Congenital Hemolytic Anemias: A Review of Recent Progress.

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Congenital hemolytic anemias are composed of various kinds of disorders and can be subdivided into three categories based on the cause, namely, membrane defects, enzyme anomalies and hemoglobin abnormalities. It certainly is a difficult task for me to review recent advances and topics on such a wide area. As I have been working mainly on enzyme anomalies and red cell metabolism, I would like to spend half of my speech about enzyme anomalies and rather limit the time to be spent for the disorders due to hemoglobin abnormalities and membrane defects.

Table. 1 Result of epidemiological survey on congenital hemolytic anemias in Japan performed in 1974 by a study group of hemolytic anemias granted by the Ministry of Health and Welfare, Japan[1].

PREVALENCE: 5.7-20.3/million Japanese/year	No. of cases*	%
Hereditary spherocytosis	201	72
Hereditary elliptocytosis	6	2
Unstable hemoglobins	3	1
Thalassemias	10	3.5
Other hemoglobinopathies associated with hemolysis	1	0.5
G6PD deficiency	6	2
Pyruvate kinase deficiency	9	3
Other enzymopathies associated with hemolysis	3	1
Congenital hemolytic anemias of unknown cause	41	15
Total	280	100%

*Nationwide survey by letter inquieries at all 8,189 hospitals (over 50 beds). Recovery of inquieries; 28%. Number of patients visited hospitals during Jan. - Sept., 1974.

First of all, the result of epidemiological survey on congenital hemolytic anemias in Japan is shown in Table 1. The survey was made in 1974 by the study group of hemolytic anemias granted by the Ministry of Health and Welfare, Japan[1]. The prevalence of congenital hemolytic anemias was estimated to be 6 to 20 per million Japanese per year. This value appears to be definitely lower than that obtained in caucasians because of the fact that in Japan, sickle cell anemia cannot be seen and both thalassemia syndromes and glucose 6-phosphate dehydrogenase (G6PD) deficiency are rare[2)3)]. Hence, hereditary spherocytosis accounts for as much as 72% of all the congenital hemolytic anemias. The prevalence of both hereditary spherocytosis and hereditary elliptocytosis is still much lower than that seen among caucasians[4)5)]. These values are of some interest from the standpoint of geographical hematology.

HEREDITARY SPHEROCYTOSIS AND OTHER HEMOLYTIC ANEMIAS DUE TO HEREDITARY MEMBRANE DEFECTS

Hereditary spherocytosis:

Although hereditary spherocytosis is common among congenital hemolytic anemias, the molecular mechanism of this disease has not yet been elucidated. The evidences of defective erythrocyte membranes in hereditary spherocytosis (HS) are shown in Table 2. Bertles[6] and Jacob and Jandl[7] have shown that the HS cells had increased permeability to sodium. In 1966, Reed and Swisher[8] and Jacob[9] observed that the red cell membrane tended to form budds which fragmented off during incubation. This causes lipid loss and decrease in surface area with concomitant increase in osmotic fragility. Membrane lipid analysis was done by Jacob and Karnovsky[10] in 1967 but no remarkable abnormality was found.

Membrane protein analysis has been hampered until relatively recen-

Table. 2 Evidences of defective erythrocyte membrane proteins in hereditary spherocytosis.

1957–64:	Increased permeability to Na^+(Bertles; Jacob & Jandl)
1966:	Lipid loss (budding and fragmentation) during incubation (Weed & Reed)
1967:	Only minor abnormality of lipid (Jacob & Karnovsky)
1972:	Evidence of abnormal membrane microfilaments (Jacob et al)
1973–75:	Defective membrane protein phosphorylation [protein kinase] (Greenquist & Shohet, Jacob et al, Yawata et al)
1974:	Relative deficiency of Ca^{2+}-ATPase (Feig & Guidotti)
1967:	Mg-ATPase deficiency (Nakao et al)
1974:	Defect in the IVb-band (Nozawa et al; Hayashi et al)

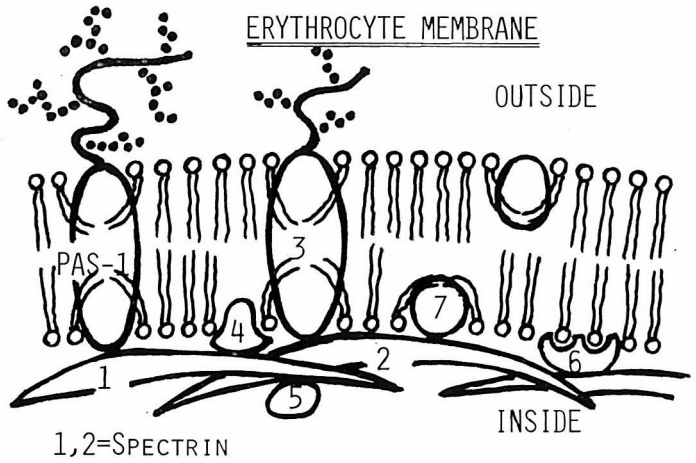


Fig. 1 Schematic representation of human erythrocyte membrane. Intrinsic proteins are embedded in a matrix of lipid bilayer while extrinsic proteins are mostly located at the inner surface of the membrane.

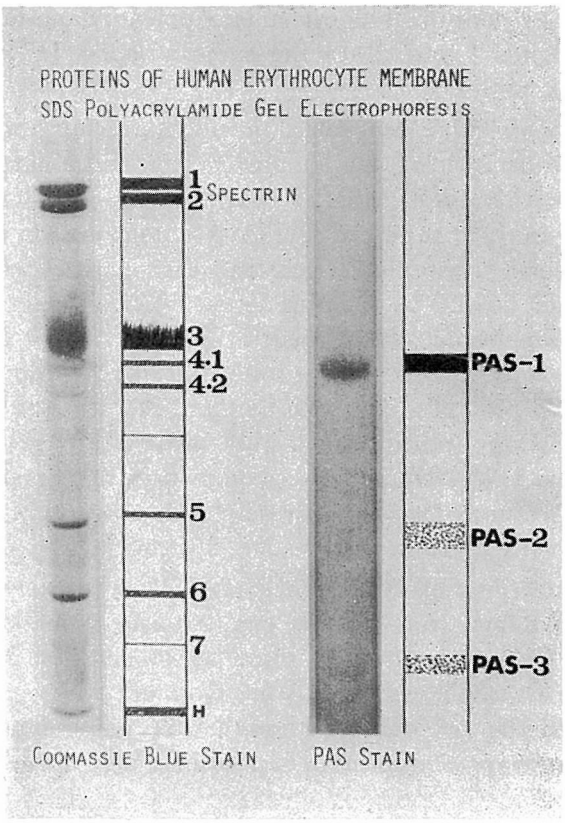


Fig. 2 Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoretic pattern of human erythrocyte membrane proteins.

tly because of the difficulty in getting soluble protein fractions. In 1968, Mazia and Ruby[11] devised a good method to solubilize membrane protein by simple dialysis of ghosts in low ionic strength media. Marchesi and Steers[12] in 1968 observed that the membrane protein solubilized in this way assumes an intriguing ultrastructure when ionic strength is reconstituted, especially in the presence of ATP and Mg^{++}. Under these conditions the membrane protein aligns into microfilaments, behavior of which is reminiscent of that seen with actin after its similar extraction from skeletal muscle. Applying these procedures to HS cells, Jacob[13] were able to obtain the evidence of abnormal membrane microfilaments in 1972. HS red cells had decreased aggregation property of solubilized protein when ionic strength was increased.

Schematic representation of human erythrocyte membrane is shown in Fig. 1. In this model, intrinsic proteins are embedded in a matrix of lipid bilayer whereas extrinsic proteins are mostly located at the inner surface of the membrane. The numerical order of the proteins in the figure are according to the nomenclature by Fairbanks et al.[14] of protein bands obtained by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Fig. 2). Bands 1 and 2 are the same as spectrin by Marchesi, a microfilamentous protein.

