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THE HISTORY OF G-6-PD DEFICIENCY

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The discovery of glucose-6-phosphate dehydrogenase (G-6-PD) deficiency was the outgrowth of studies of drug-induced hemolytic anemia. In 1926 it was found that the new 6-methoxy-8-aminoquinoline antimalarial, primaquine, produced acute hemolytic anemia in some subjects. During World War II primaquine, a more effective analog of this compound, was synthesized. This drug, too, had the capacity to produce hemolytic anemia. Using cross-transfusion studies with chromium 51 it was demonstrated that sensitivity to the hemolytic effect of primaquine was due to an intrinsic defect of the erythrocyte. The hemolytic anemia induced by this drug was found to be self-limited, because only the older members of the red cell population were destroyed. Primaquine-sensitive erythrocytes were found to be sensitive also to a variety of other drugs including certain sulfonamides and acetanilid. However, they were not destroyed by the administration of quinine, chloroquine, and many other sulfonamides. Primaquine-sensitive red cells displayed a different pattern of Heinz bodies formation than did normal cells when incubated with acetylphenylhydrazine. Inhibition of red cell metabolism with arsenite and iodoacetate caused normal cells to behave like primaquine-sensitive cells with respect to Heinz body formation. This led to investigation of the glutathione (GSH) content of red cells, and the concentration of this tripeptide was found to be diminished. Further studies revealed that the GSH of primaquine-sensitive red cells was unstable when they were incubated with acetylphenylhydrazine, and this finding led to elucidation of the primary defect as G-6-PD deficiency. In the few years subsequent to 1956, when the enzyme deficiency was first identified, it was recognized that G-6-PD deficiency was a disorder with a world-wide distribution. It became apparent that in Negro populations the enzyme deficiency was relatively mild, while it was much more severe in Mediterranean populations. Moreover, the electrophoretic mobility of the red cell enzyme was found to differ in different individuals. Study of the genetics of glucose-6-phosphate dehydrogenase also led to the development of the X-inactivation hypothesis. Glucose-6-phosphate dehydrogenase variants have been used for genetic studies and in tracing the origin of tumors.

Glucose-6-phosphate dehydrogenase deficiency represents an example of a situation in which the study of a clinical disorder led to important basic biologic information.

G6PD AS A TOOL FOR GENETIC RESEARCH

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The discovery that G6PD deficiency is the primary defect in individuals with primaquinsensitivity and/or clinical favism has been an invaluable gift for students of human inheritance.

Through extensive population and pedigree studies it was soon established that the structural gene for this enzyme is X-linked in all mammalian species so far studied and highly polymorphic in humans as well as in marsupials. The human population studies established beyond doubt that at least two deficient mutants at this locus confer to their carriers a higher biological fitness in malarial environment. In populations with a high frequency of G6PD deficiency of mediterranean type (such as Sardinia, Israel and Greece) it has been possible to use this marker for establishing the linkage relationship between the G6PD locus and many other X-linked loci such as those for deutan and protan colorblindness, Xg(a) red-cell antigen, muscular distrophy of Duchenne and Becker type, X-linked ichtyosis, ectodermal dysplasia anydrotica, hemophilia A and B. These studies led to the discovery of three very tight linkages: G6PD-deutan colorblindness, G6PD-protan colorblindness and G6PD-hemophilia A. The latter one is a linkage of medical interest, since it makes possible the detection of silent carriers of hemophilia A (most of the time impossible to classify on the basis of the sole factor VIII deficiency) in families which happen to segregate for both traits. Other population studies in Sardinia made use of the G6PD polymorphism to detect linkage disequilibrium and to estimate the frequency of X-chromosome non disjunction among parents of XXY Klinefelter patients.

To date, over a hundred mutants have been detected at the G6PD locus and for a few of them it has been demonstrated that the primary structure of the mutant polypeptide chain differs from that of the wild type in terms of a single aminoacid substitution. One of the most valuable contributions of G6PD as a tool for genetic analysis has been the demonstration that somatic tissues of heterozygotes at this locus are "mosaic", but that clones of fibroblasts derived from such individuals exhibit only one or the other of the two allelic gene products. The latter finding gave the first unequi-

vocal experimental proof in favor of the so-called Lyon-Russel-Beutler hypothesis on the random and irreversible inactivation of the X-chromosome in early embryos of normal females as well as of individuals with multiples X's. These studies were also utilized to prove the monoclonal origin of certain tumors by showing that the malignant tissues arisen in G6PD heterozygotes exhibit only one of the two allelic products. One of the latest and most rewarding applications of G6PD as a tool for genetic research is related to its use as cellular marker in linkage and complementation studies with somatic cell hybrids. The rationale for these studies is well known. Murine and human somatic cells can be fused with the help of inactivated Sendai virus and/or exposure to polyethylene glycol. The resulting hybrids undergo the progressive and preferential loss of human chromosomes. Thus, human genes can be mapped by correlating the retention or loss of human genetic markers with that of the corresponding human chromosomes. Since the X-linkage of G6PD was already established beyond doubt through pedigree analysis, this marker was the first to have been used to demonstrate the X-linkage of other human inborn errors of metabolism (such as HGPRT deficiency, PGK deficiency and alpha-galactosidase deficiency) whose X-linked pattern of inheritance was suspected but not definitely proven at the time.

Variation in the degree of somatic mosaicism in different tissues of G6PD heterozygotes has been used to estimate the size of the primordial cell pool at the time of inactivation and to spot the occurrence of somatic selection in vivo.

In turn, somatic cell genetics has proven to be a powerful tool to gather further knowledge on the genetics and biochemistry of G6PD. For instance, by using rodent human somatic cell hybrids derived from human parental cells with X-autosomal translocations, it has been possible to assign the structural locus for G6PD to the subterminal region of the long arm of the human X-chromosome. Other rodent human hybrids derived from the fusion of G6PD normal rodent cells with G6PD deficient human cells have shown the formation of normally active heteropolymeric G6PD molecules, thus indicating the potentialities of somatic cell hybridization for a novel approach to the study of the molecular pathology of the G6PD molecule via complementation analysis.

POSTTRANSLATIONAL ALTERATIONS OF NORMAL AND MUTANT G6PD MOLECULES

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Once synthesized an enzymatic protein can undergo a series of modifications varying in nature and consequences.

Sometimes these postsynthetic events are indispensable in the transformation of an inactive proenzyme into a catalytically efficient enzyme. In other cases the posttranslational modifications are mainly involved in the phenomena of molecular aging, leading ultimately to inactivation and degradation of the enzyme proteins. The modifications of G6PD in various tissues belong rather to the latter group, the enzyme being initially synthesized in a fully active form.

Two different phenomena must be distinguished:

.- a partial proteolysis of the C terminal end of the enzyme, responsible for the appearance of the erythrocyte type G6PD, and,

.- a likely covalent linkage between G6PD and a degradation product of the coenzyme $NADP^+$, responsible for the transformation into the "so called" hyperanodic forms.

The C terminal sequence of native G6PD purified from leukocytes or platelets is "Asp-Ala-Gly-Ser-Leu-Lys-Leu.

In the red cells a partial hydrolysis occurs, at the level of the glycine, serine or leucine residue. This proteolysis of the C terminal end of the enzyme is not associated with either diminished activity or altered kinetics of G6PD.

The mechanisms of the second phenomenon is totally different:

pure glucose-6-phosphate dehydrogenase is transformed into "hyperanodic forms" when incubated at acidic pH and in the presence of $NADP^+$ with excess of glucose-6-phosphate or with some "$NADP^+$ modifying proteins" purified from the same cells.

The enzyme hyperanodic forms exhibit a low isoelectric point, altered kinetic properties and high lability to heat, urea, and proteolysis. Differences between hyperanodic and native forms of glucose-6-phosphate dehydrogenase are also noted by microcomplement fixation analysis, UV absor-

in the oxidation of NADPH. This can be demonstrated in conditions of 2,3 DPG breakdown, where the pyruvate formed serves as oxidant.

