Studies on Erythrocyte Pyruvate Kinase (PK) Variant

II. Characterization of a New PK Variant (PK Ube) Found by Electrophoretic Screening

Etsuko ODA

Third Department of Internal Medicine, Yamaguchi University School of Medicine, Ube, 755, Japan

(Received September 30, 1975)

INTRODUCTION

The previous report on electrophoretic screening used for the study of genetic polymorphism of erythrocyte pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2. 7. 1. 40, PK), described a new case of PK variant that was found in a sample of 1,015 healthy adults[1]. The activity of the erythrocyte PK of this case was within the normal range and it showed fast migration in a thin layer polyacrylamide gel electrophoresis. The propositus was asymptomatic. A family study suggested the propositus to be heterozygous for the PK varient. All of the PK variant which were previously reported had been revealed by functional and physiological enzyme defects which are detectable by biochemical parameters, even in the case of an asymptomatic heterozygote[2,3,4,5,6].

The present paper describes the results of the kinetic studies for phosphoenolpyruvate (PEP), optimal pH, stability tests, nucleotide specificity test, and neutralization test of the erythrocyte PK of this case. In addition, an isoelectric focusing experiment for this abnormal PK was performed in order to verify the result of the electrophoresis.

MATERIALS AND METHODS

Heparinized venous blood specimens from the propositus, family members and normal controls were examined within 48 hours after harvesting. The specimens were passed through a cotton-wool column for separating leukocytes and then were washed with isotonic saline three times. Hemolysates were prepared using either saponin solution or water.

Erythrocyte PK was assayed by a method that was previously described[5], and the kinetic studies for PEP were performed using the method reported by Nakashima et al.[6]. The reaction mixture consisted of 200 mM Tris-HCl buffer (pH 8.0), 8 mM $MgCl_2$, 6 mM ethylenediaminetetraacetic acid (EDTA), 75 mM KCl, 2.0 mM adenosine diphosphate (ADP), 0.15 mM dihydronicotinamideadenine dinucleotied (NADH), 6 U/ml lactate dehydrogenase (LDH), and a series of different concentrations of PEP, either with or without 0.5 mM fructose 1, 6–diphosphate (FDP).

PK electrophoresis was performed by means of a thin layer polyacrylamide gel using the method of Imamura and Tanaka[7].

To test the pH dependency of the PK activity, an assay system was prepared using a 100 mM Tris-HCl buffer containing 5 mM EDTA, 8 mM $MgCl_2$, 75 mM KCl, 2.0 mM ADP, 0.15 mM NADH, U/ml LDH, and 1.25 mM PEP. The pH was determined in each cuvette solution with a pH meter TOA Model HM–9 at 37°C immediately after completion of the enzymatic reaction.

The nucleotide specificity test was performed using the procedure of Wiesmann and Tönz[8] with a slight modification[5]. For these experiments 2 mM guanidine diphosphate (GDP), uridine diphosphate (UDP), and cytidine diphosphate (CDP) were used as replacements for ADP.

A heat stability test was performed by the method of Blume et al.[8] at 53°C.

Urea stability was tested by the method of Koster et al[9], modified by using a 200 mM Tris-HCl buffer, pH 7.2, with 2 M urea at 25°C[10].

In a neutralization test, using anti-human erythrocyte PK antiserum, crude hemolysate was mixed with an equal volume of antiserum for 5 min. and then the PK activity was assayed. The antiserum was from the same batch that was used in the previous report[11].

An ATP inhibition test was performed with the same reaction mixture used in the standard PK assay system, containing various concentration of ATP in the 50 mM triethanolamine-HCl buffer (pH 7.4) at 37°C.

Erythrocyte glycolytic intermediates and adenine nucleotides were measured enzymatically according to the method by Minakami et al.[12], except for 2, 3–diphosphoglycerate (2, 3–DPG), which was assayed by the method of Rose and Liebowitz[13].

