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Altered Electrophoretic Mobility of the Human Erythrocytic Glucose-6-phosphate Dehydrogenase as a Function of their *in vivo* Ages

by

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In the pentose phosphate shunt pathway, the first oxidation step is catalyzed by glucose-6-phosphate deydrogenase [Glucose-6-phosphate: NADP oxido-reductase; G6PD, E.C. 1.1.1.49] which requires NADP as its coenzyme for maintenance of its stability (Kirkman, 1962, a). It exists largely in the form of a hexamer which magrates on starch gel electrophoresis as a single protein band (Kirkman, 1962, a, b; Yoshida, 1966): and it is reported, though unconfirmed, that it contans two different N-terminal amino acids (Chung and Langdon, 1963), suggesting that the molecule has at least two different kinds of polypeptide chain and that they might represent Kirkman and Hendrickson's (1962) active dimer.

Apart from the nature of the enzyme, its deficiency is probably the most prevalent, clinically significant, and genetically determined abnormality of man, the different forms of which are inherited as a series of X-linked mutations of recessive trait (Childs *et al.*, 1958; Chung and Langdon, 1963), manifesting varying degrees of enzyme deficiency and clinical conseuences (Beutler, 1966, 1967). Decreased red cell G6PD activity was practically demonstrated (Carson *et al.*, 1956) to be the defect underlying the well-known phenomenon of "primaquine sensitivity". Furthermore, some cases of congenital nonspherocytic anemia were formed to be due to the deficiency of the enzyme (Shahidi and Diamond, 1959).

It is a well-established fact, therefore, that the structural variants of the enzyme, recognizable by their altered electrophoretic mobilities, are also inherited as a X-linked trait.

If these two kinds of genetic anomalies are due to mutations of regulatory and structural genes, 2-deoxy-G6P as widely suggested, their two loci must be linked closely (Parker and Bearn, 1964). It is, therefore, very important to evaluate its electrophoretic behavior, since its genetic polymorphism is well reflected in the electrophoretic profile of activity (Porter *et al.*, 1964), together with other properties, such as Km's for glucose-6-phsophate (G6P)

and for NADP, rates with 2-deoxy-G6P and with galactose-6-phosphate and effect of *p*H on activity of erythrocytic G6PD (Kirkman, 1962). However, a few report (Fildes and Parr, 1968; Hopkinson *et al.*, 1963) have indicated that the *in vitro* storage of human red blood cells leads to an increased electrophoretic mobility of the enzyme, compared with the same enzyme obtained from fresh blood. Similar finding for hemoglobin derived from the fresh and stored red cells parallels the above reports (Hulsman and Dozy, 1962). The author was deeply interested in the phenomenon, the slight difference of phenotypic expression observed with the electrophoretic mobilities of the enzyme might be errorneously suggested to be of different genotypic origins.

To clarify the underlying reason of the faster electrophoretic mobility of G6PD obtained from the stored red blood cells, the present investigation was undertaken with the use of young and old human red blood cells of their *in vivo* ages, to confirm the *in vitro* findings previouly described.

MATERIALS AND METHODS

Materials:

Fresh whole donated by the healthy adult volunteers were used regardless of sex. NADP and G6P were provided by Sigma Chemical Company and EDTA by Calbiochem. DEAE-cellulose were the commercial product of Eastman Kodak. Phenazine methosulfate and nitrobluetetrazolium were purchased from Nutritional Biochemicals Corporation.

Separation of Young and Old Erythrocytes:

Heparinized whole blood were centrifuged in a narrow tube (diam, 1.0 cm) at 2,000 x g for 45 min.. The buffy white blood cell layer and supernatant plasma were removed from the remaining packed red blood cells after the procedure of Prankerd (1958) and Borun *et al.* (1957). The top and buttom one quarters were wrthdrawn from the tube with a fine suction capillary, the former being designated old (O) and latter young red cells (Y), based on the theories of Prankerd (1958) and Borun *et al.*, (1957).