Although Jacob et al. presented the evidence of abnormal microfilamentous protein in HS red cell membrane, the exact nature still remains to be proven. In this connection, it is interesting to note that Matheson and Howland[15] in 1974 reported erythrocyte deformation in patients with congenital muscular dystrophy suggesting that erythrocyte surface may reflect a systemic defect in membrane properties.
Quite recently Greenquist and Shohet[16], Jacob et al.[17] and Yawata et al.[18] found a defective membrane protein phosphorylation especially that of spectrin in HS red cells, although Zail and Hoek's result[19] is contrary. This very interesting finding means that protein kinase is defective in this disease. It is interesting in this connection to note that Roses and Appel[20] in 1973 found decreased protein kinase activity in the red cells of the patients with myotonic muscular dystrophy.

The concentrations of cyclic-AMP (cAMP), adenyl cyclase and phosphodiesterase of human red cells as compared to other tissues are shown in Table 3[21]. Concentrations of these substances in human red cell membrane are remarkably low, and one wonders whether they play an important role in the red cells. Although this point must wait further elucidation, it appears that protein kinase may play a role in maintaining red cell shape.

Table. 3 Concentration of cyclic 3':5'-AMP (cAMP) and enzymatic activities of adenyl cyclase and phosphodiesterase in various tissues[21].

	cAMP ($\times 10^{-9}$M)	Adenyl cyclase	Phospho-diesterase
HUMAN RED CELLS	<0.03	<.1	0-0.15
HUMAN PLASMA	13	—	—
$_3T_3$ FIBROBLAST	1030	58	78
RAT LIVER	960	—	—

Meanwhile in 1974 Feig and Guidotti[22] observed that the activity of Ca^{2+}-ATPase was relatively deficient in HS. Although Feig's data does not clarify the problem whether a relative deficiency of Ca^{2+}-ATPase is a primary defect or a reflection of unknown primary defect, the data does point strongly to relationship between the intracellular Ca^{2+}, the calcium pump, the fibrous membrane protein system and the deformability of the membrane. HS may be associated with an alteration in a fibrous protein in the membrane which regulates the fluidity and deformability of the membrane, or there might be a change in membrane enzyme which regulates the intracellular level of Ca^{2+}, which in turn affects the state of the membrane.

Other abnormalities that have been reported include the low Mg-ATPase activity by Nakao et al[23] and a defect in the IVb-band found by Nozawa et al.[24] and Hayashi et al.[25].

It appears quite likely that HS is a heterogenous group of disorders of different etiology because the clinical severity is different from one family to another although the intrafamilial difference is minimum.

From the foregoing, the nature of the defect in HS has been focused on the membrane protein, and either defective protein kinase or defi ciency of Ca^{2+}-ATPase appears to be more promising than others. But the controversy over the nature of the membrane protein defect must await the standardization of analytical techniques that are reproducible from one laboratory to another.

Hereditary stomatocytosis:

Hereditary stomatocytosis is also of great current interest[26-41]. This too probably represents a heterogenous group of disorders. Hereditary stomatocytosis was originally designated according to the slit-like appearance of the central pallor of the patient's red cells. But a scanning electron microscopic study revealed that stomatocytes are actually bowl-shaped cells as schematically shown on the middle-top in Fig. 3.

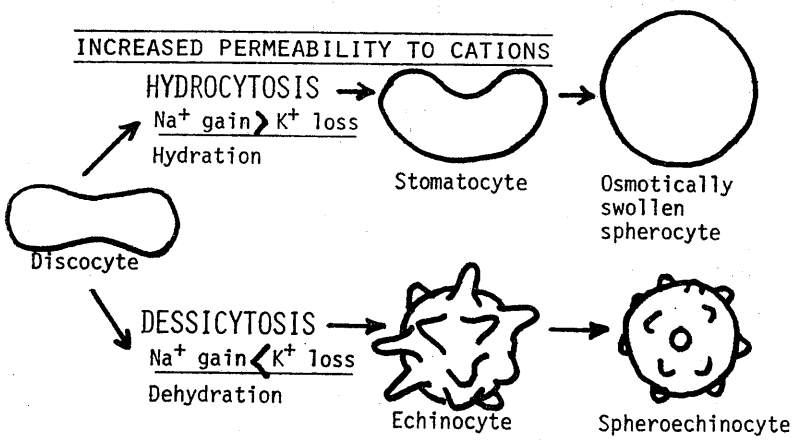


Fig. 3 Schematic representation of two forms of hereditary stomatocytosis.

Recently the concept on this disease appears to lay much stress upon biochemical lesion and some forms of hereditary stomatocytosis do not show stomatocytes but rather dessicytes[26], in other words, spheroechinocytes as shown in the right bottom of the Fig. 3 or target cells with high mean corpuscular hemoglobin concentrations (MCHC). In any event, a biochemical hallmark in these disorders is characterized by marked increase in passive sodium and potassium permeability[26)27)].

In order to compensate this cation leaks, active cation pump is increased as in the case of hereditary spherocytosis. But it seems to me that the distinguishing point in these two disorders is that membrane loss is absent in hereditary stomatocytosis while this is present in HS (Table 4). Because of this, red cell surface is not decreased in hereditary stomatocytosis.

Table. 4 Main features of hereditary spherocytosis and of hereditary stomatocytosis.

	HEREDITARY SPHEROCYTOSIS	HEREDITARY STOMATOCYTOSIS
Na-K pump	Increased	Increased
Osmotic fragility	Increased	Increased or decreased
Spherocytes	Present	Absent
Stomatocytes or target cells	Absent	Present
Lipid loss	Present	Absent
Response to splenectomy	Marked	Good-Not striking

Table. 5 Main features of two forms of hereditary stomatocytosis: Hydrocytosis and dessicytosis[31].

	HYDROCYTOSIS	DESSICYTOSIS
Increased permeability	Na>K	K>Na
Na-K pump	Increased	Increased
Cell Na+K content	Increased	Decreased
% of water	Increased	Decreased
Osmotic fragility	Increased	Decreased
Splenectomy response	Good	Not striking

Table. 6 Reported cases of two forms of hereditary stomatocytosis[26].

	Osmotic fragility	MCHC	Hb (g/dl)	RBC cations Na^+ mEq/liter	RBC cations K^+ cells)	Total lipid per cell	Remarks
DESSICYTOSIS							
Jaffé, Shohet et al	Decreased	Normal	12.6	22	75	Increased	PC*increased
Miller et al	Decreased	Normal	10	21	86	Normal	PC normal
Honig et al	Decreased	Normal	12.2	7	95		
Glader et al	Decreased	High	10	18	59		
Wiley et al	Decreased	High	12-16	9-15	79-89	Increased or Normal	PC increased
HYDROCYTOSIS							
Zarkowsky et al	Increased	Low	6.2	91-108	35-47	Increased	
Oski et al	Increased	Low	10-12	39-53	73-86	Increased	
Lo et al	Increased	Low	8-11	65	39		
Mentzer et al	Increased	Low	4-10	61-73	37-53		Defect in protein kinase
NORMAL RANGE				6-12	90-103		

*PC=phosphatidyl choline

It is not known why some cases show dessicytosis associated with decreased osmotic fragility, normal or high MCHC, low content in cations while the other cases show hydrocytosis associated with increased osmotic fragility, low MCHC, high content in cations (Table 5). It is interesting to note that high phosphatidylcholine hemolytic anemia reported by Jaffe et al.[39-41] and that of Wiley's[27] were included by Wiley in hereditary stomatocytosis (Table 6).

Mentzer et al[26] found in 1975 that protein kinase-mediated phosphorylation of erythrocyte membrane proteins is defective as in the case of HS. This means that the abnormality in this disorder appears to be in a defective membrane protein kinase rather than in the spectrin itself. According to Mentzer, protein kinase activity in a patient with this

disorder was as low or lower than that of HS, and they postulate that defective phosphorylation of membrane protein may play an equally important role in the induction of the shape changes as well as the permeability and pump efficiency defects as seen in HS. In case the primary defect in both of these diseases is defective membrane protein kinase, it seems that there must be another factor in HS which renders membrane loss with resultant spherocytosis. This point requires elucidation.