For isoelectric focusing studies, partial purification of the erythrocyte PK was performed by column chromatography using DEAE-cellulose as

described by Koler et al.[14]. Four ml of packed red cells were hemolyzed by freezing and thawing three times, and then were diluted with an equal volume of 3 mM phosphate buffer (pH 7.5). The hemolysate was dialyzed against a 3 mM phosphate buffer (pH 7.5) for 1 hour to remove other ions. The dialyzed hemolysate was then applied to a DEAE-cellulose column (40 ml) equilibrated with a 3 mM phosphate buffer (pH 7.5) containing 2 mM $MgCl_2$ and 10 mM mercaptoethanol. The column was washed with the buffer and developed with a linear gradient from 0 to 0.5 M KCl.

The eluted erythrocyte PK was collected and dialyzed to concentrate and precipitate the protein against the elution buffer which had been saturated with ammonium sulfate. The precipitate was then collected by centrifugation and dissolved in 2 ml of a 0.2% glycerol solution containing 6 mM mercaptoethanal. This solution was dialyzed against the same solution in order to remove the ammonium sulfate. The dialyzed solution was centrifuged and the supernatant was applied to the isoelectric focusing column.

Isoelectric focusing experiments were performed in a LKB 8101 colum (110 ml) at 6°C, using a 0–70% glycerol gradient. Before applying the sample to the column, prefocusing was done to reduce the focusing period. A 2% pH 5 to 8 Ampholine solution containing 6 mM mercaptoethanol, but without a sample, was electrofocused in order to obtain the pH gradient at 500 V for 12 hours and then at 1,000 V for 24 hours. After pre-run, fractions were collected and about 50 μl of the prepared sample was added to each of the fractions of pH 5 to 8. Then these fractions were transferred to the column again and isoelectric focusing was performed at 500 V for the first 12 hours and at 1,000 V for the next 24 hours.

After the run, the contents of the column were collected in 2.5 ml fractions and their PK activities and pH values were measured at 6°C. Then the fractions with the three highest PK activity levels (7.5 ml) were collected, dialyzed against a 10 mM Tris-HCl buffer (pH 8.2) containing 5 mM $MgCl_2$, 0.5 mM FDP, and 50 mM mercaptoethanol, and dialyzed against the same buffer containing saturated ammonium sulfate till the precipitate was present. After the centrifugation, the precipitate was dissolved in 0.2 ml of a 10 mM Tris-HCl buffer, and was used for electrophoresis.

All substrates, coenzymes and enzymes were purchased from Boehringer-Manheim and all other chemical reagents were of analytical grade. Deionized distilled water was used in all solutions.

RESULTS

As shown in Table 1, the erythrocyte glycolytic intermediates and the adenine nucleotides were found to be within normal limits. The characteristic pattern for erythrocyte PK deficiency, e.g. increases in 2, 3-DPG, 3-phosphoglycerate (3-PG) and PEP, and decrease in ATP, was not observed.

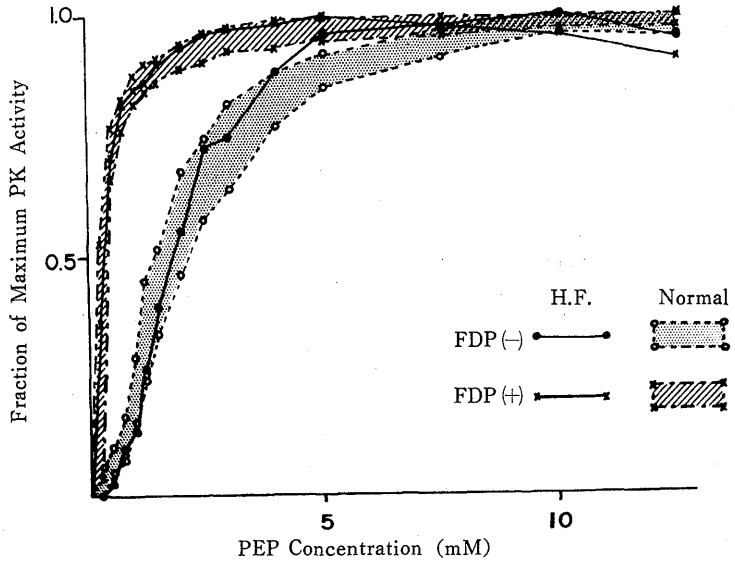

Fig. 1. Pyruvate kinase kinetic curves in the propositus. (+) : 0.5 mM FDP. (−) : absence of FDP.