Preparation of Crude G6PD:

Following the method proposed by Kirkman (1962, a), the separated erythrocytes were subjected to osmotic hemolysis by the addition of 9 vol. of water, containing 0.01 *M* NADP, and 2.7 m*M* EDTA. The lysates were contrifuged at 10,300 x g for 30 min. in order to eliminate the stroma. (Fig. 1) The resulting supernatant hemolysate was used as the starting material for the crude preparation. They were

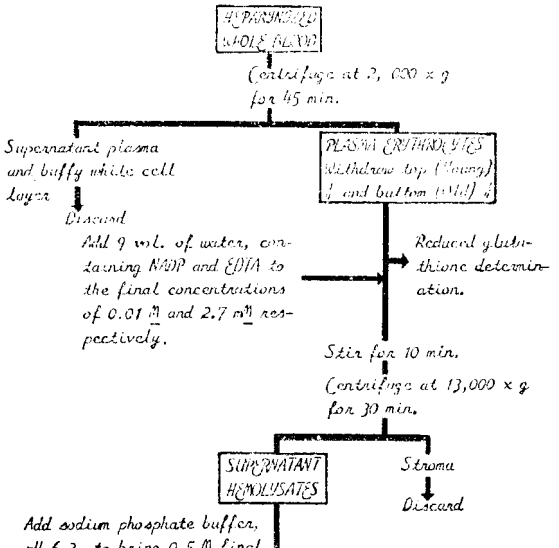

Fig. 1. Steps of sample preparation of the young and old hemolystes for G6PD assay and for lipids, glutathione and protein determination.

adsorbed batch-wise in a Buchner funnel to DEAE-cellulose, pre-equilibrated with a solution consisting of 0.02 *M* phosphate buffer, *p*H 6.3 and 7 m*M* β-mercaptoethanol. Washing the DEAE-cellulose with the same buffer, G6PD was promptly eluted with 0.5 *M* sodium phosphate buffer, 6.3 *p*H, containing 0.01 *M* NADP and 2.7 m*M* EDTA.

The elute was salted out with 70% ammonium sulfate, followed by centrifugation at 10,300 x g for 20 min.. The enzyme precipitate was dissolved in a minute amount of water, contining 0.1μ mole/ml of NADP, and 2.7m*M*/ml. of EDTA. (Fig. 2)

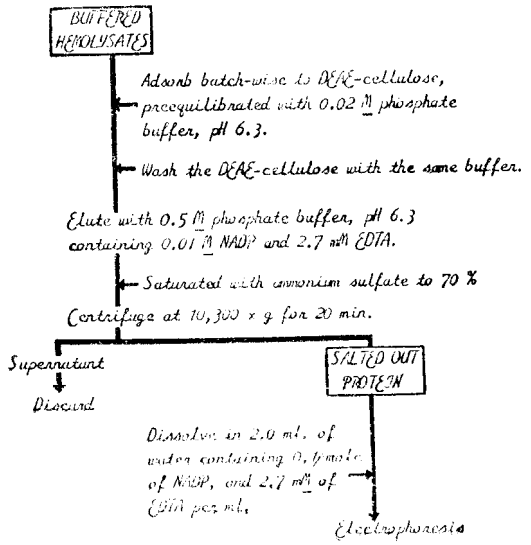

Fig. 2. Steps of crude G6PD preparation from the young and old erythroctyes.

G6PD Asay:

Acrivity of G6PD was assayed by determining the rate of reduction of NADP following the variation in absorbancy at 340 mμ. These measurements were made through a light path of 1 cm, with a UV spectrophotometer (Calbiometer) at 25°C with a reaction mixture, containing 0.1 *M* Tris buffer, *p*H 8.8, 0.01 *M* $MgCl_2$, 0.2 m*M* NADP and 0.6 m*M* G6P.

Determination of Protein:

The protein content was monitored by the method of Lowry *et al.* (1951) in order to obtain specific activities of the crude enzyme preparations. Bovine serum albumin of Nutritional Biochemicals Corp. was used as the standard.

Lipid Analyses:

Lipid was extracted with methanol and chloroform by the method of Sperry and Brand (1955) and purified by the revised method of Folch *et al.* (1954). Total lipid was gravimetrically analyzed, total cholesterol estimated colorimetrically by Schonheimer-Sperry's method (1934), and phospholipid by Fiske-SubbaRow's method (1925).

Determination of Glutathione:

The glutathione contents in young and old red cell hemolysates were estimated iodometrically by the procedure of Woodward and Fry (1932).