2) Oxidative insults

A variety of reactive chemical species such as $O_2H^{\bullet}$, $O^{\bullet}$, H_2O_2 and organic radicals have been implicated as cause of oxidative stress. Neither the reactive species nor the mechanisms of damage or for the defense against such insults have been clarified so far. Our knowledge is greatest concerning the reactions removing H_2O_2, in which both glutathioneperoxidase and catalase participate. There are two misconceptions concerning them: a) an overestimation of the relative importance of glutathioneperoxidase, which actually removes only 1/4 of the H_2O_2, and b) an overemphasis of the peroxidative reaction of catalase, which accounts for only 1/10 of total catalase activity. The powerful activity of the enzymes destroying H_2O_2 makes it very unlikely that H_2O_2 may be highly significant as damaging agent. Although that a rise of formate concentration cannot increase the rate of removal of H_2O_2 there are nevertheless astounding effects: the formation of Heinz bodies is reduced, the maintenance of GSH concentration is improved and the oxidative pentose phosphate pathway is inhibited. It would appear worthwhile to attempt a medication with formate - a harmless substance - in the prevention of hemolytic crises.

ROLE OF REACTIVE OXYGEN DERIVATIVES AND RELATED ENZYMES IN THE METABOLIC BALANCE OF REDOX INTERMEDIATES IN THE RED BLOOD CELL

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Electron donation to molecular oxygen is involved in several biochemical processes in aerobic organisms, such as energy production in mitochondria, chemical modification of metabolites by oxydases and oxygenases, and, in a wider sense, oxygen transport by respiratory proteins. In all these circumstances a variety of intermediates of oxygen reduction are expected on purely chemical grounds, according to the following simplified scheme:

$O_2 + 1\ e^- \longrightarrow O_2^-$ (superoxide)
$O_2^- + 1\ e^- \longrightarrow H_2O_2$ (hydrogen peroxide)
$H_2O_2 + 1\ e^- \longrightarrow OH$ (hydroxyl radical)
$OH + 1\ e^- \longrightarrow H_2O$ (water)

Among these derivatives H_2O is the most stable and unreactive, and all the system is driven toward its accumulation. O_2^- and H_2O_2 can be utilized in the cell by oxygenases and peroxidases with eventual formation of more H_2O, or are dismuted by superoxide dismutases and catalases, which again yield H_2O as a product.

However some of these intermediates can react with cellular components by electron abstraction, leading to oxidative damage of the cell. Aerobic cells contain enzymes and scavengers to counteract these damaging reactions. The balance of production and disappearance of reactive intermediates thus depends on both the extent of their output from any possible source and the activity and concentration of the defense systems.

In the case of red blood cells, the chain of reactive intermediates outlined in the above scheme is likely to originate from the process of oxygen binding to Hb. The chain-initiating species is liberated from Oxy Hb ($Hb^{++}\ O_2$), leaving Met Hb (Hb^{+++}) according to three possible mechanisms:

1) dissociation of O_2^- from $Hb^{+++}\ O_2^-$ which is considered by many authors to be actual electronic state of $Hb^{++}\ O_2$ (1);

2) polarization of the $Hb^{++}\ O_2$ complex and subsequent displacement of O_2^- from the resulting $Hb^{+++}O_2^-$ by anions, such as Cl^- (2);

3) reduction of $Hb^{++}\ O_2$ by various reagents (generally referred to as drugs: phenols, nitrites, 8-aminoquiloniles, phenylhydrazines etc.), with generation of H_2O_2 and subsequently O_2^- (3):

$$Hb^{++}O_2 + DH \longrightarrow Hb^{+++} + HO_2^- + D^\bullet$$

$$D^\bullet + O_2 \longrightarrow D + O_2^-$$

4) oxidation of $Hb^{++}\ O_2$ by trace metals with production of O_2^- (4):

$$Hb^{++}O_2 + Cu^{++} \longrightarrow Hb^{+++} + O_2 + Cu^+$$

$$Cu^+ + O_2 \longrightarrow Cu^{++} + O_2^-$$

According to these mechanisms, H_2O_2 and in most cases O_2^- are the primary derivatives arising from reactions of Hb O_2. Further potentially harmful species are produced by secondary reactions, namely:

$$O_2^- + O_2^- + 2H^+ \longrightarrow {}^1O_2 + H_2O_2 \qquad (a)$$

$$H_2O_2 + H_2O_2 \longrightarrow {}^1O_2 + 2H_2O \qquad (b)$$

$$O_2^- + H_2O_2 \longrightarrow {}^1O_2 + OH^- + OH^\bullet \qquad (c)$$

These reactions are all electron exchange reactions between intermediates of oxygen reduction and yield very powerful oxidizing molecules such as singlet oxygen ($^1O_2$) and hydroxyl radical ($OH^\bullet$). However they are slow in the absence of catalysts. In particular reaction (a) requires H^+ and reaction (b) and (c) are catalyzed by metal ions. At physiological pH, reaction (a) is relatively slow and it is likely that in the erythrocyte all O_2^- would enter either reaction (c) since metal catalysts are ubiquitous in cells, or would react with $Hb^{++}\ O_2$ yielding more Hb^{+++}(5). H_2O_2 also would undergo reactions (b) and (c), beside oxidation of Hb O_2, as plausible pathways under the conditions likely to exist in the red blood cell. This picture is dramatically modified by the presence in the red blood cell of enzymes that greatly enhance the rate of reactions (a) and (b), i.e. superoxide dismutase and hydrogen peroxide dismutase (better known as catalase). In the presence of such dismutases the concentration of O_2^- and H_2O_2 drops, so that reaction (c) is practically absent. Moreover the dismutases prevent formation of $^1O_2$ in both reactions (a) and (b) (6). The combined action of the two dismutases seems therefore to be an effective defense against oxidizing molecules arising from $Hb^{++}\ O_2$, as both enzymes appear to be localized in close contact with hemoglobin (7). A third defense enzyme, glutathione peroxidase, is on the other hand bound to the red cell membrane and appears to be specialized in the reduction of organic peroxides already formed in the membrane by oxidants that escape all the defense systems inside the cell (7). However it should have a role in the scavenging of

H_2O_2 itself in cases of acatalasia, as the diffusion rate of H_2O_2 is sufficiently high to allow fast reaction even with a membrane - bound enzyme.

It could be concluded that most of recent work in the field has been devoted to define the chemical reactivity of the various reactive oxygen derivatives, the effect of specific catalysts on their life time, and the role of hemoglobin and drugs as a source of oxidizing oxygen species. However a definitive demonstration of a role of these derivatives in producing membrane lipid peroxidation and hemolysis in physiological conditions is not yet available. Their interactions with vitamin E as a membrane antioxidant, though supported by many facts, are still waiting for a description at the molecular level.

On the other hand in vitro and in vivo evidence seems to support an actual role of superoxide dismutase and glutathione peroxidase in balancing the production of reactive oxygen derivatives from oxy Hb (8-11).

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RELATIONSHIP BETWEEN ENZYME PROPERTIES AND THE CLINICAL EXPRESSION IN GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PD) DEFICIENCY

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More than 100 variants of G6PD have been reported, which are distinguishable from one another by their kinetic characteristics, electrophoretic mobilities and substrate specificities. About 40 variants have normal activity or mild enzyme deficiency in red cells and therefore are not associated with any clinical manifestations. Another group of variants causes severe enzyme deficiency in red cells, but requires exogenous agents such as drugs, infections or fava beans for hemolysis to occur. Other variants are associated with chronic nonspherocytic hemolytic anemia even in the absence of exogenous agents.

