Hereditary Hemolytic Anemias Associated with Specific Erythrocyte Enzymopathies

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IT IS INCREASINGLY apparent that the hereditary hemolytic anemias of man comprise a vast and variegated spectrum of diseases, some comparatively common and some very rare, which have genetically determined aberrations in either structural or catalytic (enzyme) proteins of the erythrocyte. In some, the inborn error results in the presence of an abnormal hemoglobin; in some, such as the thalassemic syndromes, inability to synthesize adequately one or another component of globin appears to be the fundamental abnormality; in some, such as those forming the subject of this review, specific enzyme deficiencies constitute the molecular basis of anemia. In still other instances, including hereditary spherocytosis, the precise etiology at the molecular level remains undefined.

Normal Erythrocyte Metabolism

The mature human erythrocyte has lost much of the metabolic machinery characteristic of the other nucleated cells of the body. It has no nucleus, no ribosomes, mitochondria or other intracellular organelles, only ineffectual vestiges of Kreb cycle metabolism, and no capacity for oxidative phosphorylation. Beyond the reticulocyte stage, it retains no mechanism for synthesizing new protein. Some 95 percent of its dried weight is hemoglobin, which performs its crucial metabolic functions without expenditure of energy. However, its simple metabolic requirements are so geared to its restricted capabilities that it normally survives 120 days and more than 100 miles of travel in the circulation. Nonetheless, its energy requirements, while small, are still finite. It must maintain its biconcave, discoidal shape, pump sodium out of and potassium into its intracellular environment against electrochemical gradients, protect itself from oxidative stresses in its surroundings, and prevent significant conversion of its oxyhemoglobin to methemoglobin. Under normal conditions, there is slow denaturation of its crucial metabolic proteins each of which, under any given set of circumstances, has its individual biological half-life. Presumably its ultimate destruction results from attrition of its metabolic capacities and its eventual inability to provide even the austere energy requirements necessary for survival.[1-5]

The mature erythrocyte in man derives essentially all of its energy requirements from the conversion of glucose to lactate. Chart 1 indicates the available routes of glucose metabolism. Glucose, phosphorylated by hexokinase to glucose-6-phosphate (G-6-P), may traverse two main pathways. The quantitatively predominant Embden-Meyerhof pathway results in the anaerobic production of two moles of lactate for each mole of glucose metabolized. In the first half of the cycle, triose is formed from glucose, and energy is expended in the form of the conversion of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) at the hexokinase and phosphofructokinase mediated steps.

In the simplest illustration, this initial energy loss is more than compensated by the production of four moles of ATP from one mole of glucose (two of triose) traversing the terminal half of the pathway. Two moles of ATP are regenerated from ADP at the phosphoglycerate kinase step, and two more at the point of the pyruvate kinase mediated reac-

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tion. The net result is a gain of two moles of high energy ATP from each mole of anaerobically metabolized glucose. This net gain, however, may be materially reduced if triose by-passes the ATP generating phosphoglyceratekinase reaction by traversing the energy wasteful Rapaport-Luebering shunt[6,7] shown in Chart 1. The latter is a unique feature of the erythrocytes of man and certain, but not all, mammalian species. The glycolytic intermediate, 1,3-diphosphoglycerate (1,3-DPG) may