Electrophoresis:

Cellulose acetate electrophoresis was carried out according to the conventional methods. Strips were cut in 7.6×2.5 cm and soaked in a solution containing 0,05 *M*, Tris buffer, *p*H 8.8, 2.7 m*M* of EDTA, and 0.1 m*M* NADP, and mounted on microscopic slides. The crude enzyme preparation was spotted on the cellulose acetate on the cathode side of the slide, and subjected to electrophoresis at 4°C for 90 min. with a potential gradient of 13 V/cm, 0.5 mA/cm. 0.05 *M* Tris buffer, *p*H 8.8, was used in the electrophoresis, adding 7.6 mg. NADP to the cathode tank After the electrophoresis, visualization of enzyme activity on acetate strips was performed by means of formazan reaction, staining with the solu-

tion containing 0.5 *M* Tris buffer, *p*H 8.0, 3.0 mg/ml of phenazine methosulfate and 0.2 mg./ml of nitrobluetetrazolium. The staining was stopped after 1 hr. incubation in the dark by washing the the strips in 10% acetic acid for 1 hr. Densitometric evaluation of the enzymogram was carried out with electrophoretic scanner (Gelman Instrument Co.).

RESULTS

As tabulated in Table Ⅰ, lipid content was higher in the hemolysate of younger red cellls (165.0 mg. %) than in the hemolysate of older red cellls (125.5mg. %). It was an unique characteristics among classes of lipid, such as phospholipid and cholesterol. The ratios of the lipid contents of the young to the old, therefore, turned out to be more than an unity; phospholipid being 1.37, cholesterol 1.30 and total lipid 1.13 respectively.

Table Ⅰ. Lipid contents in the hemolystes of younger and older human erythorcytes.

Lipids	Younger RBC mg%	Older RBC mg%	Young/Old
Phospholipid	6.3	4.6	1.37
Cholesterol	22.3	17.1	1.30
Total lipid	165.0	125.5	1.13

The results disclosed the fact that the younger red cells were less dense than the older red cells, owing to an increase in lipid content, and were, therefore, found at the top of a centrifuged column of cells. Among the lipids analyzed, phospholipid was mostly deficit in the older cells as compared to the younger cells than total lipid and cholesterol.

It was found, as summarized in Table Ⅱ, that G6PD activities in the hemolysates of younger and older red cells were 0.171 unit/ml. and 0.126 unit/ml, showing roughly 1.3 times higher activity in the former than in the latter hemolysate.

Protein contents of both preparation were 34.2 mg/ml. and 32.6 mg/ml respectively and it was shown, therefore, that the specific activity of the younger red cell hemolysate was a littte higher (5.0×10^{-3} units/mg, protein) than that of the older red cell hemolysate (3.9×10^{-3} units/mg, protein),

There was, however, observed a little difference between the younger and older hemolysates in their reduced glutathione contents, the former being 6.39 mg. % and the latter 7.06 mg. %, being higher in the latter cells than in the former cells. The reduced glutathione contents per unit G6PD activity were 37.37 mg. % in the younger and 55.16 mg. % in the older red cells.

Electrophoretic patterns are compared in Fig. 3, in which, in all cases, there was observed a faster migration of the older preparation than the younger; and as Fig. 4 and Table Ⅲ discloses, when densstometrically scanned, the older red cell preparation was migrating about 1.09~1.25 times faster than they ounger red cell preparation of G6PD.

It was interesting to note that the enzymo-

Table. Ⅱ. Comparison of G6PD activities and glutathione contents between the hemolysates of young and old RBC.

	G6PD activity unit/ml.	Protein mg./ml.	Specific activity unit/mg. protein	Reduced glutatione mg./ml.	Reduced Glutathione /unit G6PD
Young RBC	0.171	34.2	5.0×10^{-3}	6.39	37.37
Old RBC	0.126	32.6	3.9×10^{-3}	7.06	55.16

