An Inherited Molecular Lesion of Erythrocyte Pyruvate Kinase

IDENTIFICATION OF A KINETICALLY ABERRANT ISOZYME ASSOCIATED WITH PREMATURE HEMOLYSIS

DONALD E. PAGLIA, WILLIAM N. VALENTINE, MARJORIE A. BAUGHAN, DENIS R. MILLER, CLAUDE F. REED, and O. ROSS MCINTYRE

From the Departments of Medicine and Pathology, University of California Los Angeles School of Medicine, Los Angeles, California 90024, and the Veterans Administration Center, Los Angeles, California 90073; the Departments of Pediatrics and Internal Medicine, University of Rochester School of Medicine and Strong Memorial Hospital, Rochester, New York 14620; and the Department of Medicine, Dartmouth Medical School, Hanover, New Hampshire 03755

ABSTRACT Atypical cases of heritable hemolytic anemia have been noted that conform clinically and biochemically to anemias of the pyruvate-kinase (PK)–deficient type, except for the presence of apparently adequate quantities of erythrocyte-PK activity by the usual assay procedure. Investigations of four such anomalous cases, occurring in two unrelated families, are presented. Erythrocytes contained a kinetically aberrant isozyme of pyruvate kinase (PK_2). Michaelis constants for the pathologic isozyme relative to phosphoenolpyruvate were over 10-fold greater than control values, but no kinetic abnormality was evident for the second substrate, adenosine diphosphate. PK_2 exhibited a pH optimum almost 1 U lower than the wild enzyme form (PK_1). Significant differences were also evident in the functional stabilities of the isozymes. Leukocytes were unaffected.

Family studies revealed paternal heterozygosity for quantitative PK deficiency of the usual type. Clinically normal maternal relatives and some siblings demonstrated intermediate deviations in erythrocyte-PK kinetics and reaction characteristics compatible with coexistence of normal PK_1 and kinetically abnormal PK_2. Hemolytic anemia in the propositi appeared to require simultaneous inheritance of the gene governing PK_2 production and its presumed allele resulting in quantitative PK deficiency. Both genetic defects were traced through three generations, the defective gene in both instances apparently resident on autosomes.

A revision of the PK assay technique is suggested, since catalytic inefficiency of PK_2 was manifested only at low substrate concentrations and was therefore undetectable at the relatively high phosphoenolpyruvate levels employed in the conventional assay.

INTRODUCTION

Over 100 documented cases (1, 2) of hemolytic anemia associated with hereditary deficiency of erythrocyte pyruvate kinase (PK) have appeared in the world literature since the original description in 1961 (3). A pattern of clinical and laboratory features has evolved from these studies which partially characterizes this inborn error of erythrocyte metabolism (4, 5).

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A closely related group of patients has been observed in this laboratory and elsewhere which presents a diagnostic dilemma. Because they so well conform to the accepted criteria, they are suspect as PK-deficiency problems, yet quantitative assays by conventional methods consistently fail to detect significant reductions in erythrocyte enzyme levels and, indeed, PK activity is occasionally greater than in normal cells. These findings have suggested the possible existence of an anomalous kinetic form of PK.

The present report describes four such atypical cases of severe hemolytic anemia occurring in two unrelated families. Erythrocyte PK activity in the propositi, adequate by the usual laboratory assay techniques, was found to have anomalous kinetics with markedly reduced catalytic rates at low concentrations of its substrate, phosphoenolpyruvate (PEP). Inhibitors were not demonstrable, and kinetics dependent upon the second substrate, adenosine diphosphate (ADP), were normal. Clinical expression appeared to be contingent upon simultaneous inheritance of a gene causing deficiency of the usual molecular species of PK (here designated PK_1), and a gene governing production of the pathologic isozyme (here designated PK_2). Both genetic defects were traced through three generations, the defective genes apparently residing on autosomes. Qualitative studies established further distinctions between the isozymes, most notably in regard to pH dependency and functional stabilities.

METHODS

Subjects

Kindred G included three affected siblings. 11-yr old propositus 1, a Caucasian male and the eldest child, was noted to be jaundiced shortly after birth without evidence of fetomaternal incompatibilities. Jaundice persisted for 1½ months, but exchange transfusion was not deemed necessary. Intermittent episodes of icterus, pallor, and dark urine that lasted 1–3 wk were noted at age 6 months and recurred every 2 wk–2 months. Upper respiratory infections were associated with symptom exacerbations. He was hospitalized initially in January 1960, at 4½ yr of age, with marked pallor, icterus, and hepatosplenomegaly. Laboratory studies included a reticulocyte count of 11.4%, negative antiglobulin, acid-hemolysis, and Donath-Landsteiner tests, normal levels of red-cell glutathione, and glucose-6-phosphate dehydrogenase activity, normal erythrocyte osmotic fragility, and a Dacie type-II autohemolysis pattern.

A diagnosis of congenital nonspherocytic hemolytic anemia was made, and during the next 3 yr transfusions were required every 4–6 months. In January 1964, splenectomy was performed after ^{51}chromium cell-survival studies demonstrated splenic sequestration and a $t_{\frac{1}{2}}$ of 20 days (normal, 27–36 days). The gall bladder contained numerous small stones, but a liver biopsy was normal. The spleen was enlarged (340 g) but microscopically showed only dilated congested sinusoids. Since surgery, the patient has not required further transfusions and has maintained hemoglobin between 9 and 10 g/100 ml with reticulocytosis of 10–25%. He has remained fully active and asymptomatic.

Propositus 2, a 7 yr old female, was not jaundiced during the neonatal period but has always been pale with minimally icteric sclerae. She never required transfusion and has maintained hemoglobin levels between 9 and 10 g/100 ml with reticulocytosis of 5–12%. The spleen was enlarged, extending 3–4 cm below the costal margin.

Except for slight pallor, 3-yr old female propositus 3 was well until 17 months of age, when icterus, lethargy, fever, and splenomegaly were noted. The packed cell volume at that time was 15%, and results of autohemolysis, osmotic fragility and other peripheral blood studies were similar to those of her older brother. Transfusions every 4–6 months have maintained her packed cell volume between 21 and 31%.

The fourth sibling, a 9 yr old female, and the parents and grandparents have been clinically and hematologically normal. There is no known consanguinity. The father is of English-Welsh descent, although the surname may have Norman origins. The mother is of northern German and English ancestry. The family has lived in upper New York State since the early 19th century, and all living members reside within a radius of 50 miles.

Kindred D contained one affected child. Propositus 4, a 16 yr old Caucasian male, was the product of a normal pregnancy and delivery but was jaundiced at birth. Although investigations for blood-group incompatibilities were negative, an exchange transfusion was performed. The patient remained chronically anemic thereafter with splenomegaly, reticulocytosis, and elevated serum bilirubin. Spherocytes were not evident in peripheral blood smears, and the incubated red-cell fragility test was normal. Jaundice fluctuated, being transiently worse with viral infections, but transfusions were never required.

For several years he had occasional bouts of vague right upper abdominal pain lasting several days. Initial cholecystograms were negative, but gallstones were noted on films taken in October 1966. Elective cholecystectomy and splenectomy were performed 2 months later. Preoperative physical examination was unremarkable except for jaundice and a firm spleen palpable 6 cm below the costal margin. Total serum bilirubin was 8.6 mg/100 ml (normal, 0–1.0 mg/100 ml) of which 0.7 mg/100 ml was direct reacting (normal, 0–0.25 mg/100 ml), and the reticulocyte count was 9.3%. Gallstones were present in the cystic duct and gall bladder. Common duct exploration was carried out, and a 973 g spleen was removed.

