitting of heme from the protein portion of the molecule. Indeed, evidence that the hemes of methemoglobin Köln exchange more readily than the hemes of hemoglobin A has been presented (92). Free globin is known to be extremely unstable and to readily undergo precipitation and denaturation (150). It has been suggested (60) that unstable hemoglobins result when an internal hydrophobic residue is replaced by another hydrophobic residue of different dimensions thereby distorting the molecule.

5. Types of unstable hemoglobin. Mutations involving both the *alpha* and *beta* chain of hemoglobin have been reported. Table 4 presents a listing of those hemoglobins that have been characterized.

6. Genetics of the unstable hemoglobin. The unstable hemoglobin disorders differ from most of the other hemoglobinopathies in that they are clinically expressed in the heterozygote. Thus, the mode of inheritance of the disease is dominant in most instances. In some cases no family history has been obtained, and in these it is presumed that the disorder has arisen as a new mutation.

7. Diagnosis. In the absence of drug administration the red cells of patients

TABLE 4

Hemoglobin	Peptide Chain	Residue	Substitution	Reference	Comment
Zürich	β	63	Histidine → arginine	(133)	
Köln	β	98	Methionine → valine	(48)	
Seattle	β	70 or 76	Alanine → glutamic acid	(133)	
St. Mary's	β	?		(88)	
Hemoglobin H	β4			(88)	Tetramer of normal β-chains found in α-thalassemia
Torino	α	43	Phenylalanine → valine	(8)	
Hammersmith	β	42	Phenylalanine → serine	(60)	
Gunn Hill	β	93–97	Deleted	(41)	
Genova	β	28	Leucine → proline	(152)	
Sydney	β	67	Valine → alanine	(49)	
Bibba	α	136	Leucine → proline	(108)	
Santa Ana	α	88	Leucine → proline	(137)	
Tacoma	β	30	Arigine → serine	(6)	

with unstable hemoglobins may merely appear somewhat hypochromic; but inclusion bodies and large amounts of methemoglobin may appear if the blood is incubated for several hours, particularly if incubation is carried out in the presence of an oxidant such as brilliant cresyl blue. Red cells from splenectomized patients with unstable hemoglobin generally contain large numbers of inclusion bodies. These bodies can be demonstrated most clearly after supravital staining with dyes such as crystal violet, brilliant cresyl blue, or Nile blue. Frequently they are first detected when slides stained for the purpose of carrying out reticulocyte counts are examined. In unsplenectomized patients, inclusion bodies may commonly be found during a hemolytic episode, particularly if the episode has been precipitated by the administration of a hemolytic drug.

Once the presence of an unstable hemoglobin is suspected, one of the simplest means of detecting such an abnormality is the performance of a heat-stability test. A precipitate, consisting of denatured globin, appears when hemolysates prepared from red cells containing an unstable hemoglobin are heated to 50°C for 1 hr. Under the same conditions, no precipitate is formed in hemolysates prepared from normal red cells.

Because the unstable hemoglobins rapidly undergo degradation *in vivo*, especially after drug administration, detection and identification of the abnormal pigment sometimes prove to be difficult. The techniques employed are essentially those used in investigations of other abnormal hemoglobins. On starch gel electrophoresis, pH 8.0, hemoglobin Köln, hemoglobin St. Mary's, and hemoglobin Zürich are slower than hemoglobin A, but somewhat more rapid than hemoglobin A_2. Hemoglobin Seattle cannot be separated from hemoglobin A by paper or starch block electrophoresis in a barbital buffer, pH 8.6, but separates readily from adult hemoglobin by electrophoresis, with an EDTA-Tris chloride buffer, pH 8.6, or a sodium phosphate buffer, pH 7.4 (88).

Final identification of the type of abnormal hemoglobin involved requires more complex studies such as hybridization, to determine which chain is involved, and fingerprinting to determine the precise amino acid substitution.

II. SENSITIVITY TO DRUG-INDUCED HEMOLYTIC ANEMIA DUE TO DEVELOPMENT OF ABNORMAL PLASMA FACTORS: DRUG-DEPENDENT IMMUNE HEMOLYTIC ANEMIA

A. Historical

Although many earlier efforts were made to implicate immunologic factors in drug-induced hemolytic anemia, the first clearly-documented case of such a process was reported by Harris in 1956 (82). The original case studied involved stibophen (Fuadin, Fouadin, Pyrostib; sodium antimony III bis-pyrocatechol-2,4-disulfonate). It was found that an antibody in the serum was bound to the red cells in the presence of this drug or one of its derivatives. Subsequently, other cases involving stibophen and also other drugs involving, apparently, the same type of mechanism were described. A different type of drug-induced hemolytic anemia was reported by Ley and co-workers (113) after the administration of massive amounts of penicillin. In their studies, the antibody was found to be effective against red cells that merely had been treated with a drug: it was not necessary to include penicillin in the incubation system with the antibody. Most recently a remarkable type of drug-induced hemolytic anemia has been found to occur among patients receiving α-methyldopa (Aldomet) (181a). In these patients an autoimmune hemolytic anemia occurs in which the antibody can be demonstrated to react with red cell antigen even in the absence of drug.

