THE MAMMALIAN RED CELL is a miracle of evolutionary modeling. Designed for the sole task of transporting oxygen to the tissues and carbon dioxide to the lungs, it does so without either the expenditure or gain of energy. Hemoglobin constitutes 95% of its dry weight, and only 5% of red cell constituents other than water are left to provide a plasma membrane and to carry out the functions essential for survival. Devoid of a nucleus, organelles, or any capacity for protein synthesis or oxidative phosphorylation, the erythrocyte nonetheless circulates about 120 days. Despite its inability to renew its structural and enzyme proteins, it maintains its discoidal shape, pumps cations against electrochemical gradients, synthesizes glutathione, salvages adenine nucleotides, and protects its hemoglobin against methemoglobin formation and oxidative denaturation. Each crucial protein denatures in accordance with its own biologic half-life. Attrition of its limited resources ultimately marks it for destruction, and its graveyard is the phagocytic macrophage.

The red cell supports itself through the most primitive and universal pathway of metabolism, that of the conversion of glucose to pyruvate and lactate, and the storage of a portion of the energy generated in the form of adenosine triphosphate (ATP) (Fig. 1). In the simplest situation, the anaerobic metabolism of a mole of glucose results in the net formation of 2 moles of ATP. During the first half of glycolysis, energy is expended at the phosphorylation steps catalyzed by hexokinase (HK) and phosphofructokinase (PFK). But in midglycolysis, the 6-carbon glucose splits into two trioses, each of which passes through two ATP-generating steps.
These are catalyzed, respectively, by the enzymes phosphoglycerate kinase (PGK) and pyruvate kinase (PK). In the human red cell, energy generation is complicated by the Rapaport-Luebering shunt, as a result of which 2,3-diphosphoglycerate (2,3-DPG) is formed, while simultaneously the PGK reaction is bypassed. The 2,3-DPG has an important role in regulating the oxygen-dissociation curve of hemoglobin and also provides a reservoir of triose. ATP production is modulated in accordance with body needs, and depending on the amount of glucose traversing the shunt, anaerobic glycolysis results in a net gain of somewhere between 0 and 2 moles of ATP for every mole of glucose metabolized. Also, in midglycolysis, the formation of reduced nicotinamide adenine dinucleotide (NADH) provides the obligate cofactor for methemoglobin reductase, the enzyme catalyzing the conversion of methemoglobin to functional hemoglobin. NADH is reoxidized to NAD at the terminal step in the formation of lactate.

Another metabolic sequence also available to the red cell is the aerobic, pentosephosphate (PP) shunt. The PP shunt is not concerned with ATP generation, but rather with the production of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and the formation of 5-carbon sugars from 6-carbon glucose. Its products can be returned to the mainstream of glycolysis, so that glucose metabolized via the PP shunt, and that metabolized anaerobically, ultimately travel the same common final route to lactate. Hemoglobin is subject to denaturation by noxious oxidants such as peroxides. Its chief defense against this threat resides in
glutathione (GSH), which is itself oxidized in the process of participating enzymatically in the conversion of peroxide to harmless H₂O. The percentage of glucose traversing the PP shunt normally is directly related to the rate of oxidation of GSH, and this, in turn, varies widely depending on the oxidant stresses to which the cell is exposed. The function of the shunt depends on the integrity of the mechanisms by which GSH is synthesized, by which it reacts enzymatically with peroxide, and by which it is reduced. The enzymatic reduction of oxidized glutathione requires the NADPH generated in the red cell solely by the dehydrogenases of the PP shunt. In syndromes associated with dysfunction of the Embden-Meyerhof pathway, failure of energy generation is believed fundamental in the pathogenesis of hemolysis; in PP shunt failure, oxidative denaturation of hemoglobin is the major contributor to the hemolytic process.