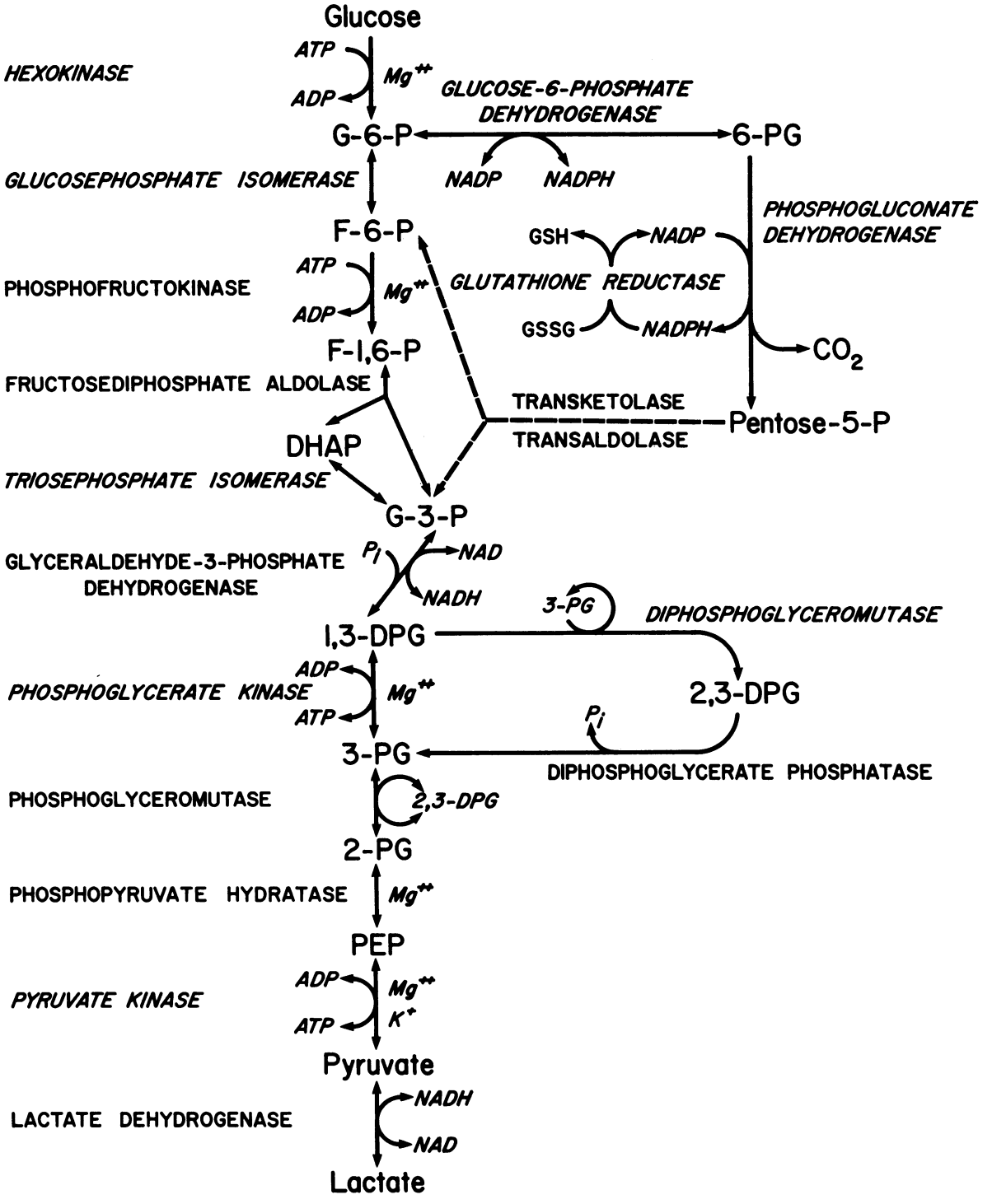

Chart 1.—The pathways of erythrocyte glycolysis.

be mutated enzymatically to 2,3-diphosphoglycerate (2,3-DPG), and the latter may accumulate to the point where at times it contains some 50 percent of the total erythrocyte organic phosphorus.[8] It, in turn, may be dephosphorylated and returned to the mainstream of glycolysis as 3-phosphoglycerate (3-PG), which is also the product of the alternative phosphoglycerate, kinase reaction of the anaerobic pathway.

It is apparent that if glucose were entirely metabolized via 2,3-DPG—that is, through the Rapaport-Luebering shunt—two moles of ATP would be lost before the formation of triose, and only two moles would be later generated (at the pyruvate kinase step only). No net gain would result from this exercise in futility. Therefore, in considering the strictly anaerobic erythrocyte metabolism of a mole of glucose, a net gain of somewhere between 0 and 2 moles of ATP will result, depending on what proportion of glucose passes through the 2,3-DPG pool. The latter may be important in regulating intracellular ATP/ADP ratios and level of inorganic phosphorus, and may also serve as a reservoir of substrate when glucose is temporarily depleted in the environment of the cell. In the anaerobic cycle, the pyridine co-factor is diphosphopyridine nucleotide (NAD) and this is cyclically reduced at the triosephosphate dehydrogenase step to NADH and reoxidized in the course of the terminal lactate dehydrogenase reaction.

While normally some 90 percent of glucose is thought to traverse the anaerobic route,[9] a significant and highly variable portion of glucose is metabolized via the so-called hexose monophosphate (HMP) oxidative shunt, likewise shown in Chart 1. Glucose-6-phosphate oxidized by this alternative pathway is converted to pentose through the action of two dehydrogenases. Pentose so generated is, through a series of enzymatically catalyzed reactions, again converted to fructose-6-phosphate (F-6-P) and glyceraldehyde-3-P (G-3-P), intermediates of the Embden-Meyerhof pathway which ultimately become lactate. It should be noted that triphosphopyridine nucleotide (NADP) is the co-factor operative in the oxidative shunt. It is reduced to NADPH by the first two dehydrogenase reactions, during which CO_2 is evolved and pentose formed. NADPH is reoxidized by enzymatic reactions outside of the strictly glycolytic metabolism. The most important of these, catalyzed by NADPH-linked glutathione reductase, maintains glutathione (GSH) in its reduced form. NADP regenerated in this manner is again available to participate in the metabolism of the HMP oxidative shunt.

While quantitatively less active than the anaerobic cycle under normal conditions, the HMP shunt is greatly stimulated by oxidative stresses resulting in glutathione oxidation, and NADPH, generated by the shunt here permits the important glutathione reductase reaction to proceed. This constitutes a protective mechanism invoked, for example, in the face of the challenge of oxidative damage resulting from the administration of certain drugs. Under such conditions, the operation of the HMP shunt may be crucial to cell survival. In view of the highly restricted metabolic resources of the erythrocyte, it is not surprising that a variety of highly specific and sharply localized inborn metabolic errors should result in impaired survival and, hence, in hemolytic anemia.

Hemolytic Anemia and Erythrocyte Enzyme Deficiency

The ultimate definition of the molecular etiology of the now recognized enzyme deficiency associated hemolytic anemias received major impetus from two main sources. The first of these resulted from a revival of interest in the known induction of severe hemolytic episodes in a significant proportion of persons receiving antimalarial agents such as primaquine.[10] Extensive studies during and after World War II culminated in 1956[11] in the recognition that "primaquine sensitivity" was conditioned by a specific deficiency of glucose-6-phosphate dehydrogenase (G-6-PD), the first enzyme of the HMP shunt pathway. For the first time the mechanism of an adverse reaction to a drug, whose administration was benign in most but catastrophic in a few, was defined at the molecular level and shown not to be on an immunological basis.

The second impetus derived from the studies of Dacie and his colleagues[12] in England. These workers were interested in certain relatively uncommon hemolytic anemias which they characterized as "nonspherocytic," in contrast to the better known disorder of hereditary spherocytosis. This heterogeneous group of hereditary anemias possessed certain clinical and laboratory features in common. No hemoglobinopathy was demonstrable, the antiglobulin (Coombs) test was negative, the osmotic fragility of fresh cells was usually normal, splenomegaly was the rule, and splenectomy (unlike the situation in hereditary spherocytosis) at best ameliorated, but did not clinically correct,

the disorder. Studies of glucose metabolism in the reticulocyte-rich, young erythrocyte populations of affected subjects in some instances suggested relatively deficient glycolysis and raised the question of some form of metabolic impairment in sugar utilization.