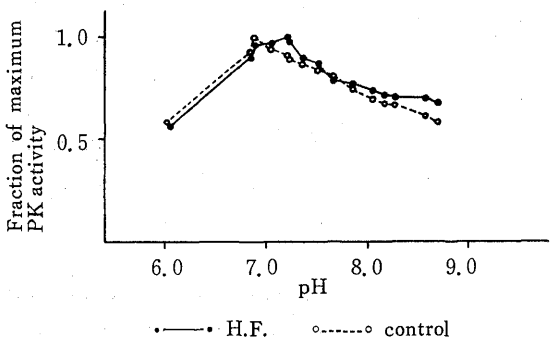

Fig. 2. pH curves of the propositus and normal control subject.

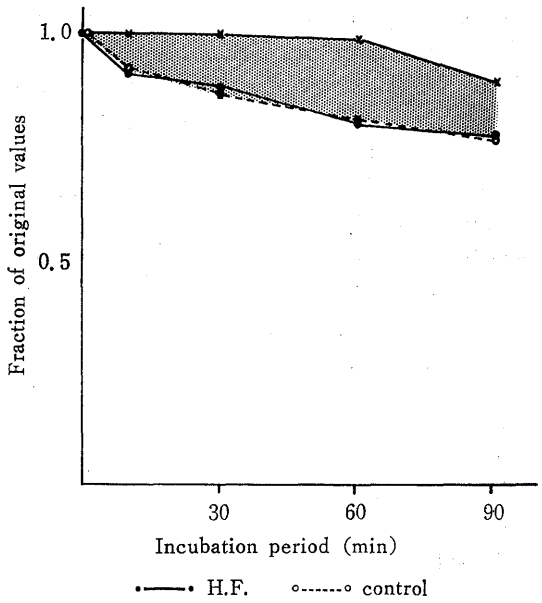

Fig. 3. Heat stability test at 53°C. Shaded area: normal range.

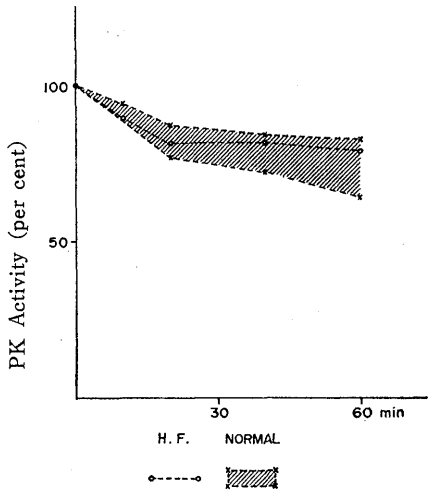

Fig. 4. Urea stability test. Urea: 2M. Temperature: 25°C. pH 7.2. Shaded area: normal range.

The result of the kinetic study for PEP is shown in Fig. 1, demonstrating a sigmoidal curve without FDP, and hyperbolic curve in the presence of FDP. The Km values for PEP of the propositus' PK were 1.73 mM without FDP (normal range 1.43–2.23), and 0.35 mM with FDP (normal range 0.21–0.35).

The optimal pH of the PK of the propositus was 7.22 and that of the normal control was 6.90 (Fig. 2).

In the heat stability test (Fig. 3), after 60 min. the erythrocyte PK

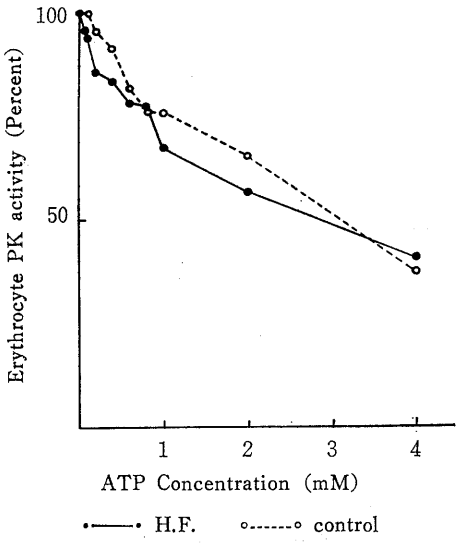

Fig. 5. ATP inhibition test.