Their remarkable energy requirements for cation transport render hereditary stomatocytes unusually vulnerable to the effects of metabolic depletion. These changes particularly become manifest *in vivo* in the splenic environment.

From the diagnostic standpoint, Wiley et al.[27] emphasized that since an increased Na^+ flux, either influx or efflux, appears to be the most consistent abnormality in this disease. Hence, measurement of Na^+ influx is of diagnostic value.

Clinically, heterogeneity in this disorder is apparent that hemolysis ranges from severe to mild or even absent from case to case. Water content of hereditary stomatocytes varies according to the sum of monovalent cations, $Na^+ + K^+$, and one factor causing hemolysis may be an abnormal cell water content which is either too high or too low. In both hydrocytes and dessicytes, deformability of the cell is impaired. Although in other hemolytic conditions dessicytosis appears to be more deleterious to cell survival than hydrocytosis, in stomatocytosis the opposite seems to be the case. Furthermore, when hydrocytosis is the predominant lesion in stomatocytosis, there is greater derangement of cation composition.

High phosphatidylcholine hemolytic anemia[39-41]:

In high phosphatidylcholine hemolytic anemia, a metabolic block

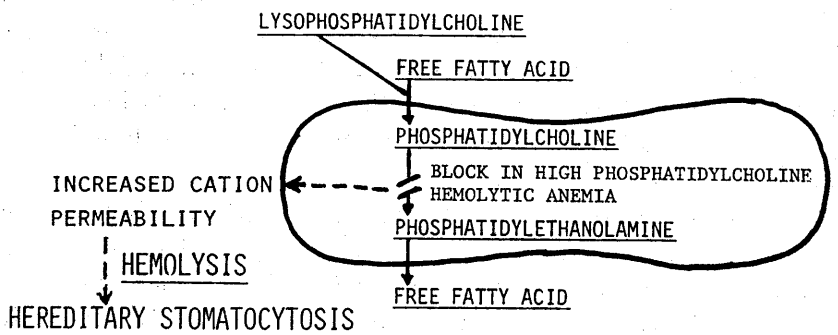


Fig. 4 Metabolic block in high phosphatidylcholine hemolytic anemia.

redides in the pathway in which actively incorporated phosphatidylcholine fatty acid is transferred to phosphatidylethanolamine prior to final release from the cell (Fig. 4). In high phosphatidylcholine hemolytic anemia, cation permeability defect as noted in hereditary stomatocytosis is thought to contribute significantly to the early demise of the red cell in this disorder[41]. However, how the defect in lipid metabolism which is thought to be primary to this disorder causes the cation permeability defect is unknown. Recent rapid advance in membrane biochemistry will hopefully soon clarify the defective lipid and protein interaction in this disorder.

Congenital hemolytic anemia with extreme microcytosis:

Another interesting congenital hemolytic anemia due to membrane defect was reported by Wiley and Gill[42] in 1976, which was called congenital hemolytic anemia with extreme microcytosis. The disease is characterized by extreme microcytosis with the mean corpuscular volume of only 25 cu. μ. and the membrane Ca^{2+} content was increased which appeared to cause reduced deformability with resultant fragmentation. Recently Zarkowsky et al.[43] reported 3 cases with similar red cell morphology as congenital hemolytic anemia with thermal sensitivity, because the erythrocyte morphology resembled that associated with thermal injury. Zarkowsky et al. postulate that the abnormally shaped cells are probably the result of membrane budding occuring at body temperature as suggested by the fragmentation of the patients' cells when incubated 37°C.

HEMOGLOBINOPATHIES AND THALASSEMIAS

Molecular basis of hemoglobinopathies and thalassemias is shown in Table 7. Single amino acid substitution has been the most common form of hemoglobinopathies. Recently, chain termination mutation and

Table. 7 Molecular basis of hemoglobinopathies and thalassemias.

Point mutation	
Single amino acid substitution	Sickle cell anemia
Chain termination mutation	Hb Constant Spring
Frame shift mutation	Hb Wayne
Unequal crossing over	
Triplet deletion	Hb Tochigi
Fusion gene	Hb Lepore
Gene deletion	α-Thalassemia (hydrops fetalis)

more interestingly, frame shift mutation have been found, while in severe form of thalassemia gene deletion was discovered. Thalassemia appears to be the most exciting field in molecular biology right now.

Thalassemias:

Until recently it was thought that, except for the rare Lepore hemoglobins, there are no structural variants which cause the clinical picture of thalassemia. This is not the case, however, and intriguing types of hemoglobin variants called chain-termination mutant and frame shift mutation have been discovered[44]. Hb Constant Spring was found to have elongated α-chains with 31 extra residues attached to the end[45]. In this case, the single base change is in the terminating codon UAA which is changed to CAA as shown in Fig. 5. CAA is the code word for glutamine.

Position on Chain	137	138	139	140	141	142	143	144	145	146	147	148·····172
C.S.	Thr	Ser	Lys	Tyr	Arg	Gln	Ala	Gly	Ala	Ser	Val	Ala·····Glu
Icaria	Thr	Ser	Lys	Tyr	Arg	Lys	Ala	Gly	Ala	Ser	Val	Ala·····Glu
A	Thr	Ser	Lys	Tyr	Arg							
Wayne	Thr	Ser	Asn	Thr	Val	Lys	Leu	Glu	Pro	Arg		
C.S.	ACC	UCU	AAA	UAC	CGU	CAA	GCU	GGA	GCC	UCG	GUA	GCA·····
Icaria	ACC	UCU	AAA	UAC	CGU	AAA	GCU	GGA	GCC	UCG	GUA	GCA·····
A	ACC	UC[U]	AAA	UAC	CGU	UAA	GCU	GGA	GCC	UCG	GUA	GCA·····
"frame shift"												
Wayne	ACC	UCA	AAU	ACC	GUU	AAG	CUG	GAG	CCU	CGG	UAG	CA

Fig. 5 Chain termination mutation and frame shift mutation of α-globin chain. Hb Constant Spring (C.S.), Hb Icaria and Hb Wayne.

Actually, normal messenger-RNA (mRNA) has extra base sequ ences which is not normally translated, but in the case of Hb Constant Spring, this extra material is translated until another 'stop' coden is reached. This fanciful explanation has been confirmed recently by the discovery of two further mutant hemoglobin, Hb Wayne[46] and Hb Icaria[47]. Hb Wayne also has an elongated α-chain but in this case the chain is normal only up to position 138 and then its structure changes and it ends at position 146. This variant appears to have resulted from a process known as a 'frame shift' (Fig. 5). A frame shift is caused by the loss of a single base so that the code words get displaced, resulting in the production of a completely new sequence.

When Hb Constant Spring was discovered it was predicted that similar hemoglobin might exist which are idential in structure except that they have amino acids other than glutamine at position 142, because

it is theoretically possible for a single base change in the chain-terminating codon to code for several amino acids other than glutamine. This prediction has been confirmed in 1974 by Clegg et al.[47] with the discovery of Hb Icaria. This variant is exactly the same as Hb Constant Spring except that instead of glutamine at position 142 it has lysine as shown in Fig. 5. In these interesting hemoglobinopathies the abnormal chains are produced at a reduced rate, hence, these belong to thalassemias.

Thalassemias can be divided into two main types, α or β, depending on which chain is inefficiently synthesized. Also, both types can be further subdivided, depending on whether there is a total absence or only a partial reduction in the amounts of globin chain produced[48].