Selwyn and Dacie[13] devised an *in vitro* "autohemolysis" test whereby sterile, defibrinated blood, with and without additives such as glucose, was incubated at 37°C for 48 hours and spontaneous hemolysis was quantitated. Different patterns were observed among the cell populations from different subjects studied. In one, categorized as Type I, autohemolysis was only slightly greater than in normal blood, and was nearly, but not quite, correctible by adding glucose during the incubation. In a second, Type II, autohemolysis was usually severe, and was poorly if at all corrected by additives of glucose or adenosine. The test, although since shown to lack critical specificity, suggested the heterogeneity of these hereditary disorders. A little later de Gruchy and his colleagues[14-16] in Australia measured glycolytic intermediate levels in the erythrocytes of similar cases. In certain instances an abnormal accumulation of intermediates suggested "pile-up" behind a point of metabolic block, which appeared to be distal to triose formation. Again, a defect in the glycolytic machinery was suggested. In 1961 the first of several genetically determined erythrocyte glycolytic enzyme deficiencies associated with chronic hemolysis—that of pyruvate kinase deficiency—was defined by Valentine, Tanaka and Miwa.[17,18]

Classification (Table 1)

At present a number of hemolytic anemias in which the inborn error has been identified are recognized. These enzymopathies may be categorized as follows:

(1) Enzymatic deficiencies in the oxidative HMP shunt pathway. G-6-PD deficiency has been widely studied.[10,19] A few reports[20,21] of possible association of hereditary hemolytic anemia with partial 6 phosphogluconate dehydrogenase (6-PGD) deficiency are available, but a cause-effect relationship here is uncertain.

(2) Enzymatic deficiencies in non-glycolytic enzymes. Chronic hemolytic anemia associated with erythrocyte glutathione reductase (GSSG-R) deficiency has been observed by Löhr and Waller.[22] Carson and co-workers[23] have reported a milder deficiency of the same enzyme associated with susceptibility to primaquine hemolysis but not with chronic anemia. Harvald and his associates[24] have reported hereditary hemolytic anemia in two young men with a deficiency of adenosine triphosphatase (ATPase). This was regarded as a probable cause of the anemia. Necheles and coworkers[25] have recently described a relatively severe deficiency of the enzyme glutathione peroxidase in a male subject who exhibited a hemolytic reaction after transfusion, and a probable mild, compensated hemolytic disorder after recovery. Glutathione peroxidase catalyzes the oxidation of glutathione by peroxides, and evidence suggests it is of importance in protecting cellular proteins from peroxidative denaturation. Oort, Loos and Prins and their colleagues[26-28] have extensively studied a kindred in which certain members have a moderately severe hemolytic process and nearly complete absence of erythrocyte glutathione. The inborn error is thought to be inability to fully synthesize the tripeptide glutathione.

(3) Deficiencies in enzymes of the anaerobic glycolytic pathway. Hemolytic disorders associated with inherited deficiencies of the enzymes pyruvate kinase (PK),[17,18,19,29,30] triosephosphate isomerase (TPI),[31-33] hexokinase (HK),[34,35] glucosephosphate isomerase (GPI),[36,37] phosphoglycerate kinase (PGK),[38a-b] and of 2, 3-diphosphoglyceromutase (2, 3-DPGM)[39-42] are now established.

HMP Oxidative Shunt Pathway Enzyme Deficiencies

G-6-PD Deficiency

The fact that the antimalarial agent pamaquine is capable of causing hemolytic episodes in certain persons had been known since 1926.[43] In 1948 Earle and associates[44] noted that about 10 percent of American Negroes were susceptible, while hemolysis was relatively rare in Caucasians in this study. Similar findings were reported for primaquine by Hockwald and co-workers[45] in 1952, and in 1954 Dern and co-workers[46] clearly demonstrated an intrinsic erythrocyte abnormality in susceptible subjects. Chromium-labeled erythrocytes from patients with "primaquine sensitivity" were rapidly destroyed after transfusion into normal persons when primaquine was given to the recipient. Conversely, normal erythrocytes, isotopically labeled and transfused into primaquine-sensitive subjects, exhibited unimpaired survival when the drug was subsequently administered.

TABLE 1.—*Erythrocyte Enzymopathies Associated with Hereditary Hemolytic Anemia*

Deficient Enzyme	*Reaction Catalyzed*
A. *HEXOSEMONOPHOSPHATE-SHUNT ENZYME DEFICIENCIES*	
1. Glucose-6-phosphate dehydrogenase:	$\text{G-6-P} + \text{NADP} \overset{\text{G-6-PD}}{\longleftrightarrow} \text{6-PG} + \text{NADPH}$
2. 6-Phosphogluconate dehydrogenase:	$\text{6-PG} + \text{NADP} \overset{\text{6-PGD}}{\longrightarrow} \text{Ru-5-P} + \text{CO}_2 + \text{NADPH}$
B. *NONGLYCOLYTIC-ENZYME DEFICIENCIES*	
1. Glutathione reductase:	$\text{GSSG} + 2\ \text{NADPH} \overset{\text{GSSG-R}}{\longleftrightarrow} 2\ \text{GSH} + 2\ \text{NADP}$
2. Adenosine triphosphatase:	$\text{ATP} \xrightarrow[\text{Na}^+\ \text{K}^+\ \text{Mg}^{++}]{\text{ATP-ase}} \text{ADP} + \text{P}_i$
3. Glutathione peroxidase:	$2\ \text{GSH} + \text{H}_2\text{O}_2 \overset{\text{GSH-Px}}{\longrightarrow} \text{GSSG} + 2\ \text{H}_2\text{O}$
4. Glutathione synthetase:	(a) $\text{Glu} + \text{Cyst} \xrightarrow[\text{ATP}\ \text{Mg}^{++}]{\text{GS-I}} \gamma\text{-Glutamyl Cyst} + 2\ \text{ADP} + 2\ \text{P}^{\text{I}}$
	(b) $\gamma\text{-Glutamyl Cyst} + \text{Gly} \xrightarrow[\text{ATP}\ \text{Mg}^{++}]{\text{GS-II}} \text{GSH}$
C. *GLYCOLYTIC-ENZYME DEFICIENCIES*	
1. Pyruvate kinase:	$\text{PEP} + \text{ADP} \overset{\text{PK}}{\underset{\text{K}^+\ \text{Mg}^{++}}{\longleftrightarrow}} \text{Pyruvate} + \text{ATP}$
2. Triosephosphate isomerase:	$\text{DHAP} \overset{\text{TPI}}{\longleftrightarrow} \text{G-3-P}$
3. Hexokinase:	$\text{Glucose} + \text{ATP} \xrightarrow[\text{Mg}^{++}]{\text{HK}} \text{G-6-P} + \text{ADP}$
4. Glucosephosphate isomerase:	$\text{G-6-P} \overset{\text{GPI}}{\longleftrightarrow} \text{F-6-P}$
5. Phosphoglycerate kinase:	$\text{1,3-DPG} + \text{ADP} \overset{\text{PGK}}{\underset{\text{Mg}^{++}}{\longleftrightarrow}} \text{3-PG} + \text{ATP}$
6. Diphosphoglyceromutase:	$\text{1,3-DPG} \xrightarrow[\text{3-PG}]{\text{DPGM}} \text{2,3-DPG}$