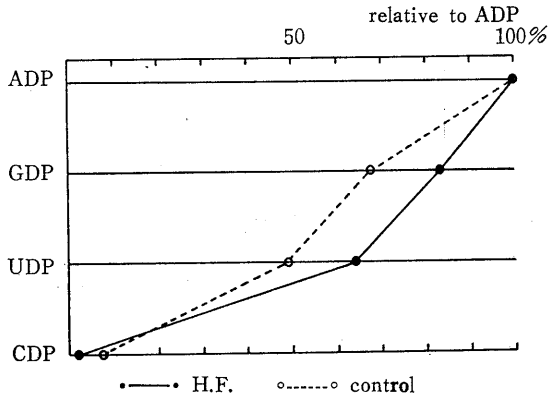

Fig. 6. Nucleotide specificity test.

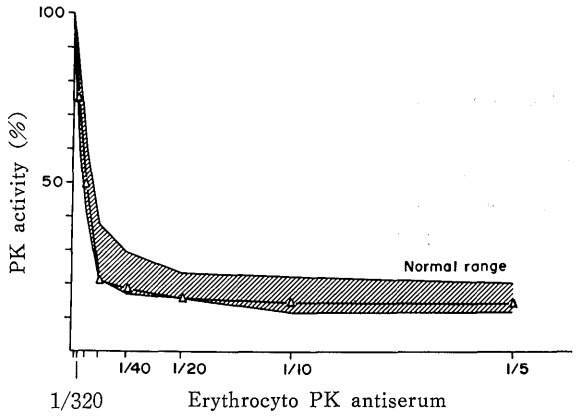

Fig. 7. Neutralization test by antihuman-erythrocyte-PK serum. Shaded area: normal range. Antiserum was diluted with 0.5% bovine serum albumin (BSA) to 5, 10, 140, 160, 320, and 640 times the original volume by serial dilution. One tenthml of hemolysate was mixed with 0.1 ml of each diluent, and after 5 min. at room temperature, PK activities were assayed. Hemolysate mixed with an equal volume of 0.5% BSA without antiserum was assayedas 100% activity.

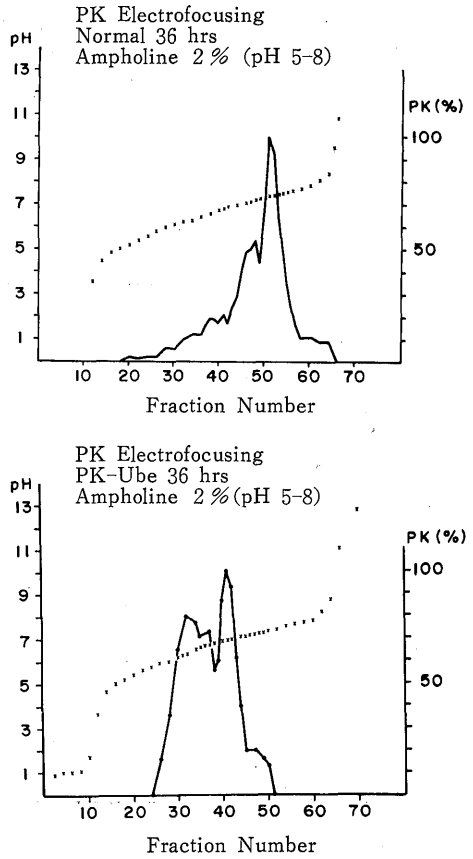

Fig. 8. Results of electrofocusing of the propositus and normal control subject.