There was a pronounced postoperative drop in total

serum bilirubin to 1.2 mg/100 ml (0.4 mg/100 ml direct reacting) and a rise in the reticulocyte count to a maximum of 25%. Reticulocytes gradually declined to preoperative levels, and hemoglobin values rose to 13 g/100 ml 2–3 months after surgery. This was not sustained and by the 6th postoperative month, serum bilirubin had again risen to values approaching preoperative levels. His condition has since remained stable and transfusions have not been necessary.

The parents, both of English extraction, and two male siblings were clinically normal. None of the maternal or paternal relatives was known to be affected. There was no consanguinity nor known relationship to kindred G.

Procedures

Routine hematologic studies were performed by standard methods (6). Autohemolysis of sterile defibrinated blood incubated at 37°C was determined by the Selwyn-Dacie technique (7) as modified by Young, Izzo, Altman, and Swisher (8). Osmotic fragility was measured by the method of Shen, Ham, and Fleming (9) according to the modification of Young et al. (8). Determination of erythrocyte 2,3-diphosphoglycerate (2,3-DPG) was performed according to Bartlett (10). Methods for the measurement of glucose utilization, lactate production, and ATP stability have been detailed in a previous report (11).

Enzyme activities in erythrocytes and leukocytes were determined on homogenates of saline-washed cells separated by sedimentation in polyvinylpyrrolidone-citrate and counted in quadruplicate as described previously (12). Pyruvate kinase was assayed by a modification of the method of Bücher and Pfleiderer (13), which has been detailed in earlier reports (12, 14). All values for erythrocyte PK were corrected for reagent-blank activities as well as for PK contributions by contaminating leukocytes on the basis of simultaneous assays performed on washed leukocyte suspensions. In all experiments white-cell corrections were less than 10% of observed activity maxima. Reagent-blank activities have been found to be virtually negligible when the assay system exclusively employed crystalline rabbit-muscle lactate dehydrogenase [1] with less than 0.003% contaminating PK activity.

Assay procedures as reported elsewhere were also performed for hexokinase, glucosephosphate isomerase, phosphofructokinase, fructosediphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, triosephosphate isomerase, phosphoglycerate kinase, phosphoglyceromutase, phosphopyruvate hydratase (enolase), lactate dehydrogenase, glucose-6-phosphate dehydrogenase (G-6-PD), phosphogluconic dehydrogenase, glutathione stability, glutathione reductase, glutathione peroxidase, glyoxalase, and other combined enzymes of the terminal pentose-phosphate pathway (15–19).

Excepting glyoxalase determinations and the 20°C assay temperature for glutathione peroxidase, all spectrophotometric assays were performed at 37°C by following pyridine nucleotide absorbance changes at 340 mμ in a Beckman DU Spectrophotometer, model 2400, coupled to a model 2000 Gilford Multiple-Sample absorbance recorder. Enzyme units (EU) were defined as the number of micromoles of pyridine nucleotide converted per minute by 10^{10} cells and were calculated on the basis of a molar absorptivity of 6.22×10^{-3} at that wave length (20).

Michaelis constants (K_m) were determined by simultaneous analyses of erythrocyte and leukocyte PK activities in the standard assay systems at 12–16 concentrations of substrate varying between 0.01 and 9.0 mmoles/liter for PEP and between 0.05 and 2.0 mmoles/liter for ADP. Final pH in the reaction system was 6.9 ± 0.1. Variations in PEP levels were performed at a constant ADP concentration, 0.4 mmole/liter, whereas studies on the effect of ADP variations were conducted at 1.5 mM PEP. Potassium and magnesium concentrations were held constant at 75.0 and 8.0 mmoles/liter, respectively, and K_m for these ions was not determined. Corrections were always made for reagent-blank activities determined at each substrate concentration. These were found to be relatively independent of substrate levels, varying by no more than plus or minus 50% of the very small standard-assay reagent-blank correction. Additionally, erythrocyte PK activities were corrected for contamination with leukocyte PK on the basis of concurrent white-cell assays at each concentration of PEP and ADP. Substrate concentrations coinciding with half-maximal PK activities were taken directly from best-fit linear coordinate curves, which defined K_m with greater reproducibility than did extrapolations of double-reciprocal Lineweaver-Burk plots.

RESULTS

Hematologic studies. Table I presents results of standard hematologic measurements on immediate family members obtained at times of enzyme analyses and averaged over the 1 yr study period. Whereas the propositi exhibited moderate to marked anemia, at least one sibling and both parents in each family appeared normal by conventional criteria, excepting perhaps the mother in kindred G who showed progressive signs of mild anemia as she entered the third trimester of her 5th pregnancy. Leukocyte values ranged within normal limits in all family members studied.

Red-cell morphologic abnormalities were qualitatively comparable among all four propositi and did not vary significantly from the nonspecific changes usually seen in congenital nonspherocytic hemolytic anemias. Slight anisocytosis and poikilocytosis with moderate polychromatophilia and macrocytosis were common features. The latter was reflected by slight alterations in red-cell

[1] A grade. Calbiochem, Los Angeles, Calif.

TABLE I

Mean Peripheral Red-Cell Values and Autohemolysis Data

	Hb	PCV	RBC	Reticulocytes	Autohemolysis			
						Additive		
					None	Glucose, 0.026 M	Adenosine, 0.018 M	ATP, 0.021 M
	g/100 ml	%	*× 10⁶/cmm*	%	%			
Kindred G								
Propositus 1 (III-6)*	8.8	28	2.67	17.8	6.0	3.5	2.0	1.0
Propositus 2 (III-4)	9.4	30	3.21	6.8	3.5	3.0	2.0	0.7
Propositus 3‡ (III-3)	9.6	31	3.74	4.4	6.5	5.5	2.0	
Repeat	6.8	21	2.60	11.4				
Sister (III-5)	14.3	44		0.5	0.8	0.1	0.2	
Mother (II-12)	11.4	35	3.79	1.4	2.3	0.3	0.3	
Father (II-11)	15.6	48	5.18	0.3	1.0	0.2	0.3	
Kindred D								
Propositus 4 (III-1)	11.1	34	3.14	18.9	6.9	3.2		0.7
Brother (III-2)	13.1	39	4.51	1.2				
Brother (III-3)	13.2	40	4.63	0.5				
Mother (II-3)	13.5	41	4.30	1.1	0.9	0.2		0.4
Father (II-2)	14.1	43	4.74	0.7				
Normal mean					1.7	0.2	0.2	0.2
Normal range					0–3.5	0–0.5	0–1.0	0–1.0

* Genealogic designation according to Fig. 6.

‡ Specimen obtained 10 days after transfusion. Repeat specimen obtained 2.5 months later.

indices. Variable numbers of irregularly contracted and fragmented cells were present along with occasional target cells. Spherocytes were not evident, and platelets always appeared adequate in quantity. Stained films of peripheral blood and erythrocyte indices from the parents and unaffected siblings appeared normal, again with the exception of the pregnant mother in kindred G, who showed changes compatible with slight hypochromic, normocytic anemia.