Abbreviations: G-6-P = glucose-6-phosphate, 6-PG = 6-phospho-3-keto gluconate, Ru-5-P = ribulose-5-phosphate, PEP = phosphoenol pyruvate, DHAP = dihydroxyacetonephosphate, G-3-P = glyceraldehyde-3-phosphate, F-6-P = fructose-6-phosphate, 1,3-DGP = 1,3-diphosphoglycerate, 2,3-DGP = 2,3-diphosphoglycerate, 3-PG = 3-phosphoglycerate, NADP = nicotinamide-adenine dinucleotide phosphate (TPN), NADPH = reduced nicotinamide-adenine dinucleotide phosphate (TPNH), ATP = adenosine triphosphate, ADP = adenosine diphosphate, H_2O_2 = hydrogen peroxide, GSSG = oxidized glutathione, GSH = reduced glutathione, Cyst = cysteine, Gly = glycine, Glu = glutamic acid, P_i = inorganic phosphorus.

The clinical events of the hemolytic syndrome were later defined in detail by Dern, Beutler and Alving.[47] Beutler then demonstrated that the erythrocytes of affected persons had a low content of glutathione, and that this rapidly declined further when primaquine was administered.[48] Further, *in vitro* incubation of primaquine-sensitive erythrocytes with drugs such as acetylphenylhydrazine resulted in pronounced and rapid disappearance of erythrocyte-reduced glutathione[49] and an abnormality in Heinz body production.[50] Heinz bodies are intracorpuscular inclusions, demonstrable by supravital staining with methyl violet or brilliant cresyl blue, and represent oxidatively injured and denatured hemoglobin.[51] Many drugs,[10] including sulfonamides, acetanalid, furadantin, and certain vitamin K derivatives, were soon found to precipitate hemolysis in like manner in sensitive subjects.

In 1956, Carson and his associates[11] demonstrated that the underlying defect was an inherited deficiency in the enzyme glucose-6-phosphate dehydrogenase. The same erythrocyte abnormality was subsequently shown to exist in all subjects susceptible to a hemolytic syndrome induced by the ingestion of fava beans or the inhalation of pollen from flowering fava bean plants.[52-55] Some subjects with G-6-PD deficiency can apparently eat fava beans with impunity,[56,57] and some factor other than enzyme deficiency appears required. Hemolytic susceptibility after fava bean ingestion is almost exclusively limited to the non-Negro type of deficiency.

It is now known that about 2 percent of subjects with G-6-PD deficiency have a chronic hemolytic

anemia in the absence of drug challenge,[58-60] and that infectious processes may[53] also cause hemolysis to some degree.

G-6-PD deficiency is thought to affect some 100 million people in a geographic distribution similar to that of falciparum malaria.[61,62] Among Caucasians it is common in Sardinians and in Sephardic or Oriental, but not in Askinazy or European, Jews; but the disorder has been found in almost all racial groups. About 11 percent of American Negroes exhibit the deficiency. Extensive studies have documented genetic polymorphism[63-67]; that is, many genetic variants, the result presumably of different mutations at the same locus, have now been observed and studied. For example, G-6-PD deficiency is readily demonstrable in the leukocytes of affected Caucasians,[68] but this is much less evident in Negroes. The disorder is X chromosome-linked.[10,69-71] Males and homozygous females have full expression of the abnormality and severe deficiency; heterozygous females have wide variability in the degree of enzyme deficiency, and in the severity of hemolytic episodes. The enzyme in erythrocytes may be quantitatively assayed and a spot test[72] and tests based on the diminished capacity of affected erythrocytes to reduce certain dyes[61,73] or methemoglobin are also available for use in the clinical laboratory or in survey studies in the field.

The mechanism of drug induced hemolysis in G-6-PD deficiency is thought to reside in the inability of affected cells to generate the necessary NADPH to maintain glutathione in its reduced form.[10] This results in impaired protection against a number of noxious oxidative challenges.

Clinically,[10,47] acute hemolytic anemia, jaundice and hemoglobinuria develop on the second to fourth day after ingestion of an invoking drug. Only the older cells are destroyed, but in severe instances as many as 30 to 50 percent of circulating cells may be hemolysed. Because hemolytic susceptibility is related to erythrocyte age, recovery usually takes place beginning at about the tenth day or a little later, even if the drug is continued. With continued drug administration, an equilibrium phase occurs in which there may be no anemia, and in which hemolysis referable to the destruction of cells attaining a critical age is detectable only by special erythrocyte survival studies. After discontinuance of the drug, about three or four months must elapse before an episode of comparable severity can be reinduced. This represents the length of time necessary to restore mean cell age to the pre-hemolytic status. Severity of hemolysis is dose related and drug blood level related, liver and kidney impairment being capable of affecting the latter. For more extensive and detailed discussions, the reader is referred to available reviews.[10]

6-Phosphogluconate Dehydrogenase (6-PGD) Deficiency

Brewer and Dern[75] and Parr and Fitch[76] have reported partial deficiency of 6-PGD in the erythrocytes of certain persons without hemolytic anemia. The enzyme activity was of the order of 50 percent of normal. In contrast, two cases of hereditary hemolytic anemia (those of Lauseckher and co-workers,[20] and of Scialom and colleagues[21] have been reported in which 6-PGD was reduced to about the same degree. It is, however, entirely possible that this moderate reduction was not related to the hemolytic process causally, and may have coincidentally been present with some other undetected etiologic factor.