With respect to α-thalassemia, before 1971, it appeared that the inheritance of Hb H disease followed a consistent pattern involving just two α-thalassemia genes, one "severe" gene called α-thal-1 gene and the other "mild" gene called α-thal-2 gene. In 1968 trace amounts of an Hb Constant Spring were noted in some patients with Hb H disease[45]. Subsequent work has shown that it occurs in 4% of the population of Thailand and is probably common in the Chinese and Malay populations of Malaysia[48,49,50] and that it has been found in Greece. Thus, both forms of Hb H disease result from the "severe" α-thal-1 gene either with a second α-thal-2 gene or with a Hb Constant Spring gene.

In thalassemias, the globin chains are synthesized either at a reduced

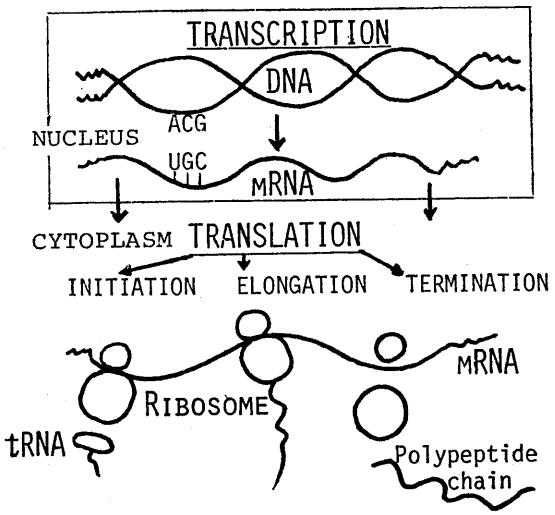


Fig. 6 Genetic control of protein synthesis.

rate or not at all. It is clear that the abnormality could be expressed either at the translational level in the cytoplasm or within the nucleus at the level of transcription of mRNA or at the gene itself as shown in Fig. 6. It has become possible to use some of the newer methods of molecular biology to study these problems. Because it is easier to study the cytoplasm of cell than its nucleus, the earliest experiments examined the processes of globin-chain initiation, translation and termination. And with the development of methods for study mRNA and DNA the trail has gradually led back to the globin genes.

The early study concerning the processes of translation have suggested that a deficiency of mRNA occurs in thalassemias[51]. In the next step, mRNA from reticulocytes were isolated and added in excess to a protein-synthesizing preparation from either frog oocyte or wheatgerm to see the production of human globin chains[52]. It has become clear from these experiments that there is either a deficiency of mRNA or an abnormal mRNA in thalassemia red cells. To distinguish between these possibilities it became necessary to measure the amount of mRNA in the human red cell.

This became possible with the development of techniques for synthesizing DNA on an RNA template using reverse transcriptase[53,54]. Using human mRNAs as a template, in 1973, Kacian et al.[53] and Housman et al.[54] were able to produce radioactive complementary DNA

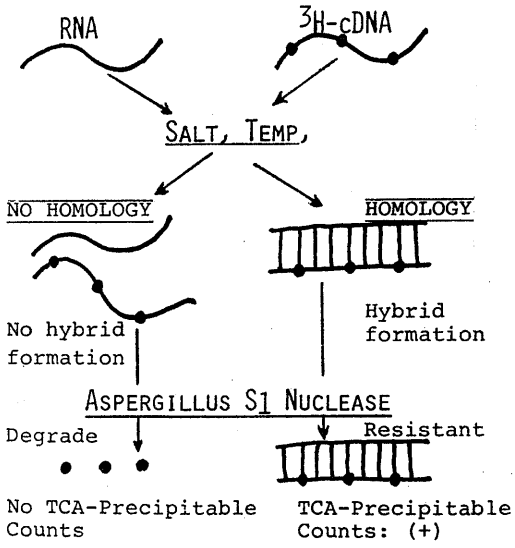


Fig. 7 Quantitation of messenger RNA using mRNA/cDNA hybridization assay.

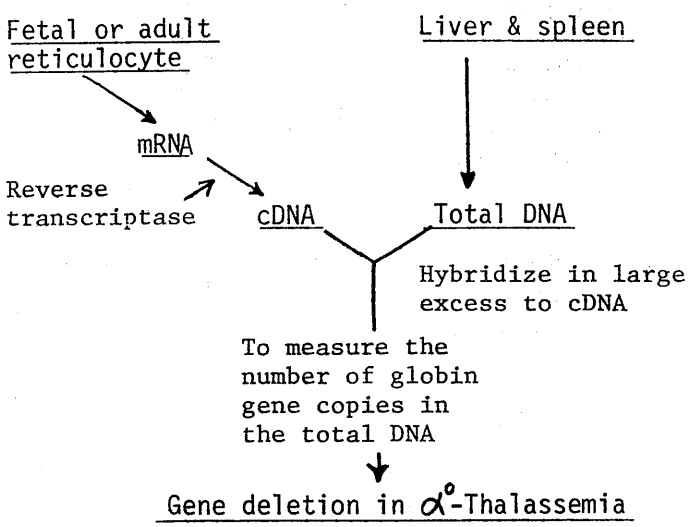


Fig. 8 Schematic representation of DNA/DNA hybridization technique which revealed α-chain gene deletion in severe form of α-thalassemia, α-thal-1 homozygote (α°-thalassemia).

(cDNA) and used these as probes to measure the amount of mRNA in thalassemic cells. It turns out that in β-thalassemic red cell precursors there is a deficiency of β-mRNA and in α-thalassemia red cell precursors there is a deficiency of α-mRNA, when examined by the cDNA/mRNA hybridization technique (Fig. 7).

In a severe form of α-thalassemia, namely α-thal-1 homozygote (α° thalassemia), no α-chains are produced and no α-chain mRNA is detectable. The question about whether α-chain genes are deleted has been answered quite recently by the technique of DNA/DNA hybridization by Ottolenghi et al.[55] and Taylor et al.[56] as shown in Fig. 8. It has been known that if total DNA is prepared from animal tissues and hybridized in large excess to cDNA made from globin mRNA, it is possible to measure the number of globin-gene copies present in the total DNA. They prepared total DNA from fetal liver and spleen which are rich in erythroblasts and complementary DNA from reticulocytes and hybridized in large excess to cDNA. The experiment clearly revealed that the α-chain genes are absent from the chromosomes of affected individuals. This exciting finding is the first time that a gene deletion has been shown to be the cause of human genetic disorder.

Then what is the molecular basis of α-thal-2? cDNA/mRNA hybridization experiments showed a genuine reduction in the amount of α-

Table. 8 Molecular defects in thalassemias[60].

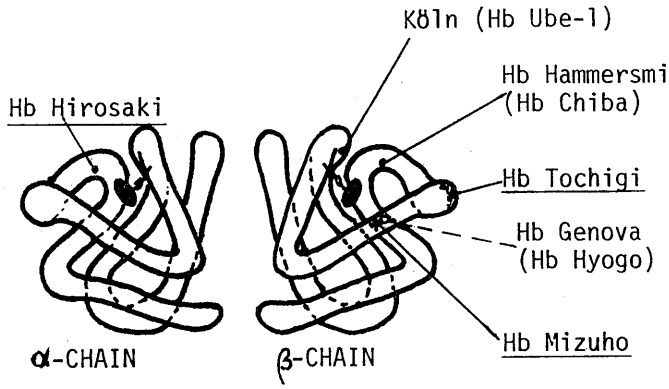
1. Gene deletion or absence of transcription (α°-& β°-thalassemia).
2. Decreased transcription (β^{+}-thal.)
3. Abnormal processing or abnormal transport of mRNA.
4. Synthesis of mRNA structurally abnormal in its untranslated regions.

chain mRNA in cells of persons with Hb H disease which is caused by one α-thal-1 gene and one α-thal-2 gene[57]. This result suggests a transcriptional defect in α-thal-2. In β°-thalassemia the absence of mRNA was confirmed[58].

Molecular defect in thalassemias is summarized in Table 8. Other than gene deletion or absence of transcription as mentioned above decreased transcription, abnormal processing or abnormal transport of mRNA and synthesis of mRNA structurally abnormal in its untranslated regions are the possibilities[59,60]. The use of the latest techniques of nucleic acid hybridization should soon allow exploration of many of these possibilities, so that we may expect within the next year or two an explanation of the molecular basis of most of the thalassemias.