Many investigations have shown that the degree of enzyme deficiency measured in hemolysates under optimal conditions does not correlate well with the clinical severity of the disease. Kinetic characteristics for the substrate and coenzyme of the variant enzymes cannot fully explain the reasons for the different hemolytic manifestations in the affected subjects. Therefore, other properties of the abnormal enzymes and the "physiologic" activity of G6PD within the intact abnormal red blood cells have to be elucidated. We can demonstrate the results in this respect on five newly characterized G6PD variants which we found recently in Germany and Switzerland. The biochemical characterization of the variants was performed on partially purified G6PD preparations according to the recommendations of the WHO scientific group. For characterization the enzymatic activity in hemolysates, the Michaelis-Menten constants for glucose-6-phosphate and NADP, the inhibition constant for NADPH, the utilization of the substrate analogues for 2-desoxyglucose-6-phosphate, galactose-6-phosphate and deamino NADP, the pH activity curves, the thermal stability and the electrophoretic mobility were used.

The clinical severity of the five variants increased in the following order: G6PD Hamburg, G6PD Aarau, G6PD Hamm, G6PD Tarsus and G6PD Bielefeld. No correlation of the clinical picture and the residual enzyme activity was

recognizable. Between the kinetic properties of the variant enzymes and the clinical severity some parallels became obvious. The variants with severe hemolytic anemia exhibited unfavourable kinetic parameters and low thermal stability.

An attempt was made to measure G6PD activity under simulated physiologic conditions according to YOSHIDA. These experiments demonstrated that variants with severe hemolytic anemia (G6PD Hamburg and Aarau) exibit very low activities under "physiologic" conditions whereas variants associated with a mild clinical course (G6PD Bielefeld, Hamm and Tarsus) are more active within the cell.

The results clearly demonstrate that enzyme activity and kinetic characteristics determined in vitro under unphysiologic conditions (at optimal pH, in the presence of high concentrations of the substrate and coenzyme, in the absence of inhibitors) often provide misleading information concerning the real physiologic activity of the enzyme in the variant cells. Kinetic analysis under simulated physiologic conditions might provide some more inshight into the molecular pathology of G6PD variants.

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GLUCOSE 6-PHOSPHATE DEHYDROGENASE DURING ERYTHROID CELL DIFFERENTIATION

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Erythroid differentiation has been investigated in a number of cells and tissues, including foetal liver, embryo yolk sac, spleen, Friend erythroleukaemic spleen cells, and bone marrow. Using bone marrow erythroid cells from rabbits made anaemic by injecting phenulhydrazine, we have investigated a number of biochemical changes associated with the differentiation of these cells, especially those related to the synthesis of nucleic acids and proteins, including several enzymes (1-3). For this purpose, the development of a suitable technique for separating cells at different stages of differentiation in sufficient quantitities for biochemical studies was essential. We have used velocity sedimentation at unit gravity to separate bone marrow erythroid cells into six distinct cell types, namely proerythroblasts, basophilic erythroblasts, polychromatic erythroblasts and reticulocytes (1). This method is especially satisfactory for cells at the later stages of development which are separable with good resolution.

The synthesis of both DNA and RNA was found to be essentially constant in terms of the polymerization of labelled precursor (thymidine or uridine) per cell up to the polychromatic erythroblast stage, when nucleic acid synthesis rapidly ceased and was no longer detectable in the immediate progeny cells, the early orthochromatic erythroblasts. On the other hand, protein synthesis in terms of labelled leucine incorporated per cell remained active in all cells (1) although it decreased more or less in parallel with the reduction in cell volume, which is characteristic of the final stages of erythroid cell development.

Haemoglobin messenger RNA is already detectable in proerythroblasts and basophilic erythroblasts (4) but haemoglobin synthesis only becomes quantitatively predominant in orthochromatic cells after nuclear condensation (2). Carbonic anhydrase activity increases in parallel with haemoglobin though quantitatively to a lesser extent. The activities of catalase and adenylate kinase also increase, but only after the final cell division (2). A number of other enzymes, including glucose 6-phosphate de-

hydrogenase, show an approx. ten-fold decrease in specific activity at the time of nuclear condensation (2). The observed loss in activity is partly accounted for by the increased dilution of the enzymes owing to the accumulation of newly synthesized haemoglobin, but some degradation evidently also occurs at this time. Thus, in the case of lactate dehydrogenase we have recently shown that there is a selective degradation after nuclear condensation of lactate dehydrogenase isoenzymes containing the M subunit by a proteinase present in lysosomes (5). When lysates of rabbit erythroid bone marrow cells were analysed for glucose 6-phosphate dehydrogenase activity after starch gel electrophoresis (0.025 M sodium phosphate, pH 7, in the presence of NADP)a minor band with a slower anodic mobility was found to be present, whereas this component was absent in similar lysates from reticulocytes. It is possible, therefore, that limited proteolytic degradation, possibly by a lysosomal enzyme as in the case of lactate dehydrogenase, may contribute to the observed decrease in glucose-6-phosphate dehydrogenase activity during the final stages of erythroid cell development.

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SEPARATION OF ERYTHROCYTES ACCORDING TO AGE AS A TOOL FOR INVESTIGATING G6PD DEFICIENCY AND OTHER ENZYMATIC DEFECTS

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Marks and Johnson (1) first demonstrated that certain enzyme activities decline with aging of the red cell. Their studies introduced the concept of "age dependent enzymes" and suggested that enzymatic activities may decline during aging to a level incompatible with cell survival. Initial observations were obtained utilizing the different osmotic resistance of cells of different ages. Better separation of intact cells of different age may be obtained by centrifugation, after the initial observation that reticulocytes tend to concentrate in the upper part of the red cell column. The difference in specific gravity with cell age can be best exploited by equilibrium centrifugation using density gradients. Both continuous and discontinuous gradients have been utilized for this purpose. Discontinuous gradients appear preferable and in our laboratory these methods have been used extensively for separation of cells of progressively increasing age for metabolic studies (2, 3). Up to few years ago, most of the gradients had been prepared with crystalline bovine albumin; more recently a different kind of material, Stractan II, an arabino galactane polymer, has been utilized for this purpose (4). This material has two advantages; the first one, that it is extremely inexpensive, and the second, that it is extremely easy to work with. By use of this technique, the rate of decline of glycolytic enzymes was systematically studied. It is apparent that all glycolytic enzymes progressively lose activity with aging of the cells, however in most cases the loss of activity is negligible and 120 day-old red cells still have more than half the activity of very young red cells. The three glycolytic enzymes which exhibit a marked rate of decline are hexokinase, pyruvate kinase and aldolase. Rate of decline of enzymatic activities measured by in vitro assays do not reflect changes in true intracellular metabolism. In fact, the assays are performed in optimal conditions, at substrate concentrations which permit maximal enzyme velocity. Within the cell, individual enzymes act only as an integral part of the entire system due to the interplay of substrates, inhibitors and activators. Therefore a better estimate of changes

in metabolic capacity with cell aging is thus obtained by studies of actual utilization of glucose and production of lactate. A significant reduction in glucose utilization to a value of one-fourth of the initial rate is apparent in 120 day-old cells. This decreased metabolic rate does not imply metabolic failure, since the minimum rate of glucose does not necessarily imply metabolic failure and the minimum consumption of glucose is unknown. Of greater importance however is the fact that stressed glycolysis (in the presence of methylene blue and increased Pi) decreases at a faster rate (5). As a consequence, while young red cells may increase their glucose utilization up to tenfold in response to stress, old red cells with a fixed low basal metabolic rate have lost most metabolic flexibility. The mechanism for this defect could be related to the peculiar rate of decline of hexokinase.