The recognition that genetically determined red cell enzyme deficiencies could, when severe, be responsible for certain hemolytic syndromes historically evolved from two chief sources. First was the investigation of hemolytic episodes associated with the administration of the antimalarial primaquine, and second, the study of so called “nonspherocytic” hemolytic anemias by Dacie and his colleagues in England. The G6PD story is reviewed in a recent article by Beutler. Suffice it only to say that X-chromosome-linked G6PD deficiency is responsible for an intrinsic defect of the erythrocyte associated with “primaquine sensitivity,” with glutathione instability, with abnormal Heinz body formation in the presence of oxidant drugs, with susceptibility in some instances to favism, and with hemolytic episodes when certain medications other than primaquine are administered. It is not one disease due to a single muted protein, but a host of related disorders having in common a mutant gene at the G6PD locus. A variety of much rarer inborn errors affecting the synthesis of GSH and its enzymatic oxidation and reduction have more recently been defined. These share with G6PD deficiency a hemolytic syndrome with a prominent component of oxidative denaturation of hemoglobin exacerbated by the administration of medications causing hemolysis in G6PD-deficient subjects.

The studies of Dacie, supported by those of Robinson and de Gruchy et al. in Australia, suggested that certain obscure hemolytic syndromes might be associated with impaired red cell glycolysis. The first enzyme deficiency of anaerobic glycolysis incriminated in the pathogenesis of hemolysis, pyruvate kinase (PK), was described by our laboratory in 1961. PK deficiency can serve as a prototype for discussing glycolytic enzymopathies associated with hemolytic anemia. Special features of other deficiencies of enzymes of the Embden–Meyerhof pathway can then be explored briefly. Finally, certain inborn errors of red cell nucleotide metabolism are also associated with hemolytic syndromes. PK catalyzes the reaction:

\[
\text{PK} \quad \text{Phosphoenolpyruvate} + \text{ADP} \rightarrow \text{Pyruvate} + \text{ATP}
\]

PK deficiency, while comparatively rare, is second only to that of G6PD as a cause of hemolytic anemia due to defective red cell enzymes. Anemia is normally moderately severe, but varies considerably. In the newborn, the accompanying jaundice at times necessitates exchange transfusion. Transfusions may or may not be required throughout life. The spleen is enlarged, and its removal may result in some partial improvement in severe cases. The reticulocyte percentage is substan-
tially increased and rises even higher if the spleen is removed. Anemia becomes temporarily more severe at times of infection or other stress, such as surgery. The rapid red cell destruction results in an increased incidence of pigment gallstones developing at an early age, as is the case in all chronic, ongoing hemolytic anemias.

Red cell PK activity in patients first investigated was found to be only a small fraction of that normally present. Further, asymptomatic parents of affected subjects and certain other family members, although clinically and hematologically normal, had biochemically detectable, partial deficiencies of PK activity, averaging about one-half that expected. This pointed to a non-sex-linked type of inheritance, in which a defective gene was inherited from each of both parents. With rare exceptions, subjects heterozygous for PK deficiency have no clinical or hematologic abnormalities. Half-normal PK activity usually permits normal red cell survival; 10% or 20% may not. Further, in PK-deficient subjects, it is often possible to demonstrate other biochemical red cell abnormalities of a secondary nature: ATP concentrations may be subnormal; the production of lactic acid from glucose in vitro may be less than expected; an abnormal accumulation of glycolytic intermediates develops upstream from the point of metabolic block, and this includes as its most prominent feature a profound increase in the concentration of 2,3-DPG.