Unstable hemoglobin hemolytic anemias:

With respect to the unstable hemoglobin hemolytic anemias, I don't think there has been much remarkable progress in the last couple of years. So, I would like to mention the unstable hemoglobins found in



Hb Hirosaki= CD1 43 Phe→Leu; Köln= FG5 98 Val→Met; Hammersmith= CD1 42 Phe→Ser; Tochigi= D7-E3 56-59 deleted; Genova= B10 28 Leu→Pro; Mizuho= E12 68 Leu→Pro

Fig. 9 Six unstable hemoglobins found in Japan.

Japan. Six unstable hemoglobins have so far been found in Japan, in which 3 turned out to be the same ones which have already been reported in Europe. These are Hb Ube-1[61] (Hb Köln), Hb Chiba[62] (Hb Hammersmith) and Hb Hyogo[63] (Hb Genova). Three others are the ones which were originally reported in Japan. These include Hb Tochigi[64], Hb Mizuho[65] and Hb Hirosaki[66] (Fig. 9).

List of 60 reported unstable hemoglobins is shown in Table 9.

Table. 9 List of known unstable hemoglobins.

α-CHAIN		β-CHAIN			
	A3 or A4	Leiden		E18	Shepherds Bush
	A11	Sogn		E18	Atlanta
	A12	Belfast		E18-19	St. Antoine
	A14-15	Lyon	Ann Abror	F1	Buenos Aires
	B5	Freiburg		F3	Tours
	B6	Riverdale-Bronx		F4	Santa Ana
	B6	Moscva		F4	Borås
	B6	Savannah	Etobicoke	F5	
	B10	Genova		F7	Sabine
	B10	St. Louis		F8	Istanbul
	B12	Volga		F8	Saint Etienne
	B12	Tacoma		F7-FG2 orF8-FG3	
	B14	Perth		or F9-FG4	Gun Hill
	B14	Castilla		FG5	Köln
	C1	Philly		FG5	Nottingham
Torino	CD1	Hammersmith		FG5	D Jelta
Hirosaki	CD1	Bucuresti		G3	Rush
	CD1-3 or CD2-4	Niteroi		G8	Casper
L Ferrara	CD5			G13	Peterborough
Hasharon	CD5			G17	Madrid
	CD7	Okaloosa	Daker	G19	
	D7-E3	Tochigi		H5	Erie
	E6	Duarte		H8	Wien
	E7	Zürich		H9	Leslie
	E10	I Toulouse	Bibba	H19	Coventry
	E11	Sydney		HC2-3	
	E11	Bristol		(elongated)	Cranston
	E12	Mizuho	γ-CHAIN		
	E14	Seattle			
	E15	Christchurch	F Pool	H8	

Cyanate in the treatment of sickle cell anemia:

I am not familiar with sickle cell anemia. So I will only briefly mention about the treatment of this disorder with cyanate[67].

Great effort has been made for its therapeutic potential for these last several years, but it has turned out to be certain that cyanate's toxicity, such as peripheral neuropathy and cataracts precludes its further clinical use at least by the oral routs. Furthermore, at present no study clearly substantiates the claim that cyanate decreases the incidence of painful crisis. On the other hand, treatment of patients by extracorporeal carbamylation of hemoglobin might be promising, but it has the obvious drawbacks of expense and commitment of time for both the patient and medical personell.

HEREDITARY HEMOLYTIC ANEMIAS DUE TO ERYTHROCYTE ENZYME ABNORMALITIES

As shown in Table 10 and Fig. 10, there are 16 erythrocyte enzyme

Table. 10 List of enzymes in which hereditary erythrocyte enzyme deficiency (anomaly) hemolytic anemia are known to exist. Lactate dehydrogenase deficiency does not cause hemolysis.

EMBDEN-MEYERHOF PATHWAY & RAPOPORT-LUEBERING CYCLE
1. Hexokinase
2. Glucosephosphate isomerase
3. Phosphofructokinase
4. Aldolase
5. Triosephosphate isomerase
6. Phosphoglycerate kinase
7. Pyruvate kinase
8. Lactate dehydrogenase*
9. 2, 3-Diphosphoglyceromutase
HEXOSEMONOPHOSPHATE PATHWAY & GLUTATHIONE METABOLISM
10. G6PD
11. Glutathione reductase
12. Glutathione peroxidase
13. Glutathione synthetase
14. γ-Glutamylcysteine synthetase
OTHERS
15. Adenylate kinase
16. Pyrimidine 5'-nucleotidase

*No hemolysis

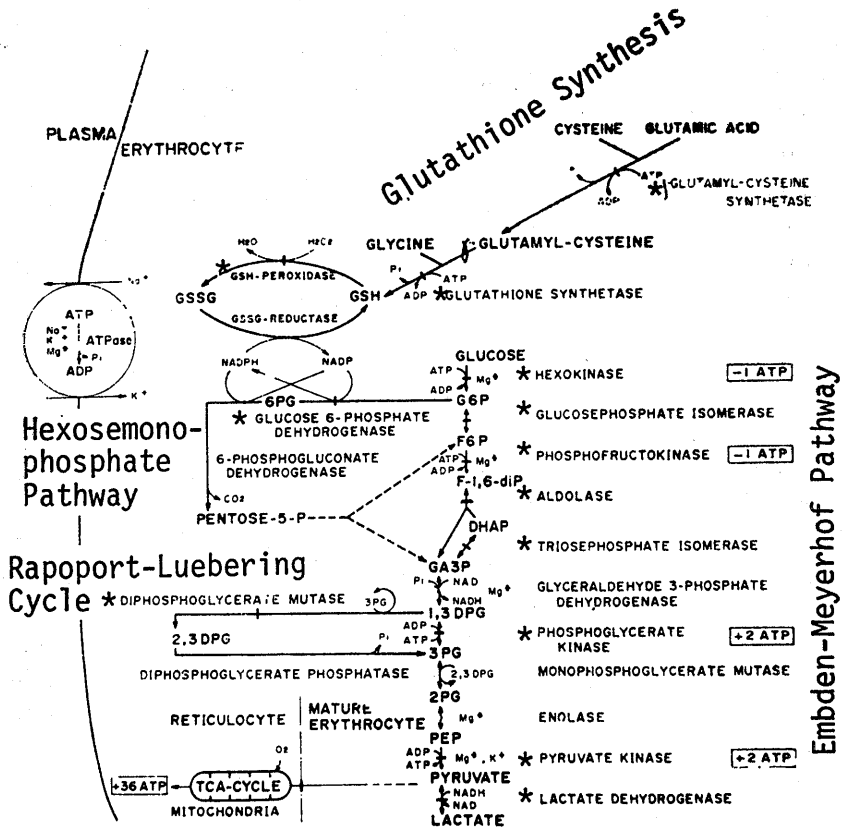


Fig. 10 Main metabolic pathways of mature erythrocytes and reticulocytes. Asterisks indicate enzymes in which hereditary deficiencies are known to exist. All these enzyme deficiencies except for lactate dehydrogenase deficiency can cause chronic hemolytic anemia.

anomalies, in which either homozygote or hemizygote have clearly been described. Except for lactate dehydrogenase deficiency[68], all these 15 enzyme anomalies are associated with hemolytic anemia. Of these deficiencies glucose 6-phosphate dehydrogenase (G6PD) deficiency is the most common followed by pyruvate kinase deficiency and the third most common being glucosephosphate isomerase deficiency. Of these deficiencies, unequivocal homozygous glutathione reductase deficiency was reported quite recently by Loos et al.[69].

I excluded the reported but generally not accepted 5 enzyme deficiencies, namely glyceraldehyde 3-phosphate dehydrogenase[70], enolase[71], 6-phosphogluconate dehydrogenase[72], 2, 3-diphosphoglycerate phosphatase[73], and adenosine triphosphatase (ATPase)[74] from the previous list[75].