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CORRELATION BETWEEN GSH AND ATP LEVELS AND MEMBRANE GLYCOPROTEIN MODIFICATIONS IN HUMAN ERYTHROCYTES

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The glycoproteins of erythrocyte membrane have been widely investigated both with respect to their structure and to their physico-chemical and functional properties (for a review see Marchesi and Furthmayr, 1976). In particular it was pointed out that, during physiological aging in the circulation, red blood cells undergo a loss of membrane sialic acid (Baxter and Beeley 1975). In the meanwhile the role of surface sialic acid as a determinant of erythrocyte life span was proved by several groups (Jancik and Shauer 1974) (Durocher et al. 1975) (Gattegno et al. 1975) (Balduini et al. 1977).

In this laboratory it was demonstrated (Balduini et al. 1974) that glycopeptides extracted by papain digestion from old erythrocyte membranes, contain, with respect to those extracted from young membranes, not only a lower amount of sialic acid, but also of galactosamine and hydroxy-amino-acids. Further investifations proved that both"ghost" membranes and intact erythrocytes are able, when incubated at 37° "in vitro", to release in the medium a glycopeptide (Brovelli et al. 1976) (Brovelli et al. 1977).
This glycopeptide showed the following properties:

- -It contains sialic acid (30-60% of the total membrane content), glucosamine, galactosamine, galactose and mainly polar aminoacids.
- - It aggregates in aqueous solutions.
- - It inhibits M - N haemagglutination.
- - Its release is pH dependent with two optima at pH 7.4 and 4.6.
- - The release from "ghost" membranes reaches its highest value after 4-5 hours of incubation at 37° C.
- - The release from intact erythrocytes reaches its highest value after 24-30 hours of incubation at 37° C.

These properties and the consideration that after incubation the SDS-polyacrylamide gel electrophoretic pattern of residual membrane glycoproteins is completely altered, seem to indicate that the two glycopeptides are a fragment of glycophorin. It can be supposed that, during incubation, an

autolytic mechanism becomes active on the membrane removing the glycophorin fragment exposed to the exterior of the cell. The same mechanism could occur during erythrocyte aging and could be responsible for the modifications of membrane glycoproteins, contributing therefore to the removal of the old erythrocyte from the circulation. Following the above considerations the study of the regulatory aspects of membrane self-digestion process was undertaken and in particular the role of some metabolites correlated with energy and redox metabolism was investigated.

The incubation of "ghost" membranes in the presence of ATP or GSH concentrations similar to the physiological ones, results in a complete inhibition of the sialopeptide release and in a quite normal aspect of the electrophoretic pattern of membrane glycoproteins. The other compouns investigated (GSSG, NAD, NADP, NADH, NADPH, DTT) did not show any effect (Brovelli et al. 1977).

When intact erythrocytes are incubated "in vitro" in Krebs-Ringer phosphate buffer, it can be observed that the autolytic process starts after 9-12 hours when a 30-50% decrease of GSH and a 90-95% decrease of ATP concentrations occur in the cell. Moreover if the precursors of GSH (8mM cysteine, 13mM glutamic acid, 24 mM glycine), adenine (0.3 mM) and glucose (1.5 mM) are added to the incubation medium, the GSH and ATP concentrations keep higher with respect to the control and the sialopeptide release after 30 hours is 30-50% inhibited. The protective effect of GSH and ATP precursors was confirmed by the normality of the electrophoretic pattern of membrane glycoproteins after incubation.

To verify if energy and redox metabolism can influence the autolytic phenomenon, experiments with erythrocytes from patients with PK or G-6-PD deficiency were recently undertaken.

Self-digestion experiments carried out using PK deficient red cells (5 cases), with a low ATP content, indicated that the sialopeptide release starts earlier with respect to the controls (after about 4 hours of incubation). Similar results were obtained by the preliminary investigations with G-6PD deficient red cells; the early sialopeptide release matches with a more rapid decrease of GSH.

From the "in vitro" evidences shown above it can be concluded that the structure of membrane glycoproteins is strictly correlated with the redox and energy metabolism of the erythrocyte. The intracellular GSH and ATP concentrations seem to control an autolytic mechanism which removes from the membrane a glycopeptide which is a fragment of glycophorin.

It can be speculated that when, during erythrocyte aging or in pathological conditions, ATP synthesis and redox metabolism fall below a critical level, a rearrangement of membrane structure and a decrease of surface negative charges occur. These membrane modifications very likely are a signal for the recognition of the cells by the haemocateretic organs and contribute to their removal from the circulation.

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GLUCOSE-6-PHOSPHATE DEHYDROGENASE VARIATION: A TOOL FOR THE STUDY OF X-CHROMOSOME INACTIVATION

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Genetic variants at the X linked locus specifying the enzyme, glucose-6-phosphate dehydrogenase (GGPD), have been very important in the investigation of X-chromosome inactivation (XCI). This developmental event (XCI) involves the random cytological and functional differentiation of the two X chromosomes in females such that one X chromosome becomes permanently inactive in each somatic cell. In this way, humans and most placental mammals achieve dosage compensation, that is, the equivalence of X linked gene expression for females with two X chromosomes and males with one X chromosome.

Since XCI is random, the egg transmitts an X chromosome which is not predetermined regarding its activation state. Therefore, the female germ cells must either never be subject to XCI, or they go through an activation reactivation cycle. The answer to this last question depends on when XCI occurs; that is, before or after germ cell differentiation. Viewed from this perspective, the investigation of XCI involves three developmental phases: differentiated somatic cells, germ cells, and the period between zygote formation and the onset of XCI.

The first definitive demonstration of an X linked gene exhibiting XCI involved human G6PD variants in fibroblast cell culture (1). Cloning of cells from heterozygotes showed expression of single alleles. The same approach was used to demonstrate that inactivation was a chromosomal event, by cloning a fibroblast culture from an individual heterozygous at both the G6PD and PGK loci (2). The first demonstration of significant variation in normal human tissues was in hair follicles from individuals heterozygous for the Mediterranean G6PD deficiency variant (3). Since that time the use of hair follicles has become an important clinical tool for diagnosing the heterozygous condition in X-linked disease (4). G6PD variants have also been extremely useful in studying tumor origin and development (5,6). The existence of mosaicism implies the possibility of selection and apparent selection in somatic cells has been shown in Mediterranean G6PD heterozygotes

(7,8).

Though XCI inactivation appears to be a stable event in differentiated somatic cells, the situation in germ cells must be different. Studies with G6PD, both in the mouse and humans, have shown that both X chromosomes are active in the oocytes (9,10). In humans this demonstration has involved the hybrid band pattern seen in G6PD heterozygous cells when both X chromosomes are active. The critical question now is whether both X chromosomes are active throughout germ cell ontogeny or whether the X chromosomes go through an activation: reactivation cycle. Some evidence, both cytological (11) and biochemical (12), suggests that the entire embryo is subject to XCI and that the germ cells undergo a reactivation at some point during their ontogeny. However, the data on this important point are controversial (13), and it is hoped that future work with G6PD variant may clear up this matter.

It is clear that the two X chromosomes in the female zygote are equivalent with respect to their activation states. The evidence is definitive that XCI has not occurred by the first four divisions and may not occur until implantation. However, it is not clear as to whether both X chromosomes at the early stages of development are active. Most gene products that have been examined at these early stages of development do not appear to increase in proportion with the growing embryo. The most likely explanation of this phenomena is that embryonic transcription of these genes is not occurring and that the observed gene products are maternal in origin. G6PD activity is an example of this type; the levels of G6PD activity remain fairly constant per embryo throughout preimplantation development in the mouse, and distributions of single embryo values show a unimodal distribution. However, two other X linked traits (α galactosidase and hypoxanthine guanine phosphoribosyl transferase) exhibit significant increases in activity levels per embryo during preimplantation development (14, 15). Studies of individual embryos for these two traits reveal bimodal distributions about the third day of development. It remains to be seen whether the two classes represent female and male embryos, and if so what bearing these findings will have on the question of dosage compensation during preimplantation development.