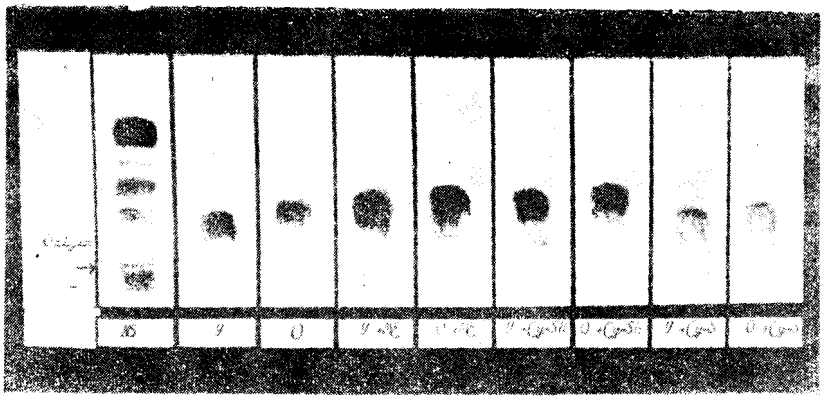

Fig. 3. Comparison of altered electrophoretic patterns of the younger (y) and older (o) human erythrocytic G6PD's preincubated with the following compounds: 8 m*M* mercaptoethanol (ME), 70 m*M* cysteine (Cy-SH), and saturated cystine (Cy-S). The electroenzymograms were visualized by means of formazan reaction. Normal serum protein was run along with the crude enzyme for reference, staining with Nigrosin.

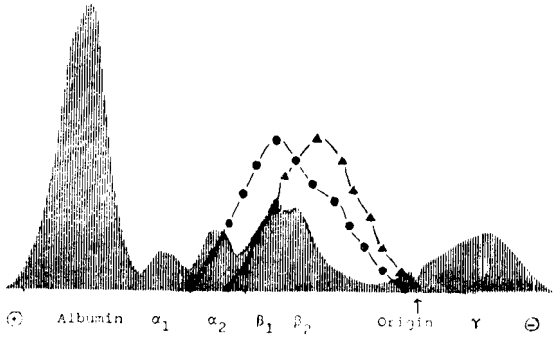

Fig. 4 Densitometical profile of the younger (▲) and older (o) human erythrocytic G6PD and of normal human serum (shadded area) for reference showing the faster electrophoretic mobility of the older erythrocytic enzyme as compared with the younger one.

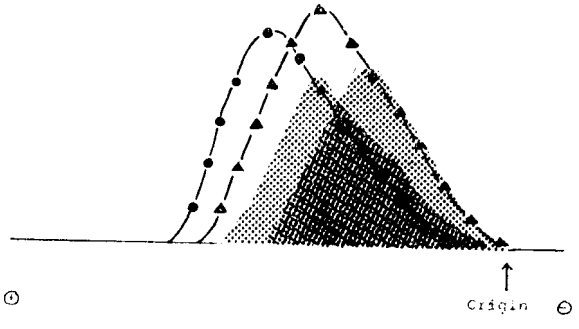

Fig. 5 Densitogram showing the slightly increased electrophoretic mobilities of the young(▲) and old (●) erythrocytic G6PD in the pre sence of 7m*M* mercaptoethanol as compared with its absence (shadded area).

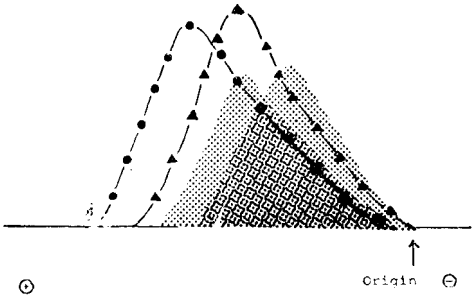

Fig. 6 Comparison of the effect of cysteine on the electrophoretic mobilities of G6PD (y▲), (o●) showing the faster migration of G6PD as compared with its absence (shadded area).

grams of G6PD under the presence of 7 m*M* of mercaptoethanol, known as enzyme stabilizers, and 70 m*M* of cysteine disclosed faster mobilities than those of G6PD alone. When cystine was added, however, to G6PD preparation, the electrophoretic mobilities were not significantly affected, though the older preparation migrated a little faster than the younger. (Fig. 5, 6, 7).

Table Ⅲ. Altered electrophoretic mobilities of the younger and older erythroctic G6PD, preincubated at 25°C for 10 min. with various additions.

Additions	Concentration (m*M*)		Migration (cm)	Ratios of (added/none)	Ratios of (old/young)
None	y*	0	3.2	1.00	1.25
	0	0	4.0	1.00	
Mercaptoethanol	y	7	5.5	1.72	1.13
	0	7	6.2	1.55	
Cysteine	y	70	5.5	1.72	1.09
	0	70	6.0	1.50	
Cystine	y	saturated	3.7	1.16	1.17
	0	saturated	4.3	1.08	

* Y:young and O:Old erythrocytic G6PD.