These are not generally accepted because of insufficient description although in some of which heterozygotes certainly exist such as in case of glyceraldehyde 3-phosphate dehydrogenase[76]. Hopefully, unequivocal homozygote for variant gene of these enzymes will be discovered in the near future.

I mentioned that except for lactate dehydrogenase deficiency all the enzyme deficiencies in Embden-Meyerhof pathway cause hemolytic anemia. Why doesn't lactate dehydrogenase deficiency cause the symptom? In case of the patient with homozygous H-subunit of lactate dehydrogenase (LDH) deficiency[68,77], LDH-1, 2, 3 and 4 bands are lacking and only LDH-5 band which correspond to liver-type is visible in all the tissues we examined including erythrocytes. Determination of erythrocyte glycolytic intermediates showed marked accumulation of glyceraldehyde 3-phosphate, dihydroxyacetone phosphate and fructose 1, 6-diphosphate indicating that glyceraldehyde 3-phosphate dehydrogenase step in glycolysis is disturbed in someway while pile up of the intermediates previous to LDH step such as phosphoenolpyruvate and 2-phosphoglycerate was not seen[77] (Fig. 11). It appears that the pyruvate which should accumulate due to LDH deficiency might diffuse out of the cell and therefore accumulation of phosphoenolpyruvate and 2-phosphoglycerate did not occur whereas relative deficiency in NAD caused by the block of LDH reaction resulted in the block in glycolysis at the glycer-

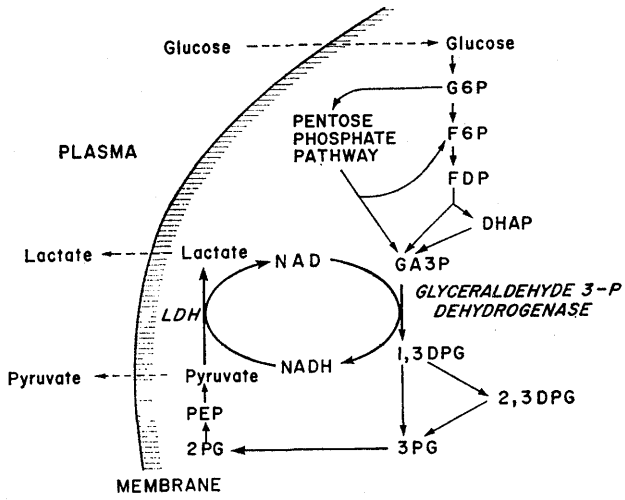


Fig. 11 Schematic representation of metabolic disturbance seen in lactate dehydrogenase deficiency. Relative decrease in NAD may play a role upon accumulation of GA3P, DHAP and FDP.

aldehyde 3-phosphate dehydrogenase step though not harmful to overall erythrocyte glycolysis as shown in Fig. 11.

Molecular basis:

In at least two of G6PD variants single amino acid substitution was found by Yoshida[78,79]. In almost all the cases of G6PD deficiency, pyruvate kinase deficiency and glucosephosphate isomerase deficiency, the characteristics of the deficient enzymes are clearly different from the normal, indicating that the deficiency is caused by structural gene mutation and not by decreased synthesis of normal enzyme protein[75]. The latter mechanism is just the same in the situation of thalassemias and it is possible that the deficiency caused by this mechanism may exist. But it appears to be difficult to state that the certain enzyme deficiency is definitely due to quantitative deficiency caused by decreased production of the normal enzyme molecule, because in most of the enzymes the amino acid sequences have not been clarified.

LDH deficiency discussed before is of some interest in this connection. As mentioned before, LDH in the propositus showed only one band, LDH-5. In case the propositus produced mutant inactive H-subunit which has the ability to hybridize with L-subunit, then the stainable bands should be detected in LDH-4, LDH-3 and possibly in LDH-2. But this was not the case. Hence it appears that in this case the deficiency is caused either by no or greatly reduced production of normal LDH-H subunit or production of mutant inactive H-subunit which lacks the ability to make hybrid with L-subunit. This point needs further study and the immunological approach seems promising.

In pyruvate kinase deficiency there is a claim that the enzyme deficiency is merely an epiphenomenon due to unknown primary cause[80,81,82], but it appears quite unlikely.

Pyrimidine 5'-nucleotidase deficiency:

In 1974, quite interesting erythrocyte enzyme deficiency, called pyrimidine 5'-nucleotidase deficiency, was discovered by Valentine et al.[83] Ben-Bassat et al.[84], Oda and Tanaka[85], Beutler et al.[86], Rochant et al.[87] reported cases subsequently and quite recently Miwa et al.[88] found three Japanese cases.

According to Valentine et al., the syndrome is characterized by marked increase in red cell basophilic stippling, total nucleotides and the patient's erythrocyte uniquely contained large amounts of pyrimidine 5'-nucleotides. As shown in Table 11, total nucleotides in patient cells were present in amount 3 to 6 times greater than normal, and more than 80% were pyrimidine-containing, while normal red cells contained less

Table. 11 Concentrations of total nucleotide, pyrimidine nucleotide and enzyme activity of pyrimidine 5'-nucleotidase in erythrocytes of patients with pyrimidine 5'-nucleotidase deficiency[83].

	Total nucleotide (μM/10^{10}RBC)	Pyrimidine nucleotide (%)	Enzyme activity (% of normal)
Patient R.	8.5	86	11
Tr.	8.8	83	6
Control	2.0	3	100

(By Valentine et al)

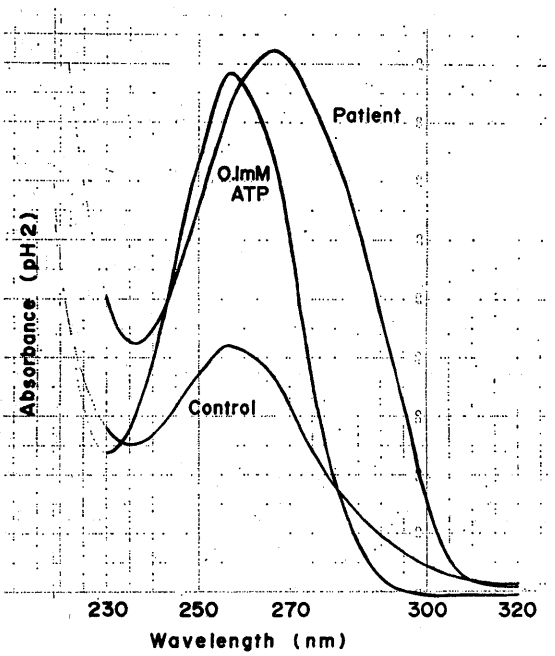


Fig. 12 Spectral pattern of deproteinized red cell extractes of patient with pyrimidine 5'-nucleotidase deficiency and of control subject.

than 3%[83]. The activity of pyrimidine 5'-nucleotidase in the patients' red cells were only 6 to 11% of that of normal subjects.

The ultraviolet spectral curve of deproteinized red cell extract of our patient is shown in Fig. 12. As Valentine et al. indicated it exibits a shift in maximum absorbance from the usual 256-257 nm which corresponds to the absorbance of ATP to approximately 266-270 nm. This is characteristic for the absorbance of pyrimidine nucleotides and provides

the basis of an easily performed screening procedure.

It is postulated that accumulation of degradation products of ribosomal RNA, namely pyrimidine nucleotides results in basophilic stippling. Pyrimidine 5'-nucleotidase is unique in that it appears to exist only in red cells. Mature human red cells are anucleated cells and lack ribosomes and hence RNA is a disused substance. It probably is necessary to remove this useless substance, and pyrimidine 5'-nucleotidase appears to be the enzyme specifically to perform this job while it does not attack adenine nucleotides which are essential substances for the red cell energy metabolism.