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BIOCHEMICAL ASPECTS OF GLUCOSE 6-PHOSPHATE DEHYDROGENASE DEFICIENCY

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Synthesis of a suitable affinity adsorbent, agarose-linked adenosine 2',5'-P_2 (1), permits the concurrent purification of a hitherto unrecognized NADP (H)-binding protein, designated FX (2, 3) and of Glucose 6-phosphate dehydrogenase (G6PD) from human erythrocytes.

FX is composed of two apparently identical polypeptide chains of NADPH (3,4). The dissociation constants towards both dinucleotide forms were determined spectrofluorometrically and found to be, $K_{NADP} = 1.8 \times 10^{-7}$ M and $K_{NADPH} = 1.0 \times 10^{-8}$ M. The Biological function of this holoprotein, which is also present in leukocytes and platelets and absent in plasma, is still unknown. FX was not identified with any of a number (approximately 30) of erythrocyte enzymes nor could it be qualified as a discrete form arising from breakdown of G6PD (5).

A solid phase radioimmunoassay was developed which enables quantitation of immunologically cross-reacting material (CRM) to FX in crude as well as in purified systems (5). A preliminary screening had shown significantly higher levels of FX CRM in erythrocytes from subjects affected by G6PD deficiency and having the Mediterranean variant of this enzyme protein compared with normal subjects (5). A further analysis, besides confirming such quantitative difference on an average, revealed a normal distribution curve of FX in subjects with G6PD B, while in hemizygous males having G6PD Mediterranean there was a considerable heterogeneity of FX CRM levels (6): thus, these were scattered from normal values (in approximately 20% of the G6PD deficient subjects tested) through consistently increased figures (up to fourfold the mean of normal individuals).

A second aspect of G6PD deficiency which was investifated in our laboratory deals with the molecular mechanisms underlying the defect of catalytic activity. These were examined in two different genetic variants of the enzyme, I.E. G6PD Mediterranean and G6PD Seattle-like. Three methodological approaches were followed: 1) a highly specific solid phase radioimmunoassay of G6PD, 2) a single step, microscale purification procedure, based on the above mentioned affinity adsorbent, whereby G6PD and FX can be concurrently isolated from single individuals (3 ml volumes of erythrocytes),

and 3) fractionation of erythrocytes of different age by centrifugation on discontinuous density gradients (7). These techniques enable to measure the specific activity (catalytic activity/mg of G6PD protein) of the enzyme variant under study in lysates from whole erythrocyte populations, in lysates from erythrocytes of different age and in purified samples. The results indicated that the Seattle-like variant has nearly normal catalytic function and the related defect is accounted for by a reduced amount of enzyme protein because of an enhanced in vivo breakdown. Conversely, both an impaired catalytic efficiency and a consistently lowered amount of enzyme protein (due to a dramatically accelerated decay during erythrocyte aging) are responsible for the severe defect of activity associated with the Mediterranean variant of G6PD (8).

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GENETIC POLYMORPHISM AND KINETICS OF NORMAL AND VARIANT G6PD

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We have presented data on two aspects of the genetic polymorphism of G6PD. Firstly, we have summarized the information that comparative studies on several variants can yield with respect to the functional properties of this enzyme in vitro and in vivo. We have confirmed previously published evidence that the saturation curve of G6PD is hyperbolic with respect to one substrate, G6P; but sigmoid with respect to the other substrate, NADP. The cooperative binding of NADP is a feature exhibiting specific changes in the products of different mutations. Thus, for instance, the maximum slope of Hill's plots, i.e. the empirical n_H value, varies in 8 different variants from 1.1 to 1.8. Furthermore, we have shown that cooperativity is a sensitive function of pH, of Mg^{2+} concentration, and especially of ionic strength. The mechanism of the increase in binding affinity with increasing NADP concentration is not yet clear, but it is likely to have considerable importance in the regulation of G6PD activity in response to changes in the intracellular environment in the erythrocyte. We have also shown evidence that a point mutation affecting the primary structure of the enzyme - a single amino acid replacement - can affect its quaternary structure: specifically, the dime/tetramer equilibrium.

Secondly, we have tested the possibility that electrophoretically undetectable G6PD variants may exist in the normal population. In order to do this, quantitative determination of G6PD activity was carried out in 219 male Nigerian children of 86 mothers with known Gd genotype. The relative intrasibship difference in G6PD activity (normalized to the lowest value within the sibship) was below 0.18 in all cases but one when the children were known to have the same Gd allele (identical by descent); whereas it was higher than 0.18 in 18 out of 33 sibships where children might have had either of the two maternal Gd alleles. G6PD from 10 (8 G6PD B and 2 G6PD A) children belonging to 4 of the sibships with high quantitative variation in G6PD activity was partially purified and extensively

characterized. The 8 G6PD type B samples fell unambiguously into two classes on the basis of Km values for glucose 6-phosphate (determined at various pH values), and KCl gradient elution from DEAE-Sephadex columns. The two G6PD type A samples were also different from each other by the same criteria. We conclude that "normal" G6PD is genetically heterogeneous and that the structural Gd alleles concerned are all polymorphic in the Nigerian population.We believe this is the first instance where a human enzyme polymorphism, not associated with enzyme deficiency, is revealed by an approach other than electrophoresis.

NEXT ASPECTS OF INTRACELLULAR REGULATION OF NORMAL AND GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENT ERYTHROCYTES

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Several mechanisms recently proposed for the regulation of the hexose monophosphate shunt require the concentration of NADP to be low or that of NADPH to be high. Recent studies (1) indicate that glucose-6-phosphate dehydrogenase, the first enzyme of the hexose monophosphate shunt, of human red cells is under severe intracellular restraint even when these conditions do not exist. In human erythrocytes with low activity variants of glucose-6-phosphate dehydrogenase, measurements of the rate of oxidation of 1-^{14}C glucose show that the enzyme is operating at a rate much closer to its maximum than in normal cells. This requires that the ratio of inhibitory NADPH to $NADP^+$ be much lower in the variant cells than in the normal cells. A low NADPH/$NADP^+$ ratio in unstressed cells, deficient in glucose-6-phosphate dehydrogenase, have been demonstrated (2), and a small increase in oxidative rate, induced in vitro by naphtol (3) and in vivo by fava beans (4), caused a further decrease in NADPH level. The reason for the limitation on maximum intracellular activity is unknown. Recent studies of the hexose monophosphate shunt activity and of NADPH level of normal and glucose-6-phosphate dehydrogenase deficient erythrocytes show a prompt increase in $^{14}CO_2$ evolution and a rise in NADPH levels (5). Since the concentration of hemolysates is comparable to that of intact erythrocytes, the relief of the restraint on glucose-6-phosphate dehydrogenase through dilution-dependent dissociation from inactivator or inhibitor is excluded. The possibility that the intracellular restraint may result from compartmentalization of glucose-6-phosphate dehydrogenase and substrates or from properties of the intact membrane of the erythrocytes could be suggested.

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ELECTROPHORETIC AND IMMUNOCHEMICAL PROPERTIES OF MOUSE GLUCOSE-6-PHOSPHATE DEHYDROGENASE

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An important difference in the behavior of glucose-6-phosphate dehydrogenase (G6PD) in liver with respect to most other tissues, including erythrocytes, is that its activity can be varied by a considerable factor (up to15-fold) in response to a variety of dietary and other signals. In erythrocytes, one can readily expect the mechanism of formation of new enzyme molecules to be wholly or partly absent. This is not the case in tissues like muscle which are also noninducible, or in uterus , where enzyme activity may increase, but in response to estradiol treatment rather than to dietary changes. It seems, therefore, that the mechanisms by which a cell responds to the various activating signals are present to different extents in different tissues. Still, a more trivial explanation may exist, such as the existence of totally different kinds ("isozymes") of G6PDH in induced tissues.