Osmotic fragility of fresh erythrocytes was either normal or slightly decreased in all cases studied, and the propositi were indistinguishable on this basis from other family members. Preincubation of blood for 24 hr at 37°C effected some deviation from normal fragility patterns. Onset of propositus-cell hemolysis was either normal (two cases) or occurred at saline concentrations slightly higher than usual (two cases), but the majority of these cell populations showed substantial resistance to osmotic lysis, a characteristic shared by the mother in kindred G. In other family

members the patterns of incubated-cell osmotic fragilities were either normal or indicative of slightly increased resistance to osmotic disruption.

Studies on the effectiveness of erythrocyte glycolysis. Autohemolysis of sterile defibrinated blood from the propositi was slightly or moderately increased after 48-hr incubation at 37°C. Table I shows the extent of this in vitro abnormality and the relative ineffectiveness of glucose and adenosine as corrective agents. In the presence of neutralized ATP, autohemolysis was restored to normal levels in the three cases tested. These findings conformed to the Selwyn-Dacie type-II designation (7), the autohemolysis pattern commonly noted in quantitative PK deficiencies.

Measurements of glucose utilization and lactate production by red cells of kindred G are presented in Table II. The proband generally showed variable increases in both parameters, most likely reflecting the increased metabolic rates of young cell populations. The ratio of lactate production to glucose consumption, more reliable for comparative purposes than either value alone, was in each case within the range of normals.

Erythrocyte ATP stabilities over a 3 hr incubation period are also recorded in Table II for three propositi and their close relatives. Except for the splenectomized patient, propositus 1, who showed a 37% loss of ATP (over a 4 hr period), all were considered to be within normal limits. Additional studies on propositus 3 were expanded to include incubations up to 6 hr. Under these circumstances, intracellular ATP decreased 9.5, 23.5, and 36.3% after 2, 4, and 6 hr, respectively, indicating that

TABLE II

Metabolic Studies on Erythrocytes of the Propositi and Various Relatives

	Glucose utilization	Lactate production	Lactate/ glucose ratio	ATP levels			2, 3-DPG
				Initial	Final*	% Change	
	μmoles/ml RBC/hr			*μmoles/ml RBC*			*μmoles P/ml RBC*
Kindred G							
Propositus 1 (III-6)‡	2.61	4.87	1.86	1.67	1.05	−37	16.6
Propositus 2 (III-4)	3.47	4.15	1.46	0.98	0.98	0	11.3
Propositus 3 (III-3)	4.96	6.83	1.38	1.37	1.32	− 3.6	18.2
Sister (III-5)	2.66	5.17	1.94	1.60	1.48	− 7.1	8.9
Mother (II-12)	2.60	3.30	1.27	0.91	0.87	− 4.8	12.5
Father (II-11)	2.57	5.01	1.95	1.21	1.26	+ 4.1	10.9
Maternal Grandmother (I-6)	2.66	5.06	1.90	1.44	1.48	+ 2.4	11.8
Maternal Grandfather (I-5)	1.95	2.63	1.38	0.89	0.97	+ 9.1	11.9
Paternal Grandmother (I-4)	2.60	4.02	1.42	1.63	1.50	− 7.8	8.8
Kindred D							
Propositus 4 (III-1)							20.2
Normal range	1.57–2.23	2.37–3.91	1.49–2.11	1.15–1.67	1.28–1.50	±10.0	7.0–11.0

* Incubation time was 3 hr for all cases except propositus 1, which was 4 hr.

‡ Genealogic designation according to Fig. 6.

TABLE III

Summary of PK Determinations on Propositi and Immediate Families by Standard Assay Technique Employing 1.5 mM PEP

	Mean PK activity (EU)*		
	RBC	WBC	Reticulocytes
			%
Normal mean and SD	2.74 (0.47)	1,373 (222)	
Heterozygous PK-deficiency range	0.6–1.75		
Homozygous PK-deficiency range	0.0–0.47		
Kindred G			
Propositus 1 (III-6)‡	3.14	1,197	22.6
Propositus 2 (III-4)	1.69	1,217	5.5
Propositus 3§ (III-3)	1.44	713	4.4
Repeat	1.25		11.4
Sister (III-5)	2.73	878	0.4
Mother (II-12)	2.27	1,256	1.1
Father (II-11)	1.68	1,342	0.5
Kindred D			
Propositus 4 (III-1)	2.60	1,521	21.3
Brother (III-2)	1.79	869	1.2
Brother (III-3)	2.06	928	1.2
Mother (II-3)	1.96	1,079	1.1
Father (II-2)	1.75	1,251	0.7

* Each PK value reported for erythrocytes is the mean of two to five determinations on each of two to four separate specimens. Leukocyte values are single determinations.

‡ Genealogic designation according to Fig. 6.

§ Specimen obtained 10 days after transfusion. Repeat specimen obtained 2.5 months later.

ATP instability might be manifested only after prolonged incubations.

Absolute amounts of cellular ATP were diminished in one propositus and perhaps in the others as well, when compared with the values usually found in reticulocytes and young cells. Three propositi showed prominent elevations in erythrocyte 2,3-DPG, which suggested a metabolic impairment in terminal glycolysis.

Cellular enzyme assays. In no instance was an erythrocyte or leukocyte enzyme other than PK found to be quantitatively deficient in the propositi or their families. Assay values of the glycolytic, pentose-shunt, and other erythrocyte enzymes enumerated in the Procedures section, in Methods, were uniformly normal or elevated commensurate with the degree of reticulocytosis. Erythrocyte G-6-PD levels in all propositi were almost twice those of normal control values.

Repetitive erythrocyte-PK assays in these kindreds over a 1 yr period have been averaged in Table III. Even considering the increases sec-

TABLE IV

Erythrocyte and Leukocyte PK Michaelis Constants for PEP and ADP

Group	Patient	Genealogic designation*	Michaelis-Menton constants			
			Erythrocyte PK		Leukocyte PK	
			PEP	ADP	PEP	ADP
			mmoles/liter			
	Kindred G					
1	Propositus 1	III-6	1.25	0.10		
	Repeat		1.30	0.09	0.10	0.17
	Propositus 2	III-4	1.23	0.15	0.11	0.18
	Propositus 3‡	III-3	0.59	0.15	0.19	0.20
	Repeat		1.60		0.11	
2	Mother	II-12	0.48	0.14	0.07	0.20
	Maternal Uncle	II-15	0.71		0.07	
	Maternal Grandfather	I-5	0.83		0.05	
3	Sister	III-5	0.09		0.05	
	Father	II-11	0.18	0.10	0.05	0.19
	Paternal Grandmother	I-4	0.16		0.05	
	Maternal Uncle	II-14	0.19		0.10	
	Maternal Aunt	II-13	0.16		0.08	
	Maternal Grandmother	I-6	0.13		0.08	
	Kindred D					
1	Propositus 4	III-1	1.31	0.18	0.08	0.18
2	Brother	III-2	0.53		0.08	
	Brother	III-3	0.83		0.15	
	Mother	II-3	0.34	0.16	0.08	0.18
	Maternal Uncle	II-4	0.55		0.10	
	Maternal Grandfather	I-3	0.50		0.10	
	Maternal Cousin	III-4	0.70			
	Maternal Cousin	III-6	0.75			
3	Father	II-2	0.09	0.20	0.05	0.24
	Maternal Grandmother	I-4	0.18		0.09	
	Maternal Cousin	III-5	0.27			
	Maternal Cousin	III-7	0.27			
	Normal Controls		0.09	0.20	0.08	0.22
			0.10	0.23	0.09	0.23
			0.09	0.10	0.07	
			0.09		0.05	
			0.15			
			0.15			
			0.21			

* According to Fig. 6.