Paglia et al.[89,90] indicated that the enzyme was inhibited by very low concentration of lead, and in addition, subjects with chronic lead intoxication secondary to industrial exposure exhibited substantially decreased pyrimidine 5'-nucleotidase activity. This result is quite interesting because the basophilic stippling and premature erythrocyte hemolysis seen in chronic lead poisoning are analogous to that encountered in the hereditary deficiency of this particular enzyme.

The mechanism by which a hemolytic syndrome is produced is complex and unknown at the moment. Several mechanisms can be speculated[83]. First of all, the presence of uridine and cytidine di- and triphosphates, might act as competitive co-factors capable of occupying binding sites of enzymes such as hexokinase, pyruvate kinase and phosphoglycerate kinase, where ADP and ATP are preferred and much more efficient. The possibility of deleterious feedback inhibitions, conceivably mediated by cytidine and uridine nucleotides, had been virtually unexplored.

From the diagnostic standpoint, Ben-Bassat et al.[84] pointed out an practically important point. They noticed that after incubating the patient's blood in EDTA for more than 3 hours, basophilic stippling disappeared. Therefore unless smears made from capillary or heparinized blood are screened, this important clue can be overlooked.

Quite recently I personally heard from Valentine[91] that he found new type of hereditary hemolytic anemia apparently caused by remarkably excess amount of adenosine deaminase.

Immunological methods in the study of mutant enzymes:

Immunological methods have been increasingly applied recently in the study of mutant enzymes[92,103]. The purpose is twofold. One is to find out the diffesence in antigenicity of mutant enzymes. The other is to quantitate mutant enzyme proteins accurately.

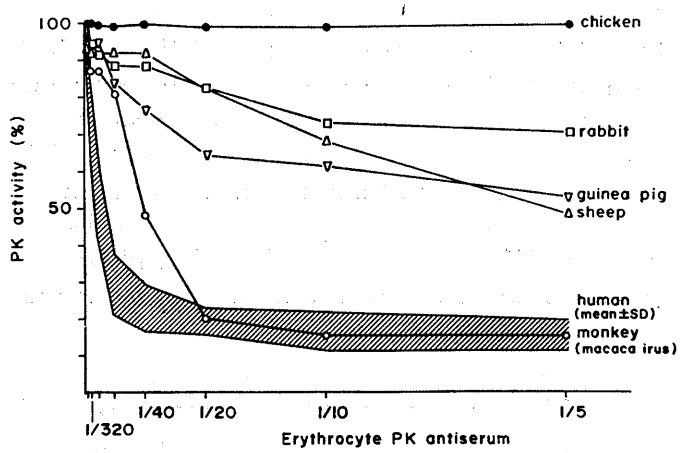


Fig. 13 Neutralization test by anti-human erythrocyte pyruvate kinase antiserum. Results of red cell pyruvate kinases obtained from various animals. Shaded area=normal range.

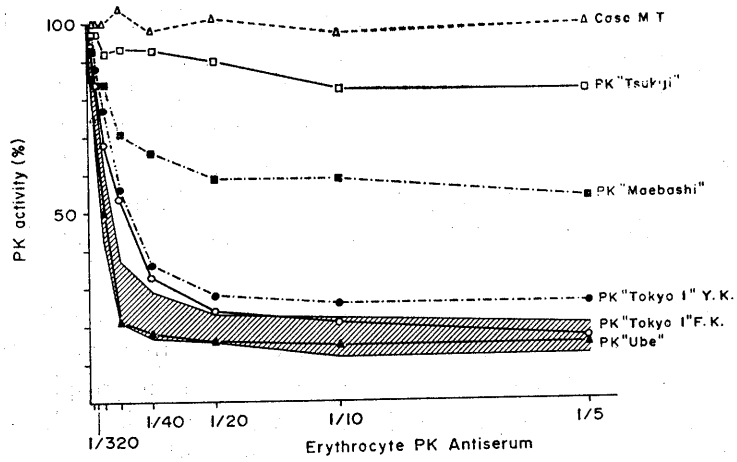


Fig. 14 Results of neutralization test by anti-human erythrocyte pyruvate kinase antiserum of patients with pyruvate kinase deficiency. Shaded area= normal range.

The result of the neutralization test of erythrocyte pyruvate kinase of animals and of human using anti-human erythrocyte pyruvate kinase rabbit antiserum is shown in Fig. 13. The hemolysates of different animals showed the varying degrees of neutralization indicating the difference of antigenicity. The result of the neutralization test of several

pyruvate kinase variants is shown in Fig. 14[101]. PK Tsukiji was scarcely neutralized, while that of PK Tokyo-I was neutralized almost to the same degree of the normal, indicating that in PK Tsukiji the antigenicity is different from the normal. It appears that the immuno-neutralization test can be used to see whether there is difference in antigenicity of the mutant enzyme.

It is quite difficult to measure directly the specific activity after purification of mutant enzyme by chemical means. Hence it is hoped that the immunological indirect measurement of the enzyme protein to the variant gene produces enzyme protein with low activity or produces enzyme protein with normal characteristics at a reduced rate. Yoshida[92] had attempted a quantitative neutralization of G6PD by specific antibody to G6PD. However, recently Nakashima and Yoshida[103] reevaluated the test, and have reached the conclusion that at least in order to measure

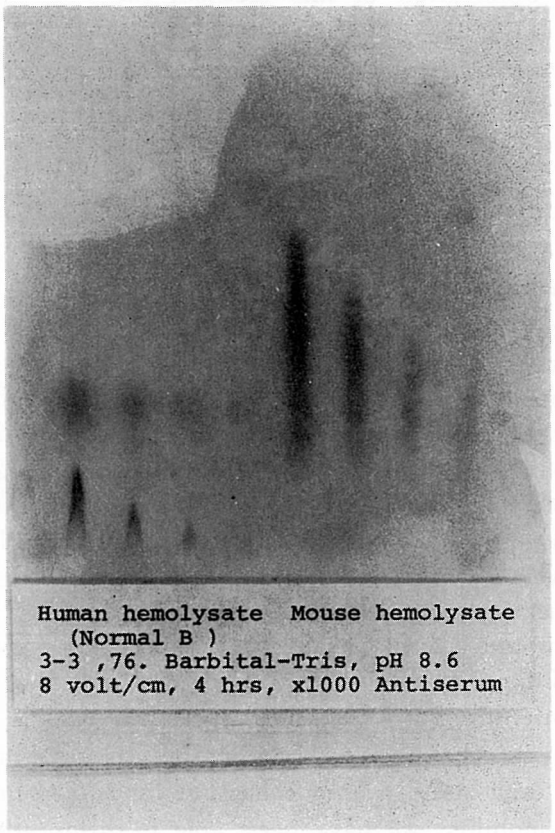


Fig. 15 Electroimmunodiffusion pattern of erythrocyte G6PD of the human and of the mouse using anti-human erythrocyte G6PD antiserum.

the enzyme protein by the neutralization test, the enzyme to be examined should have the same affinity to antibody as normal control. Furthermore, the mutant enzyme should not have non-immunological inactivation during incubation period.

Electroimmunodiffusion pattern of erythrocyte G6PD of the human and of the mouse is shown in Fig. 15. Normal human hemolysate shows linear correlation between the activity and the precipitation area, and we can quantitate a minute quantity of enzyme protein by this method. However, the G6PD of the mouse hemolysate moves faster and does not make clear precipitation areas, so that it is impossible to quantitate mouse G6PD by using anti-human G6PD serum by this method. Nakashima and Yoshida[103] obtained the similar abnormal pattern in the human G6PD variant, G6PD Manchester. An antigen with lower affinity has a tendency to make larger precipitation area than the normal because such antigen moves further along the plate as it needs more antibodies. Hence, for the application of this method, the enzyme protein should have identical antigenicity to the normal enzyme.

If the variant enzyme has an identical antigenicity it is able to measure the enzyme protein by this method. But, at least some, if not all, variant enzymes such as G6PD Manchester do have different antigenicity. That means that we have to be careful about applying the electroimmunodiffusion method or other immunological methods to quantitate the variant enzyme.