To find out whether this is the case, we examined the properties of G6PD in liver and other tissues, by a variety of electrophoretic and immunochemical techniques. The technique which proved most useful was electrophoresis of the native enzyme on acrylamide gels. The detailed investigation of the patterns obtained (Hizi and Yagil, Europ. J. Biochem. 45, 201, 1974) indicated several features which may be of considerable importance to the study of enzyme variants. The typical pattern obtained when a cytosolic preparation is electrophoresed by the classical Orenstein-Davis procedure and stained for G6PD activity can be seen in Figure 1. This pattern consists of three closely spaced bands, the intensity of which is much higher in liver homogenates from induced mice than from repressed ones. No such difference in intensity can be seen in the erythrocytes from the same mice.

At first, we assumed that the 3 bands may represent different isoenzymes in the livers. A more detailed study revealed, however, that these bands are interconvertible. Thus, when a fresh and well centrifuged preparation, protected by NADP, is examined, only the slowest band of the triplet is observed. Upon standing this is converted first into the medium band and

finally into the fast migrating band. When such "aged" preparations are incubated with 2 mM mercaptoethanol and 1 mM NADP, the slow band is reformed. This means that the relative intensities of the three bands are determined after disruption of the cells. In other words, only one form of glucose-6-phosphate dehydrogenase is present in the cytoplasm of liver, erythrocytes and other tissues examined by us, and the three bands represent postdisruptional modifications of the enzyme.

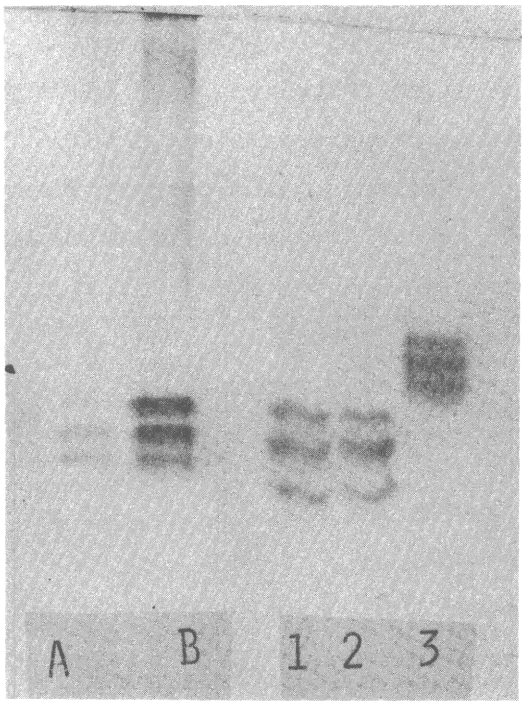
The triplet can be seen not only in mouse erythrocytes but also in human erythrocytes (band No. 3, Fig. 1). The triplet pattern can also be discerned on electrophoresis on starch gel and cellulose acetate, but the superior resolution of acrylamide is required to bring it out in detail. This may have important consequences on the analysis of variants; apparently narrower or slightly displaced bands may well represent differences in the cellular modifying system rather than of the enzyme molecule per se. It is recommended to examine variant preparations, both fresh and aged as well as under reactivating conditions, before a conclusion regarding a difference is arrived at. An example of deficient enzyme is shown in Fig. 2, where G6PD purified from a Tel Hashomer variant of the enzyme is compared with the normal enzyme. The triplet can be clearly observed in both preparations. In the variant enzyme an appreciable amount of the slower dimer band, as reported by Kirkman, Ramot and Lee (Bioch. Gen. 3:137, 1969) can also be seen.

A further approach to the characterization of enzyme in different tissues or metabolic states is by the use of specific antiserum raised against mouse liver G6PD purified by the affinity chromatographic procedure of Lamed et al. (Biochim. Biophys. Acta 304:201, 1972). One particularly useful immunochemical technique is rocket immunoelectrophoresis, first applied to G6PD by Kahn, Dreyfus and co-workers (this symposium). The area of the "rocket" formed is proportional to the amount of antigen present, so that the relative amount of antigen in induced vs. prepressed preparations can be estimated independent of the enzyme activity. Such a comparison has made it possible to conclude that the enzyme has a constant specific activity whether induced or repressed, and has confirmed the suggestion that only a single form of the enzyme exists in the mammalian tissues examined. The same technique was also used to show that the three bands obtained upon electrophoresis are antigenically equivalent.

The immunochemical approach can also be used in order to determine the rates of G6PD synthesis (Eisenbach and Yagil, in preparation). Two-dimensio-

nal analysis of the immunoprecipitate proved to be essential in obtaining reliable values for the incorporation of labeled aminoacids in G6PD molecules. In this procedure, the enzyme is electrophoresed in one dimension in the native form, and then the subunits are further separated by electrophoresis in SDS in vertical direction. In this way it can be shown that the increased levels of the enzyme in mice induced by a fatless diet are connected with a parallel increase in the rate of synthesis of the enzyme.

I hope that some of the methodologies referred to in this report will prove useful in the analysis of variant G6PD in cases of deficiency in erythrocytes.



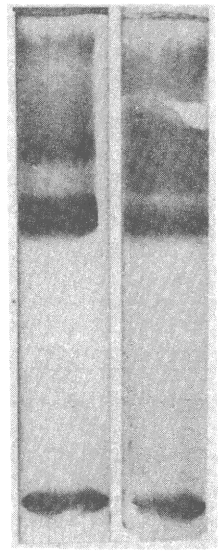


Fig. 1. Electrophoresis on acrylamide of liver and erythrocyte G6PD. The detailed procedure is described in Eur. J. Biochem. 45:201 (1974), only that a slab gel of 7.5% acrylamide was used. The sample was loaded in 40% sucrose. 0.01 mM NADP was also added to the tris-glycine solution in the upper tank. The slightly faster migration of the fast band in mouse erythrocytes is due to excess hemoglobin in this preparation, as evident before the stain.
A, repressed mouse liver, 40 µg liver, 4.1 munits loaded.
B, induced mouse liver, 40 µg liver, 26 munits.
1, erythrocytes from the repressed mouse, 0.83 µl blood, 1.83 munits.
2, erythrocytes from the induced mouse, 0.83 µl blood, 1.76 munits.
3, human erythrocytes, 1.35 µl blood, 1.72 munits.

Fig. 2. Electrophoresis on acrylamide of G6PD purified from normal erythrocytes (right) or Tel Hashomer variant (left) subject. The samples were kindly made available by Prof. B. Ramot. Resolution with purified enzyme are for some reason not as good as with homogenates, but the three bands are clearly visible on the original gel. The band at the bottom is the bromphenol blue marker.

SOURCES OF OXIDATION AND REDUCTION EQUIVALENTS IN THE RED CELL

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There are two types of stresses, which may lead to the destruction of the red cell:

1) Interference with maintenance of ATP

The resistance of the cell to changes of ATP is provided by its finely regulated mechanisms of ATP maintenance, which are part of the self regulation of the glycolytic system. Its basic features are:

a) the existence of a time hierarchy of enzymes. The enzymes differ by four orders of magnitude in their relaxation times. This means that some reactions are always near equilibrium while others are so slow that their changes may be neglected. Only some reactions need be considered for the usual metabolic changes.

b) For the control of the glycolytic flux only three nonequilibrium enzymes are important; they are the hexokinase-phosphofructokinase (PFK) system and the system of ATP-ases.

c) The ATP consumption is the major factor determining the magnitude of the glycolytic flux.

d) There exist two different mechanisms for the control of the flux, an allosteric one by means of the HK-PFK system and a stochiometric one provided by the DPG shunt.

The problems of ATP production are well understood while those of ATP consumption are not. The known transport ATPases account for no more than 1/4 of the ATP consumption.