PK deficiency is not associated with clinical abnormalities other than hemolysis, though PK is required by all tissues utilizing the Embden–Meyerhof pathway. At least three separate enzyme proteins (isozymes) are believed to catalyze the PK reaction, and these are usually designated M1, M2, and L. The liver possesses both the L and the M2 isozymes. For practical purposes, the red cell has only a single isozyme, which is closely related to the L protein of liver. The same gene may code for both liver and erythrocyte PK, which are subsequently posttranslationally modified in slightly different ways by intracellular proteolytic activity. The posttranslational modification of peptide chains by proteolysis is increasingly being recognized as a mechanism for molecular evolution. The erythrocyte and liver enzyme are immunologically identical, though their molecular weights and electrophoretic mobilities differ modestly. The liver, indeed, shares the deficiency when red cell PK is defective, but it also possesses a redundant system—the M2 isozyme—which can fill the breach when single gene failure occurs. Muscle PK, M1, is immunologically and otherwise distinct from the L-related isozyme found in red cells and in liver, while the PK isozyme of leukocytes is M2.

PK deficiency is a family of disorders whose members differ clinically, prognostically, hematologically, and in the properties of the muted enzyme protein. The enormous polymorphism of the human gene pool permits mutant genes for PK to abound, but to remain concealed in phenotypically normal heterozygotes, unrecognized until, by chance, they are clinically expressed as a hemolytic syndrome in a subject unfortunate enough to have inherited a defective gene from both his parents. Thus, in the absence of consanguinity, most subjects with hemolytic anemia secondary to PK deficiency, are doubly heterozygous for two separate mutant genes. The failure to recognize the diversity of PK mutants led to considerable puzzlement in early studies where only severe quantitative deficiency in PK activity was appreciated. A large number of mutant proteins are now characterized. These are separable in terms of catalytic activity, kinetic heterogeneity, variable isoelectric points and pH optima, electrophoretic polymorphism, variations in stability, altered behavior toward nucleotide cofactors and allosteric
modifiers, and in other ways. The majority, if not indeed the totality, of hereditary PK deficiencies now must be considered as being due to structurally mutated enzyme proteins, whose detailed characterization requires the application of a battery of sophisticated methods.

Figure 2 illustrates data from the study of a patient in whom double heterozygosity for mutant PK genes was associated with a severe hemolytic syndrome. Normally, maximal red cell PK activity is achieved at very low concentrations of substrate phosphoenol pyruvate (PEP). In the red cells of the proband in this kindred, substantial PK activity was demonstrable at unphysiologically high PEP concentrations often employed in assay procedures. But, the mutant enzyme required tenfold more substrate to attain half-maximal activity than did normal PK. The kinetics of PK activity in the red cells of the mother and certain maternal relatives who were clinically well exhibited aberrations intermediate in severity between normal PK and those observed in the patient. From the mother, there had been inherited a gene coding for a PK enzyme rendered catalytically ineffective by kinetic abnormalities; from the father, a separate mutant gene, resulting in a severe quantitative lack of PK activity at all PEP concentrations, had been inherited. The red cells of the patient, doubly heterozygous for two separate mutant genes, resulting in two separate abnormalities in PK, were profoundly catalytically defective at PEP concentrations attainable in the body.

While the precise mechanism of hemolysis associated with severe deficiencies of the red cell enzymes of anaerobic glycolysis cannot be stated with certainty, we believe that the common denominator is failure of energy metabolism. For example, PK ordinarily functions at substrate levels that are not saturating. When its activity for any reason decreases to the point where it serves as an impediment to the normal smooth flow of glycolytic intermediates, substrate concentrations must rise. When the highest achievable PK activity is still associated with metabolic blockade, maintenance of ATP concentrations inevitably begins to be impaired. This must occur at some point between zero PK activity, where glycolysis would shortly cease, and some low-level minimum activity capable of supporting the necessary flow of glycolytic intermediates from glucose to lactate. Further, a threshold effect is implied. Up to some critical point, ATP levels are maintained by compensatory increases in substrate; beyond that point, metabolic depletion should inevitably ensue. A cell population in the vigor of youth could briefly sustain
reasonable levels of ATP, and only a relatively small subpopulation of metabolically depleted cells, shortly to be destroyed, would be present at any one moment in time. It is precisely our inability to measure the metabolic events in the dying cell that creates a dilemma. We measure fundamentally the mean activities of the population that is transiently able to compensate for the metabolic handicap.