Non-glycolytic Enzyme Deficiencies

Glutathione Reductase (GSSG-R) Deficiency

Löhr and Waller[22] have reported a kindred, certain members of which had chronic hemolytic anemia associated with a relatively severe erythrocyte deficiency in the enzyme glutathione reductase. Waller and associates[77] have also reported hematologic and neurologic difficulties associated with GSSG-R deficiency. Carson and co-workers[23] have likewise noted susceptibilty to primaquine hemolysis, but not chronic anemia, in a much less severely deficient subject. Glutathione reductase reduces oxidized glutathione and concomitantly oxidizes NADPH. It interacts with the dehydrogenases of the oxidative shunt to protect against noxious oxidative processes and to maintain the level of reduced glutathione. In sufficiently deficient states this protective mechanism is impaired.

Adenosine Triphosphatase (ATPase) Deficiency

A sodium-potassium-magnesium dependent ouabain inhibitable ATPase is generally considered to play a central role in the active transport of sodium and potassium across cell membranes. Harvald and his associates[24] have briefly reported three unrelated persons, two of whom had chronic, apparently inherited, hemolytic anemia, in whom ATPase levels in the red cell approximated half-

normal values. These workers consider it likely that ATPase deficiency and the chronic hemolytic process were related. Family studies suggested a possible dominant inheritance, but normal ATPase levels in the cells of both parents of one propositus did not conform to this hypothesis. The issue was further clouded by uncertainty as to whether the transport related ATPase or, perhaps, other forms of ATPase activity as well were involved. Additional data are required before these questions can be resolved.

Glutathione Peroxidase Deficiency

The presence of glutathione peroxidase (GSH-PX) in mammalian erythrocytes was first established by Mills.[78,79] The enzyme catalyzes the *in vitro* detoxification of hydrogen peroxide. It therefore provides protection against peroxidative injury, and is linked to HMP shunt pathway metabolism through its role in glutathione oxidation. A partial deficiency in GSH-PX has been described in association with transient mild hemolysis in the neonatal period.[80] Necheles and his associates[25] have recently described an apparently homozygous state of the enzymopathy in a Puerto Rican male in whom a hemolytic reaction developed after transfusion. After recovery, mild reticulocytosis suggested persistence of a compensated hemolytic process. The cells of the patient possessed GSH-PX activity about 25 percent of normal. The red cells of both parents exhibited about half-normal GSH-PX activity. A recessive mode of inheritance was suggested. In studies with isotopically labelled glucose, hydrogen peroxide decidedly stimulated HMP shunt activity in normal subjects, but did not do so in the patient. While it is presumed this would result in increased susceptibility to drug induced oxidative injury, data on this are thus far unreported.

Glutathione (GSH) Deficiency

A series of studies in a Dutch[26-28] kindred have demonstrated moderately severe, chronic, hereditary hemolytic anemia associated with a remarkable and nearly total absence of either oxidized or reduced glutathione. In intact erythrocytes, glyoxalase activity, for which GSH is an obligatory cofactor, was zero. As expected, the affected cells were primaquine-sensitive and, in addition, chromium, even in labeling amounts, remarkably shortened cell survival *in vivo*. When radioactive glycine, glutamine, and cysteine, the amino acid components of the tripeptide glutathione, were incubated with normal cells radioactive GSH was formed. When similar incubations were performed with the affected cells no radioactive GSH could be found. Breakdown of added radioactive oxidized glutathione occurred at an equal rate in GSH deficient and normal cells. It was concluded that a defect in GSH synthesis was probably involved. It is remarkable that such low levels of erythrocyte GSH are compatible with survival, but such has been clearly demonstrated to be the case.

Deficiencies in Embden-Meyerhof Pathway Enzymes

Pyruvate Kinase (PK) Deficiency

In 1961 and 1962 erythrocytes from certain cases of hemolytic anemia, conforming in terms of the autohemolysis test to the Type II category of Selwyn and Dacie, were assayed for all glycolytic enzyme activities. All except one — pyruvate kinase, the enzyme converting phosphoenol pyruvate to pyruvate and located just above the terminal lactate dehydrogenase step — were found to have normal or greater than normal activity.[17,18] Chart 2 indicates the pyruvate kinase reaction and the essentials of the assay system.

Since that time about a hundred documented cases of hemolytic anemia with hereditary deficiency of this enzyme have appeared in the world literature, and it appears thus far that, except for

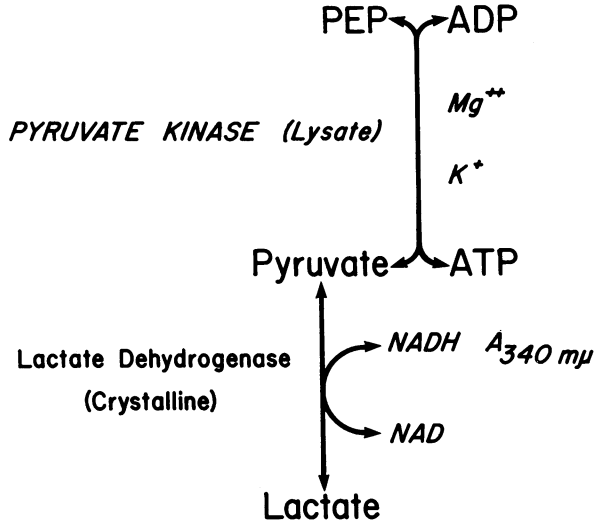

Chart 2.—Diagrammatic representation of the principles of the pyruvate kinase (PK) assay system. Substrate PEP (phosphoenol pyruvate) is provided in buffer containing ADP, Mg^{++}, and K^{+}. A dilute hemolysate provides the PK being assayed. Pyruvate generated is converted to lactate in the presence of added lactic dehydrogenase and the concomitant oxidation of NADH is followed spectrophotometrically.