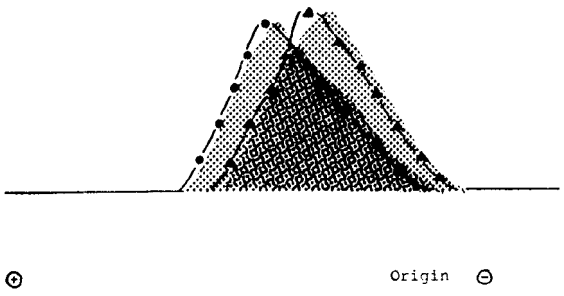

Fig. 7 Comparison of the effect of cysteine on the electorphoretic mobilities of G6PD (y▲), (o●) showing little or no alteration of mobilities as compared with its absence (shadded area).

DISCUSSION

The total lipid content, according to the present work, was higher (160.0 mg.%) in the younger red cells than the older cells (125.5 mg.%), which, of course, contributed the basic theoretical ground in separating the younger and older red cells by centrifugation.

In fact, the membrane lipid is known to play an important role in maintaining the stability of the red blood cells due to the fact that its concentration should roughly be equivalent with that of the plasma, since the lipid gradient between the two may cause disintegrity of the cell membrane.

The red cell is known unstable because of its high intracellular osmotic pressure and because of the chemical unstability of its hemoglobin. As reviewed recently (Prankerd, 1967), chain of events in the red cells to maintain osmotic and electrical equilibrium is Na^+ expulsion and K^+ uptake, and ATP (ATP ase) activity, being generated by glycolysis, contributes to the equilibrium. Lipid exchange may occur to replace its loss due to frictional stress as aging takes place *in vivo* and to maintain the physical integrity of the cell membrane, disruption of which may be prevented by hemoglobin reduction. Practically, the condition known as acantocytosis in man in which there is a deficiency of plasma β-lipoprotein and a lack of plasma lecithin, is associated with a corresponding lack of lecithin in the red cell (Ways *et al.*, 1963) and there is also a hemolytic state. The lipid as aging *in vivo* proceed appears to decrease in phospholipid content as reported by Prankerd (1958); and presumably a decrease in energy potential in the older red cells seems to be underlying cause for the present results.

The present result showing decreased content of lipids in the older red cells clearly confirms the fact that the loss of lipid associated with aging is an important factor as cells artificially

denuded of lipid survive but a short period after transfusion (Harris *et al.*, 1957).

According to several reports (Porter *et al.*, 1964), quantitative variation of red cell G6PD activity is X-linked and characterized by a genetic polymorphism in several population. Quantitative variation has also been demonstrated by few workers (Kirkman, 1962; Kirkman and Hendrickson, 1963), reporting two common G6PD phenotypes by electrophoresis.

The electrophoretic heterogeneities of G6PD were found in many populations which was evidently confirmed by Porter *et al.* (1964) to have thirteen G6PD phenotypes at present according to their combined analyses of electrophoretic and enzymatic study. But the present work elucidated that, even among red cells of *an* individual, the electrophoretic mobility of red cell G6PD varies as a function of their *in vivo* ages, the older migrating faster and the younger slower. The observation coincide with the *in vitro* observation of Fildes and Parr(1963) and Hopkinson *et al.* (1963). Hulsman and Dozy (1962) and Kunkel and Bearn (1957) also reported similar phenomenon on hemoglobins derived from fresh or stored red cells in which old hemoglobin displayed an increased electrophoretic mobility as compared to its younger counterpart. Walter *et al.* (1965) has recently observed this change in the electrophoretic mobility with the G6PD and GOT reporting that the difference in electrophoretic mobility between fresh and stored red cell G6PD was of the order of 10%, referring the similar change observed with red cell acid phosphatase reported by Hopkinson *et al.* (1963).

It seems, however, that all of the red cell enzymes does not manifest this attitude according to Walter *et al.* (1965). LDH did not reveal any alteration in electrophoretic mobility as a function of red cell ages *in vivo*.

One of the possible cause among many suggestions may be that a mixed disulfide with glutathione could be formed as a consequence of the diminished enzyme activities. such as glutathione reductase in older cells, because human G6PD is readily inactivated by heavy metals such as Hg and by p-chloromercurybenzoate, suggesting strongly the presence of one or more critical-SH groups. As Fig. 8 illustrates, oxidized glutathione present due to the diminished glutathione reductase activity as red cell ages *in vivo* may form mixed disulfide with G6PD, resulting in faster electrophoretic migration. Practically, the result obtained with cysteine in the present work seems to confirm the idea, because the younger G6PD plus cysteine resembled with the older G6PD in its migration and this suggestion is again apparent in the present paper when one take into consideration of the ratios of reduced glutathione/G6PD between the two preparations, the older red cells being higher than the younger ones. As discussed by Walter *et al.* (1965) a number of other possibilites