‡ Specimen obtained 10 days after transfusion. Repeat specimen obtained 2.5 months later.

ondary to reticulocytosis, no propositus had PK activities consistent with the usual homozygous deficiency state, although two appeared to have values compatible with heterozygosity. Comparison with our laboratory norms indicated that the father in each kindred was heterozygous for the usual quantitative PK deficiency, an observation supported by erythrocyte assays conducted on paternal relatives in kindred G which revealed seven additional instances of heterozygosity extending through three generations.

PK kinetic studies. Results of K_m determinations, summarized in Table IV, established three clear patterns of erythrocyte-PK kinetic behavior.

Whereas there were no significant individual deviations from normal controls in red-cell or leukocyte PK K_m relative to ADP, marked differences in K_m for PEP were found in erythrocytes from the propositi and certain family members. These ranged from 1.2 to 1.6 mmoles/liter for proband cells (Table IV, group 1), reflecting at least a 10-fold elevation over normal values. Studies with white-cell suspensions established that this enzyme aberration was not shared by leukocytes.

Segregation into two distinct molecular species of erythrocyte PK on the basis of kinetic behavior was further supported by the occurrence in 10 family members (Table IV, group 2) of kinetic patterns intermediate between the propositi and normal controls, a finding postulated to represent coexistence of normal (PK_1) and kinetically abnormal (PK_2) enzymes. In each kindred, the mother, maternal grandfather, and a maternal uncle demonstrated intermediate red-cell PK kinetics.

All paternal family members, in addition to some maternal relatives, displayed normal PK kinetics whether or not the individual had absolute erythrocyte activities indicative of a heterozygous PK–deficiency state on a quantitative basis (Table IV, group 3).

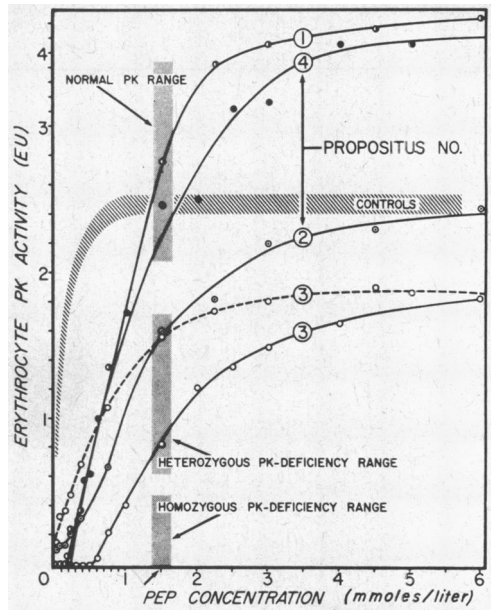

FIGURE 1 Erythrocyte-PK kinetic curves for the propositi. Points on the reaction-velocity plateau are not shown for PEP concentrations above 6.0 mmoles/liter. The cross-hatched area describes typical kinetics for normal-control erythrocyte PK. The abscissa arrow indicates the substrate level usually employed in the standard PK assay system. The broken curve for propositus 3 was derived from a posttransfusion specimen as noted in the text.

In Fig. 1, PK activity in propositus hemolysates is shown as a function of PEP concentration, demonstrating markedly reduced catalysis at low substrate levels. At the PEP concentration usually employed in PK assays (in this laboratory, 1.5 mmoles/liter), erythrocyte PK activity of all propositi was normal or slightly subnormal. K_m calculations from these data, however, revealed marked differences in kinetic behavior between normal and propositus enzyme.

To provide a common scale for comparative purposes, the data in Fig. 1 were converted from absolute values to rate-of-change curves in Fig. 2 describing the celerity of approach to maximum PK activities as substrate levels increased. These curves designated the PEP concentrations at which varying fractions of maximal PK activity occurred. Prominent slope differences, most pronounced in the initial segments, reflected the wide variations between the two isozymes in their dependencies upon substrate availability. For example, 75% maximal activity of the wild enzyme form (PK_1) occurred at PEP levels approximating 0.2–0.3 mmole/liter, whereas 2.0–3.0 mM PEP was required to effect comparable activity with the anomalous isozyme (PK_2) presumably present in propositus cells. Similarly, PK_1 catalyzed at over 70% of maximum capacity at a PEP concentration of 0.3 mmole/liter, whereas PK_2 activity was less than 10% at the same substrate level. Substrate saturation of PK_1 generally occurred between 0.5 and 1.5 mM PEP. With PK_2, concentrations of 4–9 mM PEP were required for complete saturation. Comparison with the kinetic curve of a control reticulocyte-rich specimen indicated that observed abnormalities were not simply a function of young cell populations per se.

In Figs. 1 and 2, two curves are shown for propositus 3, and similarly two K_m values for this patient are recorded in Table IV. The first, reflecting kinetic behavior directly intermediate between normals and other propositi, were derived from assays of a specimen obtained 10 days after

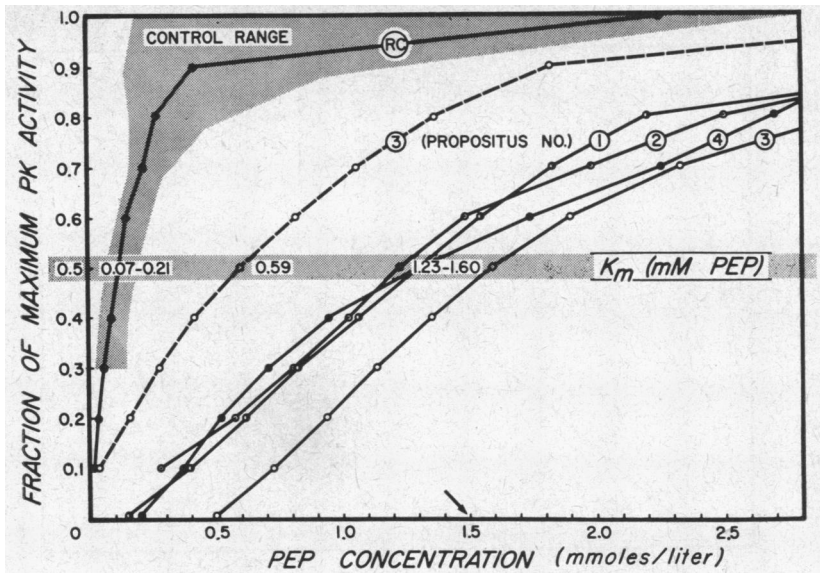

FIGURE 2 Propositus-PK kinetic curves normalized on the basis of maximum apparent activities. The shaded control range contains the mean of six representative normal controls, plus or minus two standard deviations. *RC* designates the curve derived from a specimen obtained from an unrelated patient with 31% reticulocytosis from another cause. At the half-maximal level, Michaelis constants may be read directly off the abscissa, and the value ranges are so noted. The broken curve for propositus 3 again represents kinetics of the posttransfusion specimen.

transfusion of normal whole blood. The patient presented clinically as the most severely affected of the proband yet displayed PK kinetics which in 10 other subjects were known to be phenotypically benign. Assays 2½ months later, after virtually complete attrition of the transfused cells, yielded the second curve and K_m, indicating that the original findings resulted from admixture of propositus and transfused normal erythrocytes.