G-6-PD deficiency, this is the commonest heritable disorder in the enzyme deficiency group.[29,86] Cases are of variable severity. Some patients may reach adult life partially compensated, with variable reticulocytosis and anemia and escaping transfusion. Others require transfusion from infancy and are unable to survive without donated blood. Most exhibit macrocytosis, a morphologically nondescript red cell with a scattering of cells with irregular membrane borders resembling acanthocyctes, splenomegaly and iron-containing inclusions in erythrocytes (Pappenheimer bodies) only if they have undergone splenectomy.[18]

Splenectomy only ameliorates the hemolytic process, and it was our original view that it was of little benefit. Accumulated experience[81a] has indicated, however, that removal of the spleen may be accompanied by significant improvement, sometimes with lessening or disappearance of transfusion requirements. In the severe cases of Bowman[81b,82] in an Amish population it appears that splenectomy is necessary for survival. Now we believe that splenectomy is often of distinct value and indicated, particularly when transfusions are required or anemia is significantly symptomatic. In a few cases, red cells have been morphologically bizarre,[83-85] with most cells showing numerous irregular projections of the membrane surface. Anemia is exacerbated by surgical stress and intercurrent infections.

In the commonest situation, when pyruvate kinase activity is assayed its activity is found to be greatly reduced and occasionally undetectable (though, of course, not absent) in deficient erythrocytes. Both sexes are affected, and both parents, all offspring and a number of the siblings and relatives of symptomatic subjects have on the average about one-half normal pyruvate kinase activity in their red cells. The disorder, therefore, appears to be transmitted as an autosomal recessive trait, heterozygotes being clinically and hematologically normal but biochemically detectable, and homozygotes having symptomatic hemolytic anemia.[17,18] The leukocyte pyruvate kinase activity is normal.[17,18,87,88] As would be expected with a block in terminal glycolysis, deficient cells in many instances may exhibit suboptimal glycolysis for their age,[13,30,86] may have difficulty in maintaining ATP[13,83,89-93] and NAD,[30,89,93,94] and assay of glycolytic intermediates frequently reveals abnormal accumulations of phosphoenol pyruvate, triose, particularly 2, 3-diphosphoglycerate, and even hexoses behind the point of metabolic block.[84,89,91-93,95] In such cases, pyruvate kinase, which ordinarily operates well below substrate saturation in the cell,[96] is rendered more effective by the greater availability of substrate. This compensates to some extent for the low enzyme activity characteristic of the disorder. As a result, young cell populations of homozygotes may still glycolyse actively and reasonably effectively maintain ATP levels. In some instances, defective ATP maintenance can be demonstrated by *in vitro* incubations, and similar studies may show inability to cycle NAD to NADH and back adequately. In certain cases, however, defective ATP maintenance and pronounced intermediate accumulation may not be demonstrable.[30,97,98]

It must be remembered that only metabolically affluent young cells, still capable of compensating for their glycolytic deficiency, are available for assay. As the cell ages, however, increasing accumulation of intermediates proximal to the deficient enzyme and eventual inability to generate ATP and cycle NAD are to be expected.

In most cases, the kinetics of residual enzyme activity in homozygotes has been normal. Again, such has not been universally true, and genetic polymorphism in the case of pyruvate kinase deficiency, as in G-6-PD deficiency, is becoming increasingly apparent. Instances of pyruvate kinase deficiency and abnormal enzyme kinetics have been reported by Waller and Löhr,[95] by Boivin and Galand,[99] and by Miwa.[100] Hsu, Robinson and Zuelzer[98] have intensively studied a kindred in which apparent homozygosity for pyruvate kinase activity existed in certain members with sharply differing clinical symptomatology and glycolytic intermediate patterns. In some members the characteristic hemolytic syndrome was present. In others, equally severely affected in terms of enzyme assay levels, there were no symptoms and, at most, a mild, fully compensated hemolytic process. The coexistence of two genes, undifferentiable as to their effect on pyruvate kinase in the usual assay, but with decidedly different clinical expression, was postulated.

In our laboratory, four cases of severe hereditary hemolytic anemia in two unrelated families with clinical and biochemical features of erythrocyte pyruvate kinase deficiency, but with assay values in either the normal or high heterozygote range have recently been studied by Paglia and coworkers.[101] Cases with similar intermediate or normal PK assay

values, but with the usual picture of homozygous deficiency have been noted by others.[86,88,94,100,102,103] In both families, the apparent dilemma was resolved by the finding that affected subjects had inherited from one side the "usual" gene resulting in pyruvate kinase deficiency, and from the other a gene resulting in a pyruvate kinase with grossly aberrant kinetics. The latter was characterized by remarkable catalytic inefficiency at low concentrations of its substrate, phosphoenol pyruvate, such as exist within the metabolizing erythrocyte. At high substrate concentrations (such as those employed for convenience in the usual assay) essentially no difference was evident. Heterozygosity for the kinetically abnormal gene was traceable by kinetic studies through one side of three generations in each of the two families. Not only was the K_m for the abnormal pyruvate kinase some ten times greater than that of the normal enzyme, but also differences in pH optimum and heat and storage stability were readily demonstrable. Heterozygotes had enzyme kinetics intermediate between normal subjects and the affected propositi.

The syndrome of pyruvate kinase deficiency hereditary hemolytic anemia may thus clearly result from a quantitative catalytic deficiency or, as in the above cases, from a qualitative defect of the same enzyme. With further studies it is likely that additional evidence for genetic polymorphism will be forthcoming. Sachs and coworkers[103] have likewise reported pyruvate kinase deficient hemolytic anemia in association with an enzyme catalytically inefficient at low substrate concentrations.