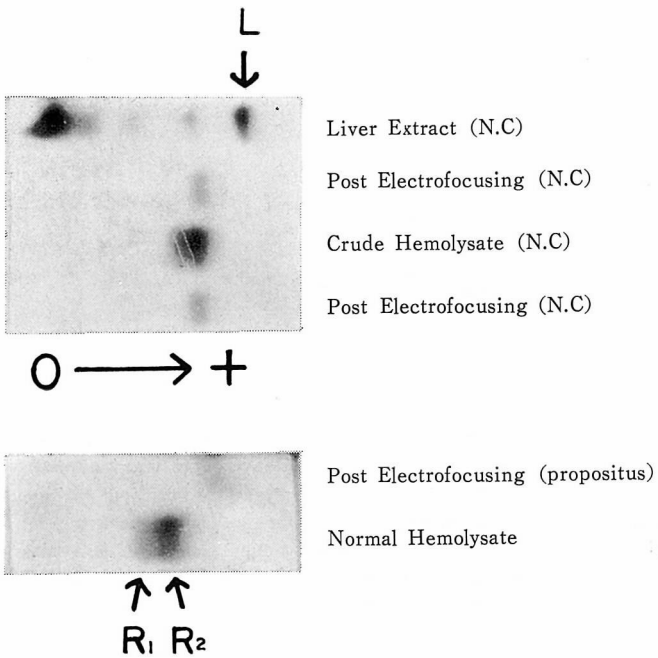

Fig. 9. Thin layer polyacrylamide gel electrophoresis of pyruvate kinase. Supporting media: 3.34% acrylamide gel. Buffer: 10 mM Tris-HCl, 5 mM $MgSO_4$, 0.5 mM FDP and 50 mM mercaptoethanol, pH 8.2. Voltage: 27 V/cm. Time: 5 hours. N.C.: normal control subject.

Table 1. Erythrocyte glycolytic intermediates and adenine nucleotides

	Propositus H.F.	Normal range mean±S.D.
Glucose*	5264	5020 ±1100
Glucose 6-phosphate (G6P)	56.3	34.8± 8.2
Fructose 6-phosphate (F6P)	13.7	9.9± 3.3
Fructose 1,6-diphosphate (FDP)	4.3	12.1± 6.2
Dihydroxyacetone phosphate (DHAP)	17.2	16.4± 5.2
Glyceraldehyde 3-phosphate (GA3P)	5.7	6.0± 3.4
2,3-Diphosphoglycerate (2,3DPG)	3997	4740 ± 568
3-Phosphoglycerate (3PG)	29.5	50.6± 11.6
2-Phosphoglycerate (2PG)	6.5	8.2± 3.2
Phosphoenolpyruvate (PEP)	10.1	13.9± 4.5
Pyruvate*	56.1	83.0± 26.1
Lactate*	807	1240 ± 399
ATP	1378	1280 ± 270
ADP	300	398 ± 191
AMP	11.6	28.9± 17.0
NAD	71.9	68.8± 13.4

mnmoles/ml Red Cell. *nmoles/ml Whole Blood.

activity levels of the propositus and the normal control were 80.5% and 81.6% of their initial levels, respectively.

In the urea stability test (Fig. 4), which was performed at 25°C using 2 M urea, the propositus' PK activity after 60 min. was found to be 78.6% of its starting level. This value was within normal limits.

Fig. 5 showed the ATP inhibition test. The propositus' PK activity level decreased to be 41.2% of its initial value when the ATP concentration reached 4 mM.

In the nucleotide specificity test (Fig. 6), GDP, UDP and CDP were used as replacements for ADP. The PK activity levels of the propositus were found to be 83.0%, 64.2%, and 1.9%, respectively relative to that of ADP, when these chemicals were used. These values did not reveal abnormalities.

In the neutralization test (Fig. 7) using anti-human-erythrocyte-PK serum, the propositus' erythrocyte PK was neutralized markedly and its activity decreased to 20% of its initial level.

As a result of the isoelectric focusing experiments on the erythrocyte PKs of the propositus and normal subjects at 6°C, pI values of 6.90 and 7.36, respectively were obtained (Fig. 8). Also the effluent, collected after the isoelectric focusing of the propositus' PK, revealed faster migration to the anose of a thin layer polyacrylamide gel electrophoresis than that of a normal subject (Fig. 9).