B. Clinical findings

Drug-dependent immune hemolytic anemia may have a sudden, explosive onset. Table 5 lists the drugs that have been reported to cause the three types of immune hemolytic anemia precipitated by drug administration. Cases of anemia induced by stibophen, *p*-aminosalicylic acid, and chorinated hydrocarbon have ended fatally, and severe anemia and anuria have been reported in many other patients treated with these agents. Poikilocytosis and anisocytosis, spherocytosis, and increased osmotic fragility of the red cells have all been observed.

C. Mechanism of hemolysis

The type of antibody formed and mechanism of attachment to the red cell appears to differ in the three types of drug-dependent immunohemolytic anemias.

1. "The Fuadin type." In these patients the serum contains an antibody that causes agglutination or lysis or a positive antiglobulin test only in the presence of drug. The positive antiglobulin test is generally of the non-*gamma* (complement) type. The mechanism by which such antibody is formed in some individuals is not clearly understood. Ackroyd and Rook (1) have suggested that the drug may form an antigenetic complex with a substance on the erythrocyte surface and that this complex may prove to be antigenic. An alternative hypothesis (158) is that drug-protein complexes form in the plasma and that the erythrocytes are damaged by passive absorption of the circulating complexes. In either case, fixation complement probably results in lysis of the red cell *in vivo*.

TABLE 5

Drugs implicated in immune hemolytic anemia

Drug	Reference
Stibophen	(82)
Trade name or common name: Fuadin	
Chemical name: Sodium antimony III bis-pyrocatechol-2,4-disulfonate	
Para-aminosalicylic acid	(89)
Trade name or common name: PAS	
Benzylpenicillin sodium	(113)
Insecticides	(131)
Isonicotinic acid hydrazide	(76)
Trade name or common name: Isoniazid	
Chlorpromazine	(114)
Trade name or common name: Thorazine	
Aminopyrine	(9)
Trade name or common name: Pyramidon	
Dipyrone	(112)
Trade name or common name: Novaldin	(112)
Quinidine	(73)
Quinine	(132)
Phenacetin	(132)
Antistine	(121)
Salicylazosulfapyridine	(156)
Sulfisomidine or Sulfisoxazole	(76)

2. "The penicillin type." Hemolytic anemia after administration of penicillin differs from the "Fuadin type" in that very large quantities of the drug are required to produce the reaction and that the substance appears to be firmly bound to the erythrocyte surface. Penicillin-treated cells will develop a positive anti-*gamma*-G antiglobulin test when they are exposed to the serum of a sensitized patient. Indeed, Swanson and his associates (167) have found that more than 90% of patients receiving penicillin produce antipenicillin antibodies, but that only those rare patients who develop *gamma*-G antibodies develop hemolytic disease.

3. "The α-methyldopa type." α-Methyldopa produces a most extraordinary and most interesting hemolytic disorder. Here the antibody usually has specificity within the Rh blood-grouping system. The presence of drug is not necessary. Thus, the disease is essentially distinguishable from idiopathic autoimmune hemolytic disease. About 20% of unselected hypertensive patients who received this drug developed a positive antiglobulin test of the *gamma*-G type. The reason for this is not at all clear, but it may be that the drug alters red cell antigens in a way that results in their being transformed into "foreign" bodies. Indeed, it has been reported, recently, that α-methyldopa will bind to and cross-link proteins with red blood cells and alter the antigenicity of both (154). Alternately, the drug may, in some way, alter the body's response to its own antigenicity. If so, this reaction might yield important basic information regarding the secrets of immune tolerance.

D. Genetics

Virtually nothing is known about the genetics of drug-dependent immune hemolytic anemia. No clear familial pattern has been noted, although, as with other abnormalities of immunologic reaction, it is quite possible that a hereditary pattern will emerge when the underlying defects are more clearly understood.

E. Detection

Detection of drug-dependent immune hemolytic anemia depends upon demonstration of the antibody by suitable techniques *in vitro*. Studies that may prove helpful include a direct Coombs' test, and an indirect Coombs' test, with the patient's serum and normal donor cells, either in the presence of the suspected offending drug or after treatment of the cells with the offending drug (as in the case of penicillin). As indicated above, antiglobulin tests carried out with both anti-*gamma-G* globulin serum and non-anti-*gamma* globulin serum may be of interest.

SUMMARY AND CONCLUSIONS

When certain drugs that are not toxic to the majority of the population are given to a few sensitive persons, marked hemolytic anemia occurs. Three distinct types of mechanism that render red cells sensitive to drug-induced hemolysis have been delineated. A lack of certain enzymes, which serve to maintain glutathione in the reduced form or which synthesize glutathione, renders the cell sensitive to hemolysis by a group of "oxidant" drugs. Abnormalities in the globin portion of the hemoglobin molecule affecting either the *alpha* or *beta* chain near the site of the attachment of heme give rise to instability of hemoglobin and result in sensitivity to the hemolytic effect of the same group of drugs. Finally, immunologic mechanisms of three separate types have been implicated in sensitivity to drugs. In such persons antibodies develop in response to either a normal red cell component or to a drug-red cell complex and this leads to premature destruction of the red cell.

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