Acquired erythrocyte enzyme deficiencies involving PK, glucosephosphate isomerase (GPI), and phosphofructokinase (PFK), have now been described in certain dyserythropoietic syndromes, including preleukemia and sideroblastic and other refractory anemias with cellular marrows. In some instances, the altered erythropoiesis is associated with diminished or abnormal production of the enzyme protein; in others, the deficiency is posttranslational and reversible by dialysis, by treatment with reagents reducing sulfhydryl groups, or by partial purification of erythrocyte enzyme. In the latter groups, postsynthetic modification of enzyme protein by small molecules occurring abnormally in plasma or perhaps intracellularly has been postulated.

A number of rarer deficiencies involving enzymes of the Embden-Meyerhof pathway are now well defined (Fig. 1). When severe, all are associated with hemolytic syndromes with one exception. Severe deficiency of lactate dehydrogenase (LDH) has been described in a Japanese kindred in the absence of hemolytic anemia or other clinical manifestations. In part, this reflects the ability of both pyruvate and lactate to diffuse from the red cell and be excreted or metabolized elsewhere. While it might have been suspected that inability to reoxidize NADH via LDH might seriously disrupt the delicate glucolytic machinery, apparently other mechanisms of NADH oxidation are sufficiently compensatory. In addition, Rosa and colleagues have recently described a subject whose red cells lacked more than traces of 2,3-DPG. Both 2,3-DPG mutase and 2,3-DPG phosphatase activities were undetectable, confirming that both activities are dependent on a single protein. Hemolysis was absent, but there was moderate polycythemia secondary to the unfavorable shift in the oxygen dissociation curve of hemoglobin occasioned by lack of 2,3-DPG.

Hexokinase (HK) catalyzes the reaction:

\[
\text{Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{Glucose-6-phosphate} + \text{ADP}
\]

HK activity is very cell-age dependent, and characteristically, is increased several fold in erythrocyte populations of young mean cell age. HK activity in the cells of severely deficient patients is even less than that of normal cells. So, on the HK scale of aging, patient reticulocytes are old before their time. In metabolic terms, red cell progeria is present, and the prematurely senescent red cells are marked for premature destruction. Despite the reticulocytosis, glucose and fructose are phosphorylated at rates below that of normal blood, but mannose, which is phosphorylated by a separate kinase, and both glucose and fructose-6-phosphates are metabolized at high rates comparable to other young erythrocyte preparations.

Some red cell enzymopathies have clinical expression in tissues other than the erythrocyte. Triosephosphate isomerase (TPI) catalyzes the reaction:

\[
\text{Dihydroxyacetone phosphate (DHAP)} \xleftarrow{\text{TPI}} \text{Glyceraldehyde-3-phosphate (G3P)}
\]
While hemolytic anemia is present when TPI deficiency is severe, the severe neurologic deficit usually leading to death in childhood is far more ominous. Some TPI-deficient subjects have expired inexplicably, probably from cardiac arrests or arrhythmias. The enzyme activity is very low in plasma, in spinal fluid, leukocytes, skeletal muscle, and probably in all the body tissues. In the highly consanguineous kindred in which TPI deficiency was originally identified, three genetically determined abnormalities—heterozygosity for hemoglobin S (HbS), G6PD deficiency, and both hetero- and homozygosity for TPI deficiency—coexisted in various permutations. Homozygosity for TPI deficiency was by far the most serious and was associated with early death.

Phosphoglycerate kinase (PGK) deficiency is characterized by both hemolytic anemia and by an atypical neurologic syndrome. It alone of the Embden-Meyerhof pathway enzymopathies is X-chromosome linked. Males have full expression of the disorder, while females possess a cellular mosaic—one population of normal red cells and one just as deficient as those of hemizygous males. When erythrocytes are separated on the basis of density, the older cells are the most dense. Enzyme activities decline variably as the red cell ages and is consigned to a denser population. However, in female patients heterozygous for PGK deficiency, there is a paradoxical rise in activity in the older denser population. The reason lies in the mosaicism. The less dense, reticulocyte-rich fraction of patient cells contains many young erythrocytes contributing virtually no PGK activity. These do not survive to be numbered in the more dense population, thus creating the illusion that the denser cells have actually augmented their PGK activity.