DISCUSSION

As described in the previous report[1], this PK variant was found by electrophoretic screening for the study of genetic PK polymorphism. This erythrocyte PK revealed faster migration to the anode in electrophoresis than those of normal subjects and its activity was within normal limits, being 5.74 $U/10^{10}$ red blood cells RBC . The propositus showed no symptom of hemolytic anemia and seemed to be heterozygous for both mutant PK allele and normal PK allele.

By isoelectric focusing, the pI value of the erythrocyte PK of the propositus was found to be 6.90, with that of the normal subject being 7.36. In other words, the pI value of the propositus' PK was shifted to the acidic side as compared to that of normal subjects. This result is consistent with the fact that the propositus' PK of the crude hemolysate revealed a faster migration than those of normal subjects on electrophoresis. The sample collected after the isoelectric focusing revealed the same abnormal electrophoretic pattern as that of the propositus' PK from the crude hemolysate. As shown in Fig. 9, L-type PK of the normal

control subject migrated forward the anode 129% faster than the migration of the normal erythrocyte PK-R_2. The migration of the propositus' PK was faster than the normal erythrocyte PK-R_2 by 119% after isoelectric focusing. Hence, the obtained pI value of the propositus' PK is not that of L-type PK or M_2-type PK but that of the erythrocyte PK. Thus, it can be concluded that the propositus has an abnormal PK with an abnormal electric net charge.

Two heterozygotes with similar fast migration in electrophoresis of the erythrocyte PKs have been reported previously[6,15]. One was the father of PK Kiyose and the other was the mother of PK Sapporo. The erythrocyte PKs of these two cases were normal in activity, but revealed high Km levels for PEP in kinetic curves. In nucleotide specificyty tests, both PKs showed an increased affinity for GDP, UDP and CDP, that is 114%, 138% and 15%, respectively in the mother of PK Sapporo, and 135%, 111%, and 26%, respectively in the father of PK Kiyose. The mother of PK Sapporo revealed signs of mild hemolysis such as jaundice and reticulocytosis. The propositus' PK can be distinguished from the PKs of the mother of PK Sapporo and the father of PK Kiyose because the PK of the propositus was within the normal ranges of the Km for PEP and of the nucleotide specificity test. The propositus' PK could not be distinguished from the normal PK with the present parameters except by electrophoresis and isoelectric focusing. This PK molecule seems to differ only in net charge from the normal PK, but this does not appear to have any deleterous effects on its functions. For other glycolytic enzyme variants there have been many reports of electrophoretic variants with normal functions[16,17,18,19]. This case is the first PK variant which can be distinguished from the normal PK only by electrophoresis or isoelectric focusing. It has been designated "PK Ube".

SUMMARY

Erythrocyte pyruvate kinase (PK) with an abnormal electrophoretic pattern was found by screening, using a thin layer polyacrylamide gel electrophoresis. The pI value of this pyruvate kinase was found to be 6.90 by means of isoelectric focusing, at 6°C. While the pI value of normal erythrocyte pyruvate kinase was found to be 7.36, at 6°C. This partially purified pyruvate kinase showed the same pattern as the fresh hemolysate of the propositus upon electrophoresis. The propositus' PK showed no other abnormality in activity, determinations of glycolytic intermediates, adenine nucleotides, optimal pH, heat and urea stability

tests, nucleotide specificity test or neutralization test. Since there were previously no PK variants found to have electrophoretically abnormal mobility and normal functions, this variant was designated "PK Ube".

ACKNOWLEDGEMENTS

I should like to thank Professor S. Miwa and Instructor K. Nakashima for their valuable guidance and encouragement, and Mr. Thomas A. Bowers for his review of English.

The study was financed in part by grants from the Ministry of Education (Nos. 837010, 857049).

A part of this report has been published in British Journal of Haematology 1975, 29: 157 by Miwa et al.