Triosephosphate Isomerase (TPI) Deficiency:

In 1965[31,104] and 1966[32] another highly consanguineous kindred of mixed Caucasian and Negro ancestry was studied. In members with hemolytic anemia resembling that of pyruvate kinase deficiency, a separate deficiency—that of triosephosphate isomerase—was demonstrated. In the process of anaerobic glycolysis, aldolase converts hexose diphosphate into two triose isomers, 3-phosphoglyceraldehyde and dihydroxyacetone phosphate. These are interconverted by the isomerase deficient in the erythrocytes of these subjects.

The very small number of subjects thus far studied, with the exception of one apparently unrelated case, derive from a population group with a high degree of consanguinity. While morphologically the findings are very similar to those observed in the erythrocytes of pyruvate kinase deficiency, the total syndrome, clinically and biochemically, presents many differences:[31,32,33a,33b]

- The autohemolysis test resembles that seen in hereditary spherocytosis. This is to say that substantial autohemolysis, well corrected by additives of glucose and adenosine, is observed.[31]
- Dihydroxyacetone accumulation is a prominent biochemical feature.[33a,33b]
- The enzyme deficiency is present in leukocytes, muscle and cerebrospinal fluid[33a] and possibly (though this is not yet certain) in other body tissues.
- An atypical, severe and progressive neurologic deficit, involving peripheral nerves and central nervous tissue, has been noted in the small number of affected children who have survived the first few months of life.[31,32,33a,104] This is clearly not related to kernicterus, and quite possibly may be the clinical manifestation of the isomerase deficiency in nervous tissue.
- Two unexplained sudden deaths in affected children have led to clinical suspicion (thus far unconfirmed) that cardiac muscle dysfunction may at times be a significant feature.[33a]

It is of tangential interest that sickle cell trait and G-6-PD deficiency have been demonstrated in the same kindreds, with a variety of combinations of these inherited metabolic errors and TPI deficiency.[32] Again, in TPI deficiency both sexes are involved, heterozygotes and homozygotes are definable, and an autosomal recessive mode of inheritance is indicated.[31,32,104]

Hexokinase (HK) Deficiency:

A single kindred has been extensively studied in which one child has had hemolytic anemia since birth, and in which erythrocyte, but not leukocyte, hexokinase activity is extremely low.[34,105] Erythrocyte hexokinase activity must be evaluated in the context of the fact that of all the glycolytic enzymes it exhibits by far the greatest increased activity in reticulocytes and young erythrocytes. Hexokinase activity in reticulocyte-rich cell populations is severalfold—up to ten times or more—greater than in populations of mixed age in normal blood. In the case of the propositus, despite pronounced reticulocytosis and a very young mean erythrocyte age, red cell hexokinase activity was at the lower limit of normal, and six to eight times

less than in simultaneously assayed populations of comparably young control blood. Morphologically, the hemolytic anemia was essentially similar to that observed in other erythrocyte enzyme deficiency states. Autohemolysis was modestly increased, partially corrected by glucose and better corrected by adenosine. Both glucose and fructose were phosphorylated at low rates, but mannose and galactose were metabolized much more rapidly. In both parents and three siblings, values for hexokinase were either distinctly low or at the extreme lower limits of the normal range, suggesting heterozygosity for the deficiency and, again, an autosomal recessive mode of inheritance. Presumably the patient's erythrocytes, already hexokinase-deficient in youth, with maturation and further attrition of the already impaired enzyme, soon are rendered incapable of survival.

Glucosephosphate Isomerase (GPI) Deficiency:

Four children in two unrelated kindreds[36,37,106] have now been shown to have severe hereditary hemolytic anemia associated with a deficiency of glucosephosphate isomerase, the second enzyme of the anaerobic glycolytic pathway, catalyzing the interconversion of the isomers glucose-6-phosphate and fructose-6-phosphate. Leukocytes in all four children share the deficiency. Other body tissues have not been adequately studied but, if affected, no evidence of clinical dysfunction exists. The propositi all exhibit a severe hemolytic syndrome. Splenectomy has proved of possibly some benefit, though pronounced reticulocytosis and hemolysis persist.

Again, both parents in each family and certain other family members have clearly intermediate GPI activity in their erythrocytes, and an autosomal recessive inheritance pattern again pertains. Studies with isotopically labeled glucose indicate striking impairment of recycling of fructose-6-phosphate, derived initially from pentose formed via the oxidative shunt, back through the partially blocked isomerase reaction to glucose-6-phosphate and thence again into the HMP shunt pathway. Starch gel electrophoretic studies,[107] interestingly, suggest that in the first case studied, the maternal and paternal enzyme patterns are not identical. Since both possess apparent heterozygosity for glucosephosphate isomerase deficiency, it may be that two separate mutated genes, both resulting in quantitatively deficient isomerase activity but electrophoretically different GPI protein, have been inherited by the propositus. Again, additional studies are required.

Phosphoglycerate Kinase (PGK) Deficiency:

We[38a,38b] have recently had the opportunity of studying in conjunction with colleagues from other laboratories the erythrocytes and leukocytes of a Chinese boy with lifelong hemolytic anemia. Both red and white cells exhibited severe deficiency of PGK. The hematological findings were essentially identical with those observed in other glycolytic enzyme deficiencies. There was evidence of mental retardation and behavioral aberrations, but it is not known whether or not these are related to the enzymopathy. Thus far, analysis of glycolytic intermediates is incomplete. The case is of particular interest since PGK activity was severely reduced to an estimated 4 to 5 percent of normal. The ability of erythrocytes to glycolyze and survive in these circumstances implies extensive utilization of the Rapaport-Luebering shunt or of unknown metabolic pathways. The genetics are at present obscure. The mother of the patient has a mild, compensated hemolytic process with reticulocytosis of 7 to 10 percent and suggestively low activity of PGK. The possibility of X-chromosome linkage is under consideration but unproved.

Very recently Kraus and coworkers[38a] reported an apparently similar case, characterized by chronic hemolytic anemia and PGK deficiency most evident in older erythrocytes separated by density gradient techniques. The patient had no known living relatives and genetic data could not be obtained.