The human red cell also possesses enzymatic machinery other than that of glycolysis. The maturing reticulocyte requires a mechanism for ridding itself of organelles and nucleic acids. When RNA is degraded, the end-products are ribonucleotides containing the purines, adenine and guanine, and the pyrimidines, cytosine and uracil. The pyrimidine-containing nucleotides are now unwanted for they can compete with preferred ATP and ADP in crucial reactions where they are highly inefficient. However, as long as they are phosphorylated, they cannot diffuse through the cell membrane. Nature has therefore provided the red cell with a specific enzyme, a pyrimidine-5'-nucleotidase, capable of dephosphorylating the ribonucleotides of cytidine and uridine. Once dephosphorylated, the resulting nucleosides are free to diffuse from the red cell and be metabolized or excreted elsewhere.

Hemolytic anemia secondary to hereditary nucleotidase deficiency and transmitted in an autosomal recessive manner has now been identified in several kindreds. One of its morphologic hallmarks is large numbers of red cells with prominent basophilic stippling on the stained blood film (Fig. 3). Under the electron microscope, stippling appears to be due to aggregations of undegraded or partially degraded ribosomes. In the near-absence of nucleotidase activity, the accumulated ribonucleotides retard the rate of ribosomal degradation. The morphologic counterpart of this phenomenon is the conspicuous basophilic stippling evident on the stained blood film. A second easily identifiable hallmark of the syndrome can serve as a screening test for nucleotidase deficiency. The ultraviolet spectra of deproteinized extracts of normal cells displays an absorption peak at about 258 nm, corresponding to that of adenine nucleotides. The extracts of nucleotidase-deficient red cells possess a several fold greater total nucleotide
Fig. 3. Basophilic stippling on stained blood film of subject with red cell pyrimidine-5’-nucleotidase deficiency.

content than those of either normal or reticulocyte-rich blood. Moreover, the peak absorption is not at 258–260 nm, but shifted to the neighborhood of 270 nm. When chromatographed, about 80% of the ribonucleotides contain cytidine and uridine. These are virtually undetectable in normal erythrocytes. Although the precise mechanism of hemolysis is not known, pyrimidine nucleotides can compete with the far more effectual adenine nucleotides in crucial reactions, such as those catalyzed by HK, PGK, and PK. Hence, they are a potential monkey wrench in the delicately geared glycolytic machinery upon which the red cell is so dependent. Knowledge as to all the mechanisms by which the pyrimidine ribonucleotides may influence metabolic events is, however, incomplete.

The similarity between the conspicuous basophilic stippling seen in some cases of severe lead intoxication and that routinely observed in severe nucleotidase deficiency, suggested that red cell nucleotidase might be inhibited by lead. The enzyme was, indeed, exquisitely sensitive in vitro, $10^{-5}\, M$ lead producing 50% inhibition. In 15 subjects with documented lead overburden, erythrocyte nucleotidase activity was reduced in all. In none of the first subjects studied was nucleotidase deficiency of such severity as to be associated with the unique intracellular accumulations of pyrimidine ribonucleotides. However, in 10 additional cases of more severe lead intoxication, substantial accumulations of pyrimidine nucleotides were demonstrable. When lead concentrations approach 200 µg/dl of packed red cells, lead-induced nucleotidase deficiency mimics in all essential respects the genetically determined lesion (Fig. 4).
An alternate type of inherited enzymopathy, one in which there is an excess of an enzyme activity, can also result in hemolytic anemia in man. Our laboratory has investigated a large kindred in which hemolytic anemia is inherited as a mendelian dominant trait in 12 of 24 family members spanning 3 generations. Red cell ATP concentrations are only about half those expected, and this poverty of ATP is believed causative for the disorder. In contrast to the entirely normal activity of adenosine deaminase (ADA) in unaffected family members, the red cells of subjects with hemolysis contain 45–70 times the ADA activity of normal erythrocytes (Fig. 5).