Comparable in vitro studies were performed on mixtures of normal cells with equal numbers of cells from propositus 1. Results of these experiments, shown in Fig. 3, indicated not only that artificial cell mixtures could yield intermediate kinetic characteristics, but further that enzyme inhibition was not a primary mechanism in modifying the kinetics, since cell combinations behaved essentially as the sum of the isolated components without apparent influence upon one another.

Rate-change curves derived in the same manner for other members of kindreds G and D are presented in Figs. 4 and 5, respectively. Erythrocytes from paternal relatives yielded normal kinetic patterns whether or not the individual was heterozygous for quantitative PK deficiency. Directly intermediate curves, similar to those noted for in vitro and transfusion mixtures of normal and proband cells, were demonstrated by the same siblings and maternal relatives who had intermediate K_m values (Table IV), supporting the hypothesis that both PK_1 and PK_2 isozymes were present in these erythrocytes.

In view of the relative lability of PK preparations and the sensitivity of K_m (PEP) measurements to assay parameters, direct comparison with K_m (PEP) values reported in the literature seems tenuous. Such constants for human erythrocyte PK have been shown to vary with environmental concentrations of magnesium (21), monovalent cations (21), ATP (21), ADP (22), and with pH, and partial purification (23). Under comparable conditions, however, there is good agreement among reported values and our determinations on normals (21, 23–25). Considerably less variation exists for reported K_m values relative to ADP (22–28), and no distinction among our cases could be made on this basis.

With incidental exceptions (21, 22), kinetic studies on partially purified PK, usually from rabbit skeletal muscle, have indicated that K_m for

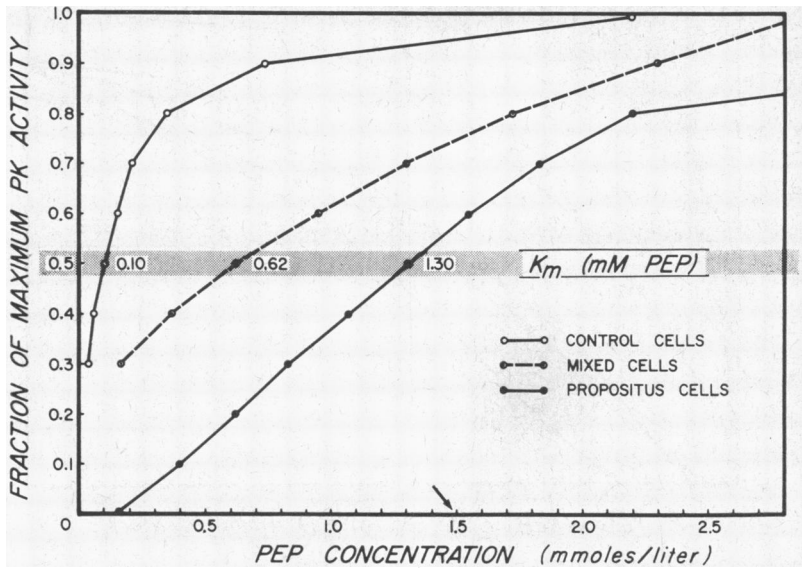

FIGURE 3 PK kinetic curve for mixture of normal erythrocytes with equal numbers from propositus 1. Solid curves describe the kinetics for the individual components of the cell mixture.

either PEP or ADP was independent of concentration of the other substrate (24, 27, 28). That human erythrocyte PK may be unique in this regard was suggested by the findings of Campos, Koler, and Bigley (22), who demonstrated that each K_m was apparently a dependent variable of the second substrate's concentration. While this may have been a function of the concentration

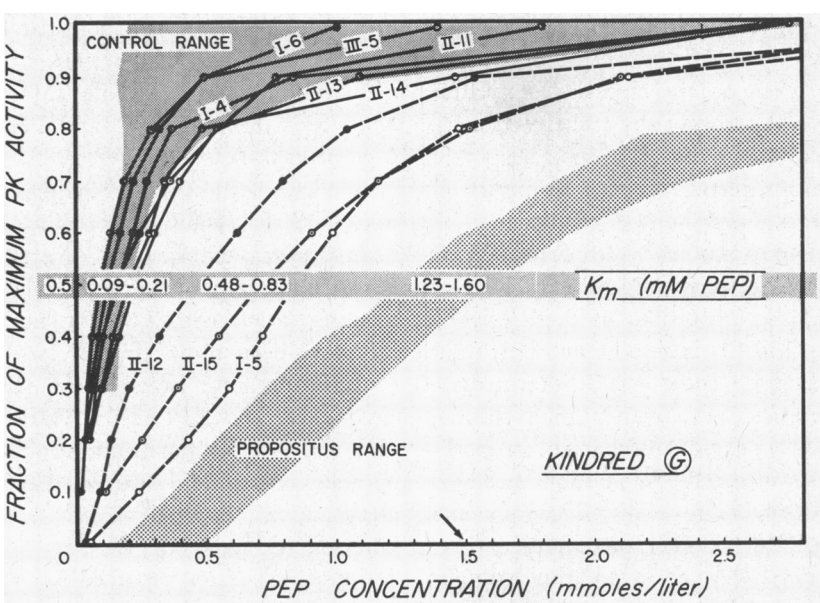

FIGURE 4 Erythrocyte-PK kinetic curves for kindred G. The range of all propositus values from Fig. 2 is included in the heavily stippled area. Relatives are individually designated according to genealogic position as shown in Fig. 6. Normal and intermediate kinetic patterns are shown by solid and broken curves, respectively.

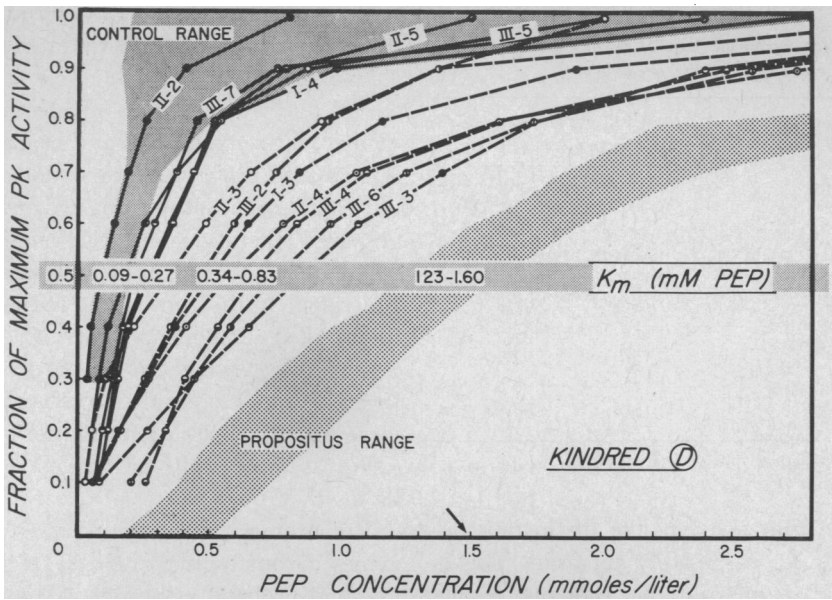

FIGURE 5 Erythrocyte-PK kinetic curves for kindred D. Value ranges and designations are the same as those indicated in Figs. 2 and 4.

ranges studied or the high magnesium-ion levels employed (21), these findings, if confirmed, would suggest a mechanism of enzymatic conversion possibly different from that suspected to prevail with PK from leukocytes and other sources (24, 29). As suggested by the investigators, it would be of particular pertinence to consider this phenomenon in relation to studies with mutant enzyme forms such as PK_2. As yet, we have not been able to establish whether this effect is demonstrable with either PK_1 or PK_2 isozymes under our assay conditions.