REFERENCES

1) Oda E.: Studies on Erythracytu pyruvate kinase (PK) variant I. Electrophoretic screening for the study of genetic PK Polymorphism. *Bull, Yamaguchi Med. Sch*, 23 : 59–66, 1976.
2) Tanaka, K.R., Valentine, W.N., and Miwa, S.: Pyruvate kinase (PK) deficiency hereditary nonspherocytic hemolytic anemia. *Blood*, 19 : 267–295, 1962.
3) Hanel, H.K., Harvald, B., Mor, G., Christensen, N., and Decker, T.: A case of haemolytic anaemia due to pyruvate kinase deficiency. *Scand. J. Haemat.*, 4 : 53–60, 1972.
4) Paglia, D.E., Valentine, W.N., Baughan, M.A., Miller, D.R., Reed, C.F., and McIntyre, O.R.: An inherited molecular lesion of erythrocyte pyruvate kinase. Identification of a kinetically aberrant isozyme associated with premature hemolysis. *J. Clin. Invest.*, 47 : 1929–1946, 1968.
5) Miwa, S. and Nishina, T.: Studies on pyruvate kinase (PK) deficiency. 1. Clinical, hematological and erythrocyte enzyme studies. *Acta Haem. Jap.*, 37 : 1–16, 1974.
6) Nakashima, K., Miwa, S., Oda, S., Tanaka, T., Imamura, K., and Nishina, T.: Electrophoretic and kinetic studies of mutant erythrocyte pyruvate kinase. *Blood*, 43 : 537–548, 1974.
7) Imamura, K. and Tanaka, T.: Multimolecular forms of pyruvate kinase from rat and other mammalian tissues. 1, Electrophoretic studies. *J. Biochem.*, 71 : 1043–1051, 1972.
8) Blume, K.G., Arnold, H., Lohr, G.W., and Beutler, E.: Additional diagnostic procedures for the detection of abnormal red cell pyruvate kinase. *Clin. Chim. Acta*, 43 : 443–446, 1973.
9) Koster, J.F., Staal, G.E.J., and Von Milligen-Boersma, L.: The effect of urea and temperature on red blood cell pyruvate kinase. *Biochim. Biophys. Acta*, 236 : 362–365, 1971.
10) Miwa, S., Nakashima, K., Ariyoshi, K., Shinohara, K., Oda, E., and Tanaka, T.: Four new pyruvate kinase (PK) variants and a classical PK deficiency. *Brit. J. Haemat.*, 29 : 157–169, 1975.
11) Shinohara, K.: Immunochemical studies of pyruvate kinase (PK) molecules: Abnormal PK of human and PK of different species. *Acta Haem. Jap.*, 38 : 9–18, 1975.
12) Minakami, S., Suzuki, C., Saito, T. and Yoshikawa, H.: Studies on erythrocyte glycolysis. 1. Determination of the glycolytic intermediates in human erythrocytes. *J. Biochem.*, 58 : 543–550, 1965.
13) Rose, Z.B. and Liebowitz, J.: Direct determination of 2, 3-diphosphoglycerate. *Anal. Biochem.*, 35 : 177–180, 1970.
14) Koler, R.D., Bigley, R.H., and Stenzel, P.: Biochemical properties of human erythrocyte and leukocyte pyruvate kinase. in Beutler, E. (ed.) *Hereditary disorders of erythrocyte metabolism.* Grune & Stratton, New York and London, 1968.

15) Miwa, S.: Hereditay hemolytic anemia due to erythrocyte enzyme deficiency. *Acta Haem. Jap.*, 36 : 573–615, 1973.
16) Boyer, S.H., Porter, I.H., and Weilbacher, R.G.: Electrophoretic heterogeneity of G6PD and its relationship to enzyme deficiency in man. *Proc. Nat. Acad. Sci.*, 48 : 1868–1876, 1962.
17) Yoshida, A.: Human glucose–6–phosphate dehydrogenase: purification and characterization of Negro type variant (A^+) and comparison with normal (B^+). *Biochem. Genet.*, 1 : 81–99, 1967.
18) Detter, J.C., Ways, P.O., Giblett, E.R., Baughan, M.A., Hopkinson, D.A., Povey, S., and Harris, H.: Inherited variations in human phosphohexose isomerase. *Ann. Hum. Genet.*, 31: 329–338, 1968.
19) Reys, L., Manso, C., and Stamatoyannopoulos, G.: Genetic studies on southeastern Bantu of Mozambique. 1. Variants of glucose–6–phosphate dehydrogenase. *Amer. J. Hum. Genet.*, 22 : 203–215, 1970.