Unlike the nucleated cells of the body, the red cell cannot synthesize the adenine ribonucleotides de novo from small precursor molecules. The maintenance of the...
adenosine phosphate pool therefore depends on the availability of salvage pathways. One of these involves adenosine, which on phosphorylation, becomes AMP, which is convertible in the cell to ADP and ATP. Small amounts of adenosine are formed in various tissues and diffuse into the plasma where they are available to the free-floating red cell. However, adenosine is the substrate for two competing red cell enzymes. Adenosine kinase (ADK) phosphorylates adenosine and provides a viable mechanism for replenishing the crucial adenosine phosphate pool. The ADA converts adenosine to inosine, rendering it no longer a precursor of adenine nucleotides. When enormously increased ADA activity is present, most adenosine is deaminated and very little can be salvaged by ADK for renewal of the adenine nucleotide pool. The diminished red cell ATP, on reaching crucially low concentrations, no longer can support viability. Kinetically, electrophoretically, and in terms of its reaction with inhibitors, the increased ADA activity in this syndrome appears derived from simple overproduction of a normal enzyme. Dr. William Osborne of the University of Washington has purified the enzyme in this kindred to homogeneity. By all criteria thus far applied, including its specific activity, no abnormalities are demonstrable. The inherited lesion, therefore, appears to involve regulation of production of this specific enzyme protein, but its molecular and genetic basis is still a mystery. Interestingly, the ADA activity of cultured skin fibroblasts and the leukocytes is comparable to that of normal controls. The converse situation, ADA deficiency, is associated with severe immunologic incompetence and not with hemolytic anemia. In the contrasting ADA deficiency, ATP and deoxy ATP are increased. Here, ADK acts virtually unopposed.

While certain obscure hemolytic and neurologic syndromes can now be removed from a diagnostic wastebasket and firmly defined at a molecular level, perhaps there are more basic rewards to be derived from experiments of nature involving genetically determined lesions of a single molecular species. Such experiments are impossible to reproduce in pure form in the laboratory. They permit insight into the effects of molecular sequences disturbed at a single point and into the often unforeseen and unpredictable negative and positive feedback and feedforward mechanisms that fine tune metabolism. New gene markers have been identified. The enzymopathies provide testimony to the profound polymorphism of the human gene pool and support the somewhat oversimplified thesis that evolution in the last analysis is largely the substitution of one amino acid for another. Certain idiosyncratic reactions, formerly considered “allergic” or on an immunologic basis, are now known to be dependent on a deficiency of a single mutant protein. Ingenious use of knowledge concerning mutant enzymes and isozymes has in some instances permitted confirmation of the clonal origin of certain neoplastic and non-neoplastic disorders. Recognition that the red cell is a fertile source of biopsy material has enhanced the range of inborn errors readily diagnosed by simple and safe procedures. Freely floating, capable of being biopsied repeatedly and safely by venepuncture, easily separated in pure culture from the other components of blood, the red cell mirrors aberrations not only affecting its own integrity but also impairing the function of other body tissues. Galactosemia and actalasemia, for example, are readily diagnosed by hemolysate assays of the enzymes whose deficiency is causative. Yet neither disorder appreciably affects the red cell itself. There are many other examples.
In closing, it may be predicted that additional disclosures concerning the molecular basis of human disease will be forthcoming from this cellular nomad who, metabolically speaking, starts with little and ends with less. Under normal circumstances, its life of bartering oxygen and carbon dioxide is remarkably successful; in the presence of genetically determined handicaps, its best is none too good.

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The Stratton Lecture. Hemolytic anemia and inborn errors of metabolism

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