Modification of the standard PK assay method. For the patient in each kindred initially studied (propositi 1 and 4), intersection of normal and propositus activity curves in Fig. 1 occurred at PEP concentrations very close to that employed in the routine PK assay system, i.e., 1.5 mM PEP. Because of this fortuitous crossover, the in vitro test for PK activity as performed in our laboratory was unable to differentiate PK_1 from PK_2. The test procedure was subsequently modified by assaying hemolysates simultaneously at high and low PEP concentrations, e.g., 1.5, or 2.0 and 0.3 or 0.4

TABLE V
Ratio of PK Activity at 0.4 mM PEP To That at 2.0 mM PEP

Subject group	Proposed genotype	Ratio of PK activities at 0.4 and 2.0 mM PEP		
		Mean	Range	No. of determinations
Propositi	$PK_1^A PK_2$	0.16	0–0.23	8
Relatives with intermediate PK kinetics	$PK_1 PK_2$	0.39	0.22–0.55	15
Relatives with heterozygous PK deficiency	$PK_1 PK_1^A$	0.78	0.67–0.89	3
Relatives with normal PK kinetics	$PK_1 PK_1$	0.82	0.75–0.86	8
Normal controls	$PK_1 PK_1$	0.80	0.69–0.96	16

TABLE VI

PK Activities in Mixtures of Propositus and Normal-Control Erythrocytes

Relative proportions of cells in mixture		PK activity (ΔA_{340} mμ/10 min)*	
Propositus cells	Control cells	1.5 mM PEP	0.3 mM PEP
All	0	0.150	0.030
5	1	0.150	0.040
2	1	0.150	0.050
1	1	0.150	0.060
1	2	0.150	0.070
1	5	0.140	0.080
0	All	0.150	0.090

* Accuracy of reported values is ±10–15%.

mM PEP. With this revision, the distinction between PK_1 and PK_2 was readily apparent. This is exemplified by data in Table V, showing results of modified PK assays on normal control and proband erythrocytes along with those from other family members. Ratios of enzymatic reaction rates at two substrate concentrations appeared to be a distinctive feature of the isozyme involved and independent of absolute PK levels which varied widely. Segregation of cases on the basis of differences in these ratios, except for an occasional equivocal value, correlated exactly with groupings obtained by more elaborate K_m determinations. PK activity ratios for different combinations of PEP concentrations, viz., 0.3 : 1.5, 0.4 : 1.5, and 0.4 : 2.0 mmoles/liter yielded identical case groupings.

The sensitivity of the modified assay method was demonstrated by data recorded in Table VI. Hemolysates of normal control and propositus cells were mixed in varying proportions and their resultant PK activities were determined at 1.5 and 0.3 mM PEP. Measurable activity at the higher substrate concentration was constant for all combinations. At 0.3 mM PEP, however, variations in hemolysate proportions as little as 16% produced detectable alterations in observed PK reaction rates. This is approximately the resolution limit of the spectrophotometric assay method per se.

With the modified assay technique, other available family members were screened for erythrocyte PK activities. Findings suggestive of altered PK kinetics or low absolute activity levels were investigated further by K_m determinations. Results of family surveys are incorporated in the pedigrees shown in Fig. 6.

PK inhibition studies. Experiments cited previously, in which normal and proband erythrocytes were mixed in varying proportions, failed to detect any inhibitors in propositus cells to account for the observed alterations in PK kinetics. In each instance, PK activities in hemolysate mixtures were equivalent to the sums of activities contributed by the isolated components. These findings were independent of varying cell proportions up to 5 : 1 in favor of either normal or proband cells, and they were unaffected by preincubation of the mixtures, either as intact cells or lysates, up to 30 min at 25 or 37°C.

Preparatory to attempts to separate the isozymes chromatographically, lysates of mixed normal and proband erythrocytes, as well as hemolysates from relatives with intermediate PK kinetics, were deionized by dialysis or gel filtration in 25

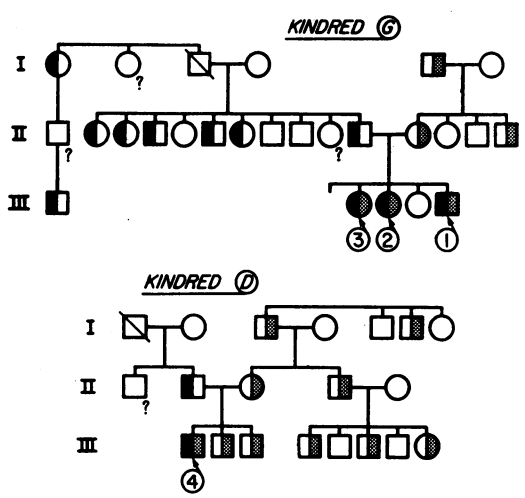

FIGURE 6 Genealogies for kindreds G and D. Generations are designated by Roman numerals with individual positions numbered consecutively from left to right. Males and females are shown as squares and circles, respectively. The propositi are indicated by numbered arrows. Deceased individuals and those not studied are noted by diagonal lines and question marks, respectively. A partially blackened symbol indicates heterozygous deficiency for the usual molecular form of erythrocyte pyruvate kinase, PK_1. Heterozygosity for the anomalous isozyme, PK_2, indicated by partial shading, is isolated to the proband and their siblings and maternal relatives. Position III-2 in kindred G has been filled by the recent birth of a male infant whose cord-blood PK assayed at 1.79 EU with a K_m (PEP) of 1.1 mmoles/liter.

separate procedures. Lysates were prepared by dilution of saline-washed erythrocytes in 4–9 volumes of 0.005 M phosphate buffer, varying in different experiments from pH 6.6 to 7.6, followed by triplicate freezing and thawing in a Dowanol and dry-ice mixture. Hemolysates (approximately 20 ml) were equilibrated at 4°C either by dialysis against large volumes of the appropriate buffer (3–5 liters changed three times over 8 to 12 hr) or by passage through a 1.5 × 25.0–cm column of Sephadex G-25 previously equilibrated with the same buffer. Without exception, PK kinetics in the hemolysates were unaltered by such deionization procedures. Some decrease in total PK activity was occasionally observed after the more prolonged dialyses, but again activities in mixtures were equal to the sums of activities in the unmixed components identically treated.

Studies on pH effects. Erythrocytes from two propositi were studied for pH dependency of their PK activities. Cells from four normal controls and three family members with intermediate kinetics were similarly studied. The assay system was prepared using triethanolamine-HCl (TEA) buffers of identical molarity but varying pH, and pH was determined in each cuvette solution with a Beckman Expandomatic pH meter, both before and after initiation of the enzymatic reaction with hemolysate.

Prevailing pH was found invariably to be lower than that of the primary buffer. It was apparent that the standard assay system, containing TEA in a final concentration of 8.3 mmoles/liter, possessed insufficient buffering capacity to maintain pH at 7.5 as supposed. Actual values ranged between 6.8 and 7.1, the variations occasioned by different batches of PEP.