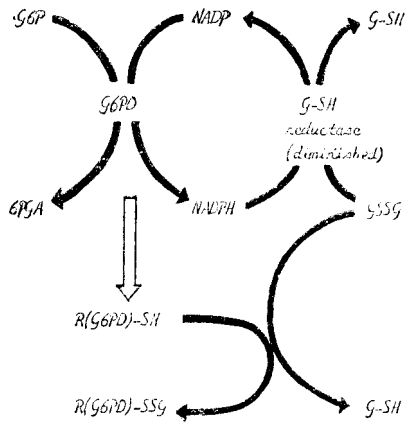

Fig. 8 A possible mechanism of a mixed disulfide* formation between G6P and oxidized glutathione in the older red cells resulting from diminished activity of gultathione reductase as aging take place.

may have been operated. A possible explanation of these faster mobility of older red cell G6PD might be isoenzymic nature, constituted of the slower and faster migrating isoenzymes.

Actually, Boyer and his associates (1962), using starch-gel electrophoresis with discontinous borate buffer systems, reported two prominent bands of enzyme activity, and other workers have also described minor bands of activity when other buffer systems were used. (Kirkman and Hendrickson, 1963; Kirkman *et al.*, 1964; Nance, 1964; Porter *et al.*, 1964).

If the author's former explanation is of true picture, this altered migration would not be observed with those enzymes not possessing such-SH group in readily accessible position on its surface. And the phenomenon might also serve as an indicator of this type of different molecular structure of some enzymes having similar substrate speccificties but derived from different species. Generally thiols have been used as stabilizing agents during isolation and purification of enzymes, of which integrity is dependent on their thiol groups. Cysteine, reduced glutathone and 2-mercaptoethanol produced a marked activation of yeast aldeyde dehydrogenase (Black, 1955), to name an example among many others. Present results obtained with cysteine and 2-mercaptoethanol not only enhanced the G6PD activity but also accelerated its electrophoretic mobility.

It may be that the enzyme-mercaptoethanol or cysteine complex would give rise to a mixed disulfide linkage between glutathione which migrates faster on electrophoresis as in the present results, making the younger and olger G6PD's migrate faster in any cases than G6PD alone.

As is well understood, the above molecules not only protected the integrity of the enzyme, but also, stimulated reduction of $NADP^+$ to NADPH.

Even after addition of serum protein an additional G6PD band appeared resulting from disulfide bridge formation between G6PD derived from red cell and serum protein, possibly its cystein side chains (Rosenszagn *et al.*, 1970).

Therefore, older human red cell G6PD seems to show a decreased catalytic activity and alterations in its properties, including the present results with regard to red cell lipid content change and faster electrophoretic mebility.

CONCLUSION

Human red cells were separated, by centrifugation by virtue of their density gradient due to lipid contents, into the younger and older cells.

Lipid analysis confirmed that the older red cells have lower content than their younger conterparts.

Crude G6PD's derived from each younger and older red cells were subjected to cellulose acetate electrophoresis, and their activities were assayed spectrophotometrically. The activity declined apparently as aging takes place, with little or no changes in their glutathione contents.

Formazan bands on electropherograms of both G6PD's derived from both cells clearly disclosed the fact that the G6PD from the older cell migrates roughly 1.2 times faster toward anode than the enzyme from the younger cell. The alteration of the G6PD mobility as well as its activity was more accentuated with additon of 2-mercaptoethanol and cysteine.

<국문초록>

정상인 노유(老幼) 적혈구 Glucose-6-phosphate dehydrogenase의 전기 영동적 이동율 변화

서울대학교 의과대학 생화학교실

이 춘 식·김 승 원

정상인 적혈구를 지질함량에 기인하는 density gradient에 따라 원심 분리를 이용하여, 노유(老幼) 적혈구를 분리하였다.

지질 분석의결과 노쇄(老衰) 적혈구가 유약(幼若) 적혈구 보다 그 지질 함량이 적었다.

노유 적혈구로 부터 얻은 crude G6PD를 cellulose acetate로 전기 영동하는 한편 spectrophotometry로 그 활성을 분석한 결과 적혈구의 노쇄에 따라 그 G6PD 활성은 분명히 감소하나, glutathione의 양은 별 변동이 없었다.