Glycolysis and the pentose phosphate pathway are connected by G-6-P. Here there is a competition between the PFK and the G6PD. The share of the PFK is determined by positive effectors such as AMP and P_i and by the negative effector H^+ ions. The activity of the G6PD is mostly affected by the concentration of NADP. It is conceivable that the effect of pH has clinical implications which may be even more severe with G6PD mutants with lowered affinities for their substrates.

Of theoretical interest is the fact that glycolytic intermediates may serve

bance difference spectrum and fluorescence emission spectrum.

Drastic denaturation of the enzyme by urea and acid treatment did not suppress the difference of isoelectric point between native and hyperanodic forms of glucose-6-phosphate dehydrogenase.

From our data we may suggest that the conversion into hyperanodic forms could be due to the covalent binding on the enzyme of a degradation product of the pyridine nucleotide coenzyme. This modification could constitute a physiological transient step toward the definitive degradation of the enzyme.

ELECTRON MICROSCOPY OF PROTEIN MOLECULAR STRUCTURE WITH SPECIAL REFERENCE TO GLUCOSE 6-PHOSPHATE DEHYDROGENASE (G6PD).

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Electron microscopy can be used to study G6PD morphology at three structural levels:

a) the cellular level, where erythrocyte shape, and particularly where the condition and topography of its membrane are important. Macroscopic phenomena such as the distribution of G6PD within the cell and its possible attachment to the membrane, may be investigated by complexing the G6PD with specific ferritin-tagged antibody and preparing serial sections.

b) at the level of quaternary structure (e.g. Wrigley et al., J. Mol. Biol. 68, 483-499, 1972) where monomer-dimertetramer transitions are concerned using equilibrium and time-dependent conditions.

c) at finer levels in a detailed definition of tertiary structure and possible changes of that structure. Even finer details such as location of the NASP-binding site, could be studied using an NADP-specific antibody as marker (e.g. as Wrigley et al., J. Mol. Biol. 109, 405, 1977).

Studies in category b) are limited by conventional techniques of negative staining to a resolution of 30 Å at best. However current development in reducing molecular damage by staining and electron irradiation can be expected to improve this to a reliable 20-18 Å. Computer modelling in which the negative stain distribution is simulated (Wrigley et al., J. Microscopy, in preparation) should improve this to perhaps 15 Å in one or two years by permitting the integration of information from many individual molecules and so enhancing pictorial signal noise.

Studies in category c) will depend critically on the availability of crystals of the three molecular forms of G6PD. This could permit total elimination of negative stain and vastly reduced electron dosage. Combined with Fourier filtering and reconstruction procedures, this might yield information at the 10 Å level or better. Given the availability of large (>1 mm) crystals however, these problems are best transferred to X-ray crystallography with a potential of 2 Å resolution.

If the above could yield morphological features fine enough to distinguish G6PD variants (though their differences are probably below 5 Å), then

electron microscopy could be of further value in any proposed mass-screening project forming part of a public health programme. In principle, 50×10^{-9} gm of highly purified G6PD would be sufficient for morphological comparison with previously determined and computer-stored structural information. This could also offer great rapidity with the typing of several hundred samples per man-day. Limiting factors would be 1) equivalently rapid enzyme purification procedures and 2) sufficient investment in electron microscope, picture scanning and computer equipment.

BEHAVIOR OF GLYCOLYTIC METABOLITES, PYRIDINE COENZYMES, ADENINE NUCLEOTIDES, AND GLUTATHIONE DURING FAVA-BEAN CRISIS

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The levels of some glycolytic metabolites, 2,3-DPG, ATP, pyridine coenzymes, total glutathione, and the total glycolytic flux were assayed in G6PD-deficient erythrocytes

1. in steady-state conditions
2. during fava-bean hemolytic crisis
   2.1. within 24 h from ingestion (early crisis)
   2.2. between 36 and 72 h from ingestion (late crisis)
3. during mild crisis occurring almost without hemolysis.
4. The same parameters were measured also in incubation experiments at different pH-values in order to evaluate the influence of G6PD-deficiency on the glycolytic regulation.

1. Steady-state conditions

Significant differences were noted in the levels (nmoles/ml RC) of 2,3-DPG (normal (N) 5150; deficient (D) 3600), NAD (N 21;D 38), NADH/NAD (N 2.57; D 1.16), NADP (N 1;D 17), NADPH/NADP (N 30;D 2.53), and total GSH (N 2364;D 1433).

2.1. Early crisis

Significant differences were noted in the levels of NADPH (deficient controls (DC) 43 early crisis (EC) 26), NADPH/NADP (DC 2.53; EC 0.61),NADP (DC 17; EC 42), total GSH (DC 1433; EC 780), and total glycolytic flux (DC 1316; EC 1843).

2.2. Later crisis

36 to 72 h after ingestion, significant differences were remarked in the levels of 2,3-DPG (DC 3600; late crisis (LC)5900), ATP (DC 1208; LC 1697), total glycolytic flux (DC 1316; LC 2100), and NADPH/NADP (DC 2.5; LC 0.63).

3. Mild crisis

During mild crisis there was no drop in total GSH but a sharp decrease in the ratios NADH/NAD (DC 1.16 ;mild crisis (MC) 0.8), and NADP/NADP (DC 2.53;MC 0.4).

4. Incubation experiments

Lower NADH/NAD-ratios measured in G6PD-deficient red cells (see sect.1) are expected to influence and activate the glyceraldehyde 3-phosphate dehydrogenase-step. In fact, deficient red cells accumulate less dihydroxyacetone phosphate, glyceraldehyde-3-phosphate and fructose 1,6-bisphosphate at alkaline pH, and produce more lactate at all pH-values studied sofar (pH 6.80, 7.00, 7.40, 7.80, 8.00). Lower 2,3-DPG and higher intracellular inorganic P levels were measured at all pH-values.

CONCLUSIONS

The most sensitive parameters modified by fava-bean ingestion were the redox couples NADH/NAD and NADPH/NADP: total GSH dropped only when hemolysis occurred. Except for a transient and compensatory increase in post-hemolytic anemia, 2,3-DPG is constantly lower in the deficient subjects. The redox shifts correlated with the G6PD-inactivity influence glycolysis and increase the lactate output.

PREPARATION AND PROPERTIES OF RESEALED RED CELL GHOSTS

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The preparation of resealed ghosts (6,7) aims at replacing the cell contents by solutions of one's own choice. Cell contents can be removed by various forms of hemolysis, including osmotic hemolysis, to which the present paper is confined. After hemolysis, the ghosts can be equilibrated with solutions of the desired composition. Incubation at 37° C for 15-45 minutes finally leads to a recovery of the imperviousness of the cell membrane ("resealing" (2)). The present paper deals with:

(1) the maximalization of the yield of resealable ghosts in a population,

(2) the maximalization of impermeability of those ghosts in a population that can be resealed,

(3) the minimalization of the residual cell contents in resealable ghosts, and

(4) the preparation of vesicles which are surrounded by the original red cell lipid bilayer containing one or a few of the original membrane proteins.

(1) The yield of resealable ghosts is maximal if hemolysis is performed at 0° C, pH 6. Under these conditions the presence or absence of alkaline earth metal ions or complexing agents is of little consequence for the yield. If hemolysis is performed at more elevated temperatures, the presence of complexing agents at the instant of hemolysis decreases the number of resealable ghosts while the presence of Mg or low concentrations of Ca increases the yield (1, 3, 5).

(2) Resealing is a relative term. Those ghosts in the population that can be resealed may be more or less permeable to a given solute. The permeability for K is minimized by a wash of the resealed ghosts in a medium containing a complexing agent. Hemolysis in the presence of Mg or Ca (which produce a temperature-dependent effect on the yield of resealable ghosts) decreases the K permeability of those ghosts that did successfully reseal (8).

(3) A combination of the principles described under (1) with column chromatographic techniques allows the preparation of ghosts that are virtual-

ly free of hemoglobin and still can be resealed by subsequent incubation at 37° C (9).