2,3-Diphosphoglycerate Mutase (2,3-DPGM) Deficiency:

Bowdler and Prankerd[108] have reported an inferred 2,3-DPGM deficiency in association with hereditary hemolytic anemia. The inference was based on finding about 50 percent normal 2,3-DPGM in erythrocytes, a failure of 2,3-DPG to increase when glycolysis was inhibited *in vitro* with sodium fluoride, and very small increases of 2,3-DPG after incubations with glucose and inosine. The latter two findings were in contrast to those observed with normal cells.

Löhr and Waller[39] made similar inferences in subjects with decreased 2,3-DPG content of erythrocytes. The mutase itself was not measured di-

rectly. The indirect measurements upon which inferences are based leads to some reservations as to the conclusive nature of the findings.

Schröter[41,42] presented more convincing observations in a child with very severe nonspherocytic hemolytic anemia who died at age 3 months. Curiously, though, despite the evidence of a fulminating hemolytic process, the reticulocyte count was only slightly elevated, and serum bilirubin only in the neighborhood of one milligram percent. The child required almost daily transfusions and donated cells were also destroyed at a phenomenal rate despite inability to demonstrate any extracorpuscular mode of destruction. The constant presence of many foreign, homologous red cells in the infant's blood prevented meaningful enzyme assays in the child. However, 2,3-DPGM activity of the order of 50 percent of normal was demonstrated by direct methods in both parents, a paternal sister and a grandmother of the propositus. The results suggest that the propositus was homozygous for 2,3-DPGM deficiency, while the phenotypically healthy parents and affected relatives were heterozygous. The peculiar lack of more than slight reticulocytosis or bilirubinemia, and the rapid destruction of transfused cells remain an enigma, however.

Discussion

The presence of highly specific erythrocyte enzymopathies, genetically transmitted as either autosomal recessive or X chromosome-linked traits in a wide variety of the comparatively uncommon lifelong hemolytic anemias lends strong support to a direct cause and effect relationship. This is rendered still more likely by the glycolysis-related nature of observed deficiencies and the established unique dependence of the metabolically impoverished erythrocytes upon the glycolytic machinery. Still, certain unresolved questions clearly exist. If the enzymopathies interfere with glucose metabolism, why do *in vitro* studies at times indicate reasonably adequate glycolysis and ATP maintenance in the cells of affected subjects? Why are sharp phenotypic differences observed in the clinical severity of hemolysis in persons in different families (and sometimes in the same kindred) in the presence of *in vitro* enzyme assays indicating comparably severe deficiency? What are the ultimate mechanisms of hemolysis in deficient erythrocytes?

The answers are at present imprecise and inadequate. First, though, it must be recognized that *a priori* only those reticulocytes and young erythrocytes still capable of survival in the patient are available for study, and all metabolic parameters measured are mean values for this cell population. The metabolic capabilities of these young erythrocytes cannot be projected to define the metabolic status which hypothetically would exist at some later stage of aging. Reticulocytes and young erythrocytes possess many metabolic advantages which are lost with increasing maturity. They are able to prime the metabolic pipeline with glycolytic subtrates much more effectively than can older cells as a result of the relatively great activity of many of their glycolytic enzymes and, particularly, but not exclusively, hexokinase.[5,34,35] The reticulocyte still possesses Krebs cycle activity, the capacity for oxidative phosphorylation and the ability to generate ATP by mechanisms other than those relating to the conversion of glucose to lactate.

The metabolic impairment inherent in a severe enzymopathy is, while severe, partial and never complete. Compensatory mechanisms may in young cells, then, still permit survival. But a form of selective metabolic progeria exists. Enzymatically, in a sense, such erythrocytes are old before their time, and normal survival is not to be expected.

To determine precise preludes to destruction requires knowledge of the metabolic parameters of the older, moribund cell. Unfortunately, for obvious reasons, these crucial measurements cannot be made. Further, the deleterious effects of accumulated intermediates by feedback mechanisms are inadequately known and currently difficult to ascertain. In addition, far too little is known about qualitative and kinetic differences in the catalytic proteins deficient in these hemolytic states. It is clear that genetic polymorphism is in some instances present, and that qualitatively different enzyme molecules can vary, not only in catalytic capacity in a given assay procedure, but also in susceptibility to denaturation with aging and in catalytic efficiency at different substrate concentrations. In most cases the available data are inadequate for assessment of these properties.

Technological difficulties of *in vitro* assays likewise exist. Since broken cells are employed, enzyme stability in dilute hemolysates and under preparatory conditions are factors in many in-

stances insufficiently explored. The glycolytic sequences are composed of interlocking and interdependent reactions. When a critical, rate-limiting threshold of activity for any given enzyme is approached, the phenotypic consequences are conditioned by all the other interrelated reactions of glycolysis. Variations within the so-called normal range, ordinarily negligible, may, in the critical threshold zone, determine the ability or inability of erythrocytes to maintain their integrity and survive at any given stage in their maturation. Precise *in vitro* assay activities may, then, not *per se* correlate well with *in vivo* erythrocyte life span.

The mechanisms resulting in enzymopathies may well be diverse. A given genic defect may result in the actual lack of synthesis of enzyme protein or, alternatively, in the production of a catalytically inefficient protein with or without aberrant kinetics. In the case of some forms of G-6-PD and PK deficiencies, it is clear that the production of qualitatively abnormal proteins is operative. In many other circumstances there is no valid information. The possibility of the presence of enzyme inhibitors or absence of activators is also present, though less likely, since no evidence for these has been obtained to date. Finally, the existence of many instances of apparently heritable hemolytic anemia, resembling those associated with known enzymopathies, but in which a point of metabolic inadequacy has not been yet ascertainable, renders it certain that other defined instances of molecular disease will emerge in future investigation.

Note: Bibliographical references have been selected particularly to indicate historical priorities and comprehensive studies, and to document specific points. The vast medical literature in certain areas covered precludes complete documentation; hence some significant contributions have been omitted. These are for the most part available in the total bibliographies of the individual references cited.

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