Results of pH dependency studies, presented in Fig. 7, indicated that a pH range of 6.8 to 7.1 was still optimal for normal PK_1, but PK_2 from propositus cells reached peak efficiency between 6.1 and 6.4. Rapid decrescence of PK_2 activity above pH 6.5 possibly accounted for the several low values noted for some propositi and maternal relatives on the basis of standard PK assays (Table III and Fig. 1), some of which would normally be considered indicative of heterozygous PK–deficiency states.

All hemolysates behaved similarly at low pH but showed divergent characteristics above pH 6.0,

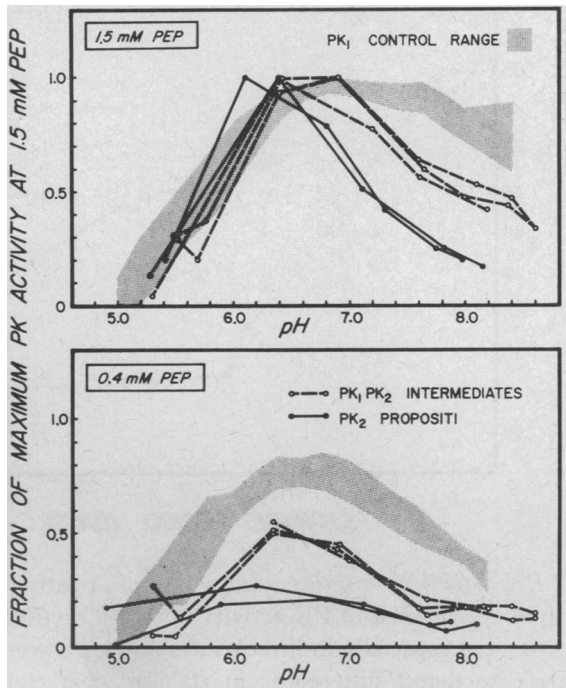

FIGURE 7 pH optimum curves. The shaded area contains the range of values from four representative normal controls. Values shown in the upper graph were determined at the PEP concentration usually employed in routine PK assays; those in the lower graph were determined simultaneously at a low substrate level. The pH reported is a mean of values determined before initiation and after completion of reaction measurements in each cuvette, a value range of less than 0.05 pH U.

when PEP concentration was 1.5 mmoles/liter. The distinct differences between pH–dependency curves derived for propositus and normal red-cell PK were accompanied again by intermediate curves in three cases postulated to represent coexistence of PK_1 and PK_2. These distinctions were clear whether or not PK activities were determined at high or low substrate levels. As shown in the lower half of Fig. 7, differences among the three groups (propositi, intermediates, and normals) at 0.4 mM PEP were apparent over the entire pH range explored.

Comparison of curves in Fig. 7 indicated further that the ratio of activities at these two concentrations was also a dependent variable of pH. Segregation of cases on the basis of activity ratios at high and low PEP levels, as proposed for the modified PK screening test, therefore required critical control of pH to ensure reproducibility as well as validity.

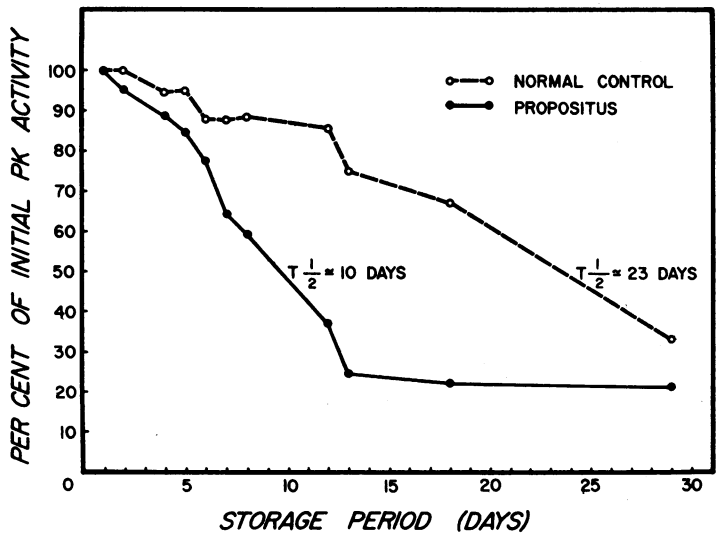

FIGURE 8 PK stability curves for saline-washed erythrocytes. Each point is the mean of four determinations on two separate cell preparations. Storage temperature was either 4°C or −20°C with no significant difference apparent between results at the two temperatures.

PK stability studies. In view of relatively rapid degradation of PK activity in stored erythrocytes, and especially in hemolysates, attempts were made to detect differences in stability characteristics of PK_1 relative to PK_2. Saline-washed red cells from normal controls, propositi, and some relatives with intermediate kinetics were stored intact or as phosphate-buffered hemolysates at 22, 4, and −20°C. Intact erythrocytes at the latter temperature were frozen rapidly in dry-ice and Dowanol mixtures before storage. PK assays at high and low substrate concentrations were conducted over a 1 month period. Magnesium in conjunction with mercaptoethanol (MCE) was also tested for ability to retard PK inactivation.

Generally, PK_2 stability was found to be less than half that of PK_1 under all comparable storage conditions. Washed-cell preparations showed identical activity losses whether preserved at 4 or −20°C. Mean curves presented in Fig. 8 combined the values determined for both temperatures and indicated a twofold or greater decay rate for propositus enzyme. At room temperature, decay rates were considerably accelerated, but a similar relative difference existed between PK_1 ($t_{\frac{1}{2}}$, 6 days) and PK_2 ($t_{\frac{1}{2}}$, 2 days) for the initial decay slopes.

Propositus and normal hemolysates equivalent to 10^5 cells/μl in 0.005 M phosphate buffer, pH 7.2, also showed pronounced differences in PK degradation at 4°C, as shown in the first part of Fig. 9. Interestingly, the rate of activity loss in propositus cells was virtually identical to that determined for the patient's mother, even though an intermediate decay curve might be expected for

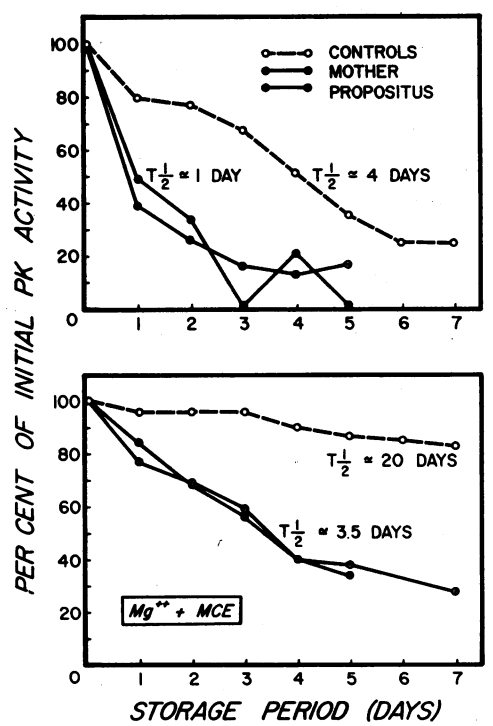

FIGURE 9 PK stability curves for buffered hemolysates. Each point is the mean of four determinations on two hemolysate preparations. Storage temperature was 4°C. Values in the lower graph were determined on identical hemolysates which additionally contained 0.01 M $MgCl_2$ and 0.001 M mercaptoethanol. Zero-time assays were performed approximately 48 hr after venipuncture.

the latter. This may have resulted from shipment delays between venipuncture and zero-time assays, which generally approximated 48 hr.