(4a) Spectrin and the majority of the red cell membrane proteins can be removed by exposing red cell ghosts to incorporated trypsin. The intrinsic membrane proteins are partially degraded, but fragments of the protein in band 3 or two other proteins still remain associated with the membrane ("enzymatic cleaning", (4)). The treated ghosts tend to form vesicles which are leaky for K but not for anions and which can be used for the study of the function of the protein in band 3 in anion transport.

(4b) Extraction with slightly alkaline solutions of Trition X-100 removes virtually all membrane proteins except the protein in band 3. Vesicles can be formed that can be used for the study of anion transport ("chemical cleaning", (10)).

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FACTS, ARTEFACTS AND FUTURE TRENDS IN ISOELECTRIC FOCUSING

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With a resolving power of at least 0.02 pH units (in pI differences) (1) isoelectric focusing (IEF) represents one of the most powerful tools today available to biochemists and genetists. In one of its most promising adaptations, IEF is performed in gel slabs, run horizontally, and the sample is applied to the gel matrix soaked in a piece of filter paper (2). Up to 40 samples can thus be handled simultaneously. IEF has already found several clinical applications. It has been successfully used in bidimensional mapping of serum proteins (3,4). In studying the polymorphism of α_1-antitrypsin (P_i system or protease inhibitor) 17 alleles have been characterized so far in human patients. Of these, the MM phenotype is the most common one, while the ZZ phenotype represents one of the most severe cases, since the carrier exhibits a severe α_1-antitrypsin deficiency associated with lung and hepatic disease. These studies have been made possible by the use of very shallow pH gradients (spanning $\frac{1}{2}$ of a pH unit) and high voltages, thus increasing the resolution while maintaining sharp protein zones (5,6).

We have applied IEF to the study of chemically modified as well as genetic variants of hemoglobin (Hb) (7-10). Our results are summarized in Table I.

TABLE I
pI's of genetically and chemically modified hemoglobins

Protein (pI)		pI	No. Residues	No. charges
HbA (7.0)	HbS ($\alpha_2^{6Glu\text{—}Val}\beta_2$)(7.25)	0.2	2	2
HbA (7.0)	HbC ($\alpha_2^{6Glu\text{—}Lys}\beta_2$)(7.4)	0.4	2	4
HbA (7.0)	HbE ($\alpha_2^{26Glu\text{—}Lys}\beta_2$)(7.4)	0.4	2	4
HbA (7.0)	HbMalmö($\alpha_2^{97His\text{-}Gln}\beta_2$)(6.9)	0.1	2	1
HbA (7.0)	HbWood($\alpha_2^{97His\text{-}Leu}\beta_2$)(6.9)	0.1	2	1
HbA (7.0)	HbA_C($\alpha_2^c\beta_2^c$) (6.9)	0.1	4	1
HbA (7.0)	$HbA_{\frac{1}{2}C}$($\frac{1}{2}$ carbamylated)(6.95)	0.05	2	$\frac{1}{2}$

It can be seen that when a spot mutation in the polypeptide chain affects an amino acid with a low or high pK, the total charge variation results in a change of 0.1 pI units per charged residue substituted (i.e. per proton lost or acquired). However, when the substitution is on a His residue (Hb's Malmöand Wood) or when the terminal $-NH_2$ is chemically modified (carbamylated Hb), the ΔpI is much smaller, since only about $\frac{1}{2}$ and $\frac{1}{4}$ of a proton, respectively, are lost per modified amino acid residue. As a rule of thumb (for proteins of at least 40 000 daltons and in the case of few amino acid substitutions) it can be stated that when the pK of a charged group lies at least two pH units on either side of the pI of a protein, the "mutant" protein will change its pI, in comparison with the "wild type", of about 0.1 pH unit/charged residue lost or acquired. Thus IEF can be used not only to separate genetic variants but also to predict, by pI measurements, the extent of amino acid substitution.

A word of caution should be stated against the indiscriminate use of IEF for the resolution of any separation problem. While it appears that in most cases, concerning protein separations, no artefacts are generated, there are entire groups of compounds which produce spectacular artefacts in IEF. Recently (11), we have demonstrated that focusing of nucleic acids (t-RNA) produces a series of multiple bands representing specific complexes among t-RNA and the carrier ampholytes which generate and stabilize the pH gradient. As the macromolecule travels towards its pI (if any) it crosses the boundaries of a stack of a multiphasic buffer (the focused ampholytes) thus generating the observed spectrum of bands. The same happens with acidic dyes (12) and with heparin (13). We have demonstrated that this phenomenon is also elicited by all polyanions (polysulphates, such as heparin, polyphosphates, such as nucleic acids and polycarboxylates, such as polygalacturonic acid) (14). Even a protein model, polyglutamic acid, generates the same artefacts and it can be anticipated that polycations (polylysine, polyarginine) and other very basic compounds, such as histones and protamine, will behave in the same fashion.

What is new in IEF? More than 10 years after its introduction, new developments in IEF are still far from reaching a plateau. One promising development, made in our department, is IEF at very low temperatures (-20 to -30° C) in modified polyacrylamide gels containing anti-freezing solvents (dimethylsulphoxide, ethylene glycol, etc.). In this system, it should be possible to detect reaction intermediates, such as enzyme-substrate com-

plexes, which dissociate too quickly at room temperature. Another promising variant is "pH gradient modifier"IEF, which consists in further flattening the pH gradient in the region of interest in order to optimize the resolution of closely adjacent protein zones (15, 16). This is achieved by adding to the Ampholine mixture appropriate amphoteric compounds (usually 50 to 100 mM) having a pI very close to the pI's of the proteins to be separated. By this technique, we have been able to fully separate HbA from HbA_{IC}, one of the minor components of the glycosylated Hb pool, whose quantitation is important in the follow-up of diabetic patients.

As summarized in Fig. 1, it would appear that there are no new electrophoretic techniques in sight, and that all the possibilities have been exploited. Yet, as indicated by the two dotted lines, it is possible to anticipate the possibility of working diagonally, by performing a sort of affino-isotachophoresis or affino-isoelectric focusing. If a suitable ligand forming a strong complex with a protein can be found, it should be possible to alter the mobility or the pI of a protein in such a way that its position in the stack or in the pH gradient will be removed from adjacent contaminants (this principle has been recently applied in "charge-shift electtrophoresis") (17,18).

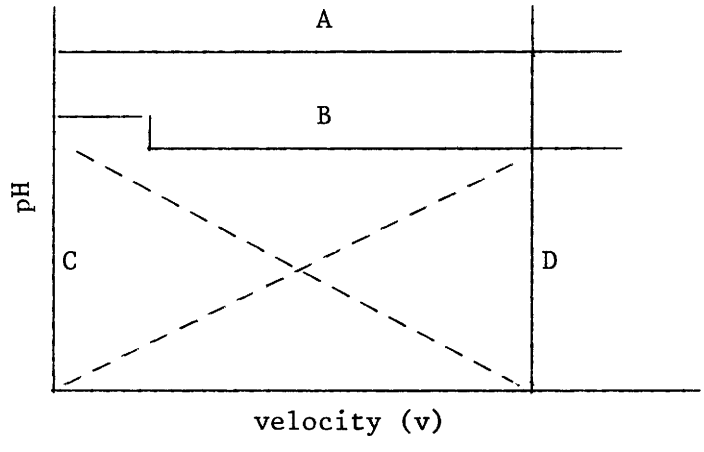

Fig. 1
Classification of electrophoretic techniques. The velocity of the protein zones is plotted against the pH in the zones. A: zone electrophoresis and moving boundary electrophoresis; B: discontinuous zone electrophoresis ; C: isoelectric focusing (at equilibrium); D: isotachophoreris (modified from R.J. Routs, Ph.D. Thesis, Technical University of Eindhoven, The Netherlands, 1971).

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