노유 적혈구 G6PD의 Formazan band를 보면 노쇄 적혈구의 것이 유약 적혈구의 것보다 약 1.2배 빨리 양극 ( ) 쪽으로 이동하였음이 나타났다.

G6PD의 활성과 그 전기영동적 이동율은 2-mercatpoethanol과 cysteine의 첨가로서 증가하였다.

REFERENCES

1. Beutler, E.; *Blood, 28, 553 (1966).*
2. Beutler, E.; *Am. J. Clin. Pathol., 47, 303 (1967).*
3. Black, S.; *The Methods in Enzymology, vol 1, ed. by Colowick, S.P. and Kaplan, N.O., New York, Academic press Inc. (1955), p. 510.*
4. Borun, E.R., William, Figueroa, W.G. and Perry, S.M.; *J. Clin. Inv., 36, 676 (1957).*
5. Boyer. S.H., Porter, I.H. and Weilbacher, R. G.; *Proc. Nat. Acad. Sc., 48, 1868 (1962).*
6. Carson, P.E., Flanagan, C.L., Ickes, C.E. and Alving, A.S.; *Science, 124, 484 (1956).*
7. Childs, B., Zinkham, W., Browne, E.A., Kimbro, E.L. and Torbert, J.V.; *Bull. Johons Hopkins Hosp., 102, 21* (1958).
8. Chung, A.E. and R.E. Langdon; *J. Biol. Chem., 238, 2309 (1963).*
9. Fildes, R.A. and Parr, C.W.; *Biochem. J., 87, 45 p* (1963).
10. Fiske-SubbaRow; *J. Biol. Chem., 66, 375 (1925).*
11. Folch, J., Leas, M., and Slonae-stanley, G.H.; *Fed. Proc., 13, 209 (1954).*
12. Harris, I.M., McAlister, J.M. and Prankerd, T.A.J.; *Clin. Sci. 16, 223 (1957).*
13. Hopkinson, D.A., Spencer, N. and Harris, H.; *Nature, 199, 969 (1963).*
14. Hulsman, T.H.J. and Dozy, A.M.; *J. Lab. Clin. Med., 69, 302 (1962).*
15. Kirkman, H.N.; *J. Biol. Chem., 237. 2364* (1662, *a*).
16. Kirkman. H.N.; *Am. J. Dis. Child., 104, 566 (1962, b).*
17. Kirkman, H.N. and Hendrickson, E.M.; *J. Biol. Chem., 237, 2371 (1962).*
18. Kirkman, H.N. and Hendrickson, E.N.; *Am. J. Hum. Genet., 15 241 (1993).*
19. Kirkman, H.N., Schettini, F. and Pickard, B. M.; *J. Lab. and Clin. Med., 63. 726 (1964).*
20. Kunkel, H.G. and Bearn, A.G.; *Fed. Proc., 16, 760 (1957).*
21. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J.; *J. Biol. Chem., 193, 265 (1951).*
22. Nance, W.E.; *Human Genetics, vol. XXIX. New York: Cold Spring Harbor Laboratory on Quantitative Biology, 415 (1964).*
23. Parker, W.C. and Bearn A.G.; *Am. J. Med., 34, 680 (1963).*
24. Porter, I.H., Boyer, S.H., Watson-Williams, E.H, Adam, A., Szeinberg, A., Siniscalco, M.; *Lancet, i 895 (1964).*
25. Prankerd T.A.J.; *J. Physiol.: 143, 325, (1958).*
26. Rozenszagn, L.A., Kolman, S., Shoham, D. and Gazith, J.; *Nature, 226, 826 (1970).*
27. Schoenheimer, R. and Sperry, W.M.; *J. Biol. Chem., 106, 745 (1934).*
28. Shahidi, N.T. and Diamond, L.K.; *Pediatrics, 24, 245 (1956).*

29. Sperry, W.M. and Brand, F.C.; *J. Biol. Chem.*, *213*, *69* (*1955*).
30. Walter, H., Selby, F.W. and Francisco, J.R.; *Nature*, *208*, *76* (*1965*).
31. Ways, P., Reed, C.F. and Hanahan, D.J.; *J. Clin. Inv.*, *42*, *1284* (*1963*).
32. Woodward, G.E. and Fry, E.G.; *J. Biol. Chem.* *87*, *465* (*19£2*).
33. Yoshida, A.; *J. Biol. Chem.*, *241*, *4966* (*1966*).