Pyruvate kinase deficiency:

Until recently the study of pyruvate kinase variants has been hampered by the lack of a suitable method of electrophoresis, one of the standard methods for differentiating the structurally abnormal enzyme protein from the normal, because of the instability of the enzyme and the lack of a suitable positive staining method. In 1972 Imamura and Tanaka[104] developed a good method using thin layer polyacrylamide gel. The electrophoresis was soon applied to one pyruvate kinase deficient subject, the liver of which clearly revealed slowmoving L-type PK as compared to the normal[105]. The red cell of this variant, PK Tokyo-I also showed the slow moving PK R_1 and R_2 while other variants such as PK Kiyose and PK Sapporo showed fast moving PK R_1 and R_2 and classical PK had no stainable PK-R[101].

Using other biochemical parameters such as substrate affinity, velocity maximum, urea stability, neutralization by antiserum as well as the electrophoretic mobility, we have been able to differentiate at least 9 PK variants[106]. Some of them are shown in Table 12.

Table. 12 Various pyruvate kinase variants. Enzyme characteristics and clinical symptoms.

	Electro-phoresis	KmPEP	Vmax	Urea stability	Clinical symptoms
PK Tokyo II	Fast	Low	Low	Stable	Mild
PK Sapporo	Fast	High	High	Labile	Mild-moderate
PK Nagasaki	Slow	High	High	Labile	Moderate
PK Maebashi	Normal	High	Very low	Labile	Moderate
PK Osaka (Classical)	Slow*+M_2	–	–	–	Severe

*PK Osaka had extremely low activity and the patient's PKs were composed mainly of M_2-type presumably due to compensatory production.

In 1975 we defined a classical type PK deficiency on the basis that the usual red cell PK (PK-R_1 and R_2) was not demonstrable by electrophoresis but instead M_2-type PK was present in hemolysate free from leukocytes and platelets, presumably by compensatory process[101].

Quite recently, we were able to detect a slow moving PK-R after partial purification, and in addition this abnormal PK-R had abnormal kinetics[107]. This means that in at least some, if not all, of the so-called classical type of PK deficiency, which had been considered to be a quantitative deficiency, were caused by the production of the variant enzyme with markedly low activity.

In collaboration with Dr. Black, we examined the blood of PK deficient Basenji dog[102]. It revealed that the erythrocyte PK of the dog was composed of only M_2. We were not able to do further examination because of the limitation of the blood specimen, but it is quite likely that if the further analysis is made using partially purified enzyme, very low active variant PK might be found.

As to the characterization of G6PD variants, standardization of procedures has been established in 1967 by a WHO scientific group[108]. As pyruvate kinase deficiency is the second most common enzyme deficiency which causes hemolytic anemia, there is a trend to standardize the procedures for the characterization of pyruvate kinase variants, and there are at least several pyruvate kinases which show different biochemical characteristics. Although it will not be easy to define a standard set of conditions that would be acceptable to all investigators, it would be most useful and hopefully, this can be accomplished fairly soon.

As compared to the characterization of G6PD variants, certain difficult problems exist. The most difficult one is that while G6PD deficiency is a sex-linked disorder and hemizygous subjects are available, pyruvate

kinase deficiency has autosomal recessive inheritance. If the patient is a pure homozygote for a single mutant gene, then it is not difficult to characterize the variant, but in case the patient is double heterozygous for two different mutant genes, it is pretty difficult to do so. This may imply that in order to characterize all of the pyruvate kinase variants it is necessary to have a good method to separate one mutant enzyme from the other. It is by no means easy.

Glader[109] reported that when pyruvate kinase deficient erythrocytes were incubated with salicylate 2 to 30 mg/100ml, there was a marked decrease in ATP. Salicylate is known to uncouple mitochondrial oxidative phosphorylation, and it is also known that viability of pyruvate kinase deficient reticulocytes depends on ATP by mitochondrial metabolism[110]. Glader's data suggest that aspirin therapy may aggravate hemolysis in patients with pyruvate kinase deficiency.

Quite recently Burge et al.[111] reported the first case of neutrophil pyruvate kinase deficiency associated with lifelong recurrent staphylococcus infections due to intracellular bacterial killing defect. The isozyme of pyruvate kinase in neutrophils must be M_2-type which is distributed in many other organs. The mutant M_2-type PK was said to be unstable with normal electrophoretic mobility but kinetic studies could not be performed. No functional defect in other organs was noticed. Since PK isozyme in red cells and leukocytes are different, it is understandable that this patient did not show hemolytic anemia. Before the discovery I was thinking that M_2-type pyruvate kinase deficiency might exist but it might not be compatible with life because M_2-type is so widely distributed in human tissues. Burge et al. postulated the mechanism of bacterial killing defect in this case as follows. Decreased lactate formation due to pyruvate kinase defect results in difficulty in maintaining acidic pH in phagocytic vacuoles. As acidic milieu is necessary for bacterial killing, pyruvate kinase defect causes recurrent staphylococcus infections.

Glucosephosphate isomerase deficiency:

Glucosephosphate isomerase deficiency is the third most common enzyme deficiency[112]. About 30 cases have already been known. Variant enzymes show different electrophoretic mobility. It is interesting to note that most cases have normal enzyme kinetics, and all the mutant enzymes associated with hereditary hemolytic anemia are thermolabile. The mechanism of hemolysis in this deficiency is schematically shown in Fig. 16. Mature erythrocytes cannot synthesize enzyme protein and hence mutant thermolabile glucosephosphate isomerase is inactivated and results in disturbance in energy metabolism with resultant early demise of the red

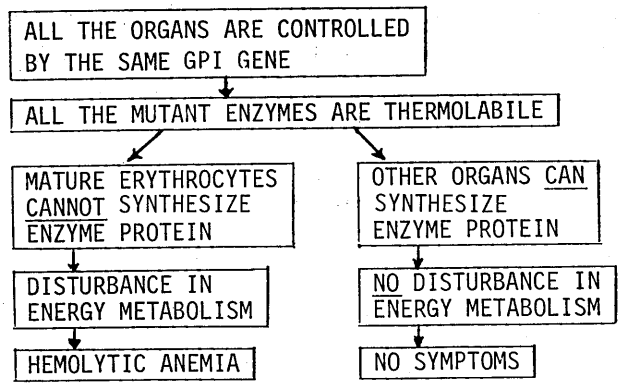


Fig. 16 Schematic representation of mechanism of hemolysis in glucosephosphate isomerase deficiency.

cells while other organs can synthesize enzyme protein to compensate for the rapid decay of the enzyme resulting in no disturbance in energy metabolism with no symptoms. However, it is interesting to note that in a particular variant, GPI Utrecht, the liver biopsy specimen showed the histologic characteristics of glycogen storage disease[113]. This appears to be due to marked functional disadvantage of this particular variant enzyme.

FUTURE PROSPECT

As mentioned before, standardization of procedures for characterization of mutant enzymes is necessary.

In two of the G6PD variants, the amino acid substitution has been shown[79,80]. But to do so a large amount of blood was required and it is almost impossible to apply it to patients with anemia. Refinement of purification procedure which requires less amount of blood is badly needed. Amino acid sequence analysis in normal enzyme such as G6PD is also necessary in order to completely understand the structure and function of the enzyme as well as to understand the mechanism of malfunction of the variant enzyme. This is now in progress in Yoshida's laboratory[114].

Preservation of mutant enzymes is also an important task. In case the isozyme distribution is the same in erythrocyte and in the skin, then skin biopsy followed by the culture of the skin fibroblasts is a good way of preservation of rare mutant enzymes. But in pyruvate kinase deficiency the isozyme distribution is different between the skin and erythrocyte and this method cannot be applied.

If we could get a good animal model such as the Basenji dog in the case of pyruvate kinase deficiency, this certainly would be a very good tool for elucidation of the mechanisms of hemolysis as well as for therapeutic trials.

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