Whereas the presence of 0.001 M MCE and 0.01 M $MgCl_2$ did not effectively alter PK decay rates in intact erythrocytes, hemolysate activities were significantly affected in all instances. The extent of PK protection by these additives is shown by comparison of the two graphs in Fig. 9, which also indicated a greater stabilizing effect by the additives on PK_1 than on PK_2.

DISCUSSION

Preliminary attempts to obtain isozyme separation by gel filtration, starch-gel electrophoresis, and column chromatography yielded equivocal results at least partially due to instability of this enzyme over the periods required for shipment and processing. Electrophoretic separations were additionally complicated by the lack of a suitable positive stain for PK. Nevertheless, proband erythrocyte PK was found to differ distinctly from the usual enzyme form on the basis of kinetic properties, pH dependency, and functional stability. In the absence of demonstrable inhibitors, these variations, virtually identical in propositi from two unrelated families, seemed sufficient in magnitude to justify the supposition that a pathologic PK isozyme (PK_2) was operative in erythrocytes of affected subjects. This supposition was supported by the frequent occurrence of intermediate degrees of variation in phenotypically normal relatives in whom erythrocyte studies indicated coexistence of both isozymes.

The diverse findings in these studies may be reconciled within the framework of a simple unifying hypothesis regarding the patterns of genetic expression in erythrocyte PK deficiency. This hypothesis presumes that erythrocyte-PK activities and kinetics common to most individuals in the population at large should occur when both contributed genes are normal, resulting in a genotype designated PK_1PK_1.

A gene, here designated PK_1^A, which produces kinetically normal but quantitatively deficient activity, when inherited in company with a normal PK_1 gene, would then effect the common heterozygous deficiency state with normal kinetics but approximately half-normal activity levels, a genotype of $PK_1PK_1^A$. Similarly, the usual homozygous PK deficiency with genotype $PK_1^APK_1^A$ should behave normally by kinetic standards if the paucity of enzyme protein permits experimental determinations. We have found this to be true in several cases so studied, as have other investigators (*22, 23, 29*).

Nearly pure production of the kinetically anomalous isozyme (PK_2) could occur when this gene is inherited along with one causing quantitative deficiency, i.e., a genotype of $PK_1^APK_2$. Occurrence of kinetic patterns which are directly intermediate between pure PK_1 and PK_2 would then be contingent upon concomitant inheritance of both (genotype PK_1PK_2), which should be distinguishable biochemically, but not clinically, from either PK_1PK_1 or $PK_1PK_1^A$. Even though results of present studies conform to an allelic hypothesis, this terminology is used only for simplicity of discussion and should not imply that allelism does in fact exist.

In normal erythrocytes, PK activity has been shown to depend directly upon PEP availability, and generally this enzyme operates far below its potential maximum (*21*). In PK-deficient cells of the usual variety, the enzyme may be more nearly saturated as PEP concentrations increase due to the partial metabolic block. Concurrently, the fall in intracellular levels of ATP, which partially competes with PEP for a common binding locus (*27, 30*), may actually increase rates of PK catalysis in the deficient cell with a consequent K_m (PEP) depression (*21*). In the case of PK_2, however, inefficiency of catalytic conversion is demonstrable in vitro even in the absence of ATP. Intracellular PEP levels would therefore be expected to elevate appreciably before effective catalysis could occur. Presumably this would have the same consequences of intermediate accumulation, such as 2,3-DPG, and altered ADP/ATP ratios as those which obtain when quantitatively exiguous PK_1 is operative at full saturation. In these propositi, then, the enzymopathy conceivably could present a significant metabolic handicap to the red cells by virtue of profound catalytic inefficiency at the low intracellular PEP concentrations normally prevalent (*31, 32*).

In the present study, as in many instances of quantitative PK deficiency, glycolysis and ATP maintenance in the cells assayed appeared reasonably adequate. It should be emphasized, however,

that in such subjects only select reticulocyte-rich populations of young cells still capable of survival are available for study. In view of the ancillary metabolic pathways available to reticulocytes, the greatly enhanced glycolytic capacities of young erythrocytes compared with older cells, and the fact that all assays yield mean values, such observations fail to answer the crucial question, i.e., how will these same cells glycolyze and maintain ATP at some later stage of existence?

It is clear that capacity for erythrocyte survival in extreme cell youth cannot be extrapolated to older cells, and hemolytic anemia by definition will be the result of diminished survival. Ultimate metabolic aberrations preceding cell destruction may be demonstrable only in moribund cells which are not selectively available for study. Measurements of glycolytic activity, ATP levels and ATP maintenance in cells a priori still capable of survival can only provide hints of metabolic events immediately proximal to cell destruction.

Adequate erythrocyte survival measurements were not available in the present cases, but lifespan shortening may be gauged clinically and in terms of measured elevations in G-6-PD activities and reticulocytes. The etiologic basis of the metabolic findings reported in Table II is thus open to some speculation, and equal argument might be made for an increase in ATP utilization as opposed to impaired formation. Such considerations aside, one point remains clear: the patterns of metabolic changes in propositus erythrocytes, as well as the clinical features presented by the proband, conformed well to those demonstrable in homozygous PK deficiency of the quantitative type.

Chronic hemolytic anemia, then, has been found to be associated both with severe quantitative depression of PK activity and with active enzyme catalytically inefficient at low substrate concentrations. This essential identity in phenotypic expression resulting from two separate aberrations in erythrocyte PK renders it unlikely that any mechanism other than deficient PK catalysis is primary in causing the observed syndrome.

Just as the general category of congenital nonspherocytic hemolytic anemias has been shown to be a heterogeneous group frequently associated with one of several erythroenzymopathies, similar heterogeneity appears to be evolving within the subcategory of PK–deficiency hemolytic anemias. Some cases reported as atypical PK deficiencies may in fact represent kinetic anomalies similar, if not identical, to those in the present study, e.g., case 6 of Loder and de Gruchy (33), cases E. H. and M. C. of Koler, Bigley, Jones, Rigas, Vanbellinghen, and Thompson (29), and the case reports of Fusco, Busch, Negrini, and Azzolini (34) and Sachs, Wicker, Gilcher, Conrad, and Cohen (35).

Unpublished studies by Miwa, Ohyama, and Kumatori [2] and by Tanaka [3] have also indicated that abnormal PK kinetics rather than a quantitative enzyme deficit may underlie some instances of the syndrome. Tanaka has noted an occasional patient with intermediate levels of red-cell PK activity whose apparent K_m (PEP) was over twice normal. In the case investigated by Miwa et al., a child with nonspherocytic hemolytic anemia was found to have elevated erythrocyte levels of PEP and monophosphoglycerates, as well as increased PK activity. The K_m (PEP) was five times greater than that of controls, and the pH optimum was also shifted to the acidic region.

There has been justifiable controversy regarding the relative importance of mutant structural-gene vs. control-gene effects in PK-deficiency anemias. The extensive work of Koler et al. (29), which established the molecular distinction between leukocyte and erythrocyte PK, additionally demonstrated apparent molecular homogeneity of this enzyme within erythrocytes, whether normal, heterozygous, or homozygous for quantitative PK deficiency. In support of the latter findings, kinetic studies of homozygotes by Wiesmann and Tönz (23) and Campos et al. (22) failed to demonstrate significant deviations from normal.

The possibility of mutant structural genes also operating in PK-deficiency states received preliminary support from Waller and Löhr (36, 37) and from Boivin and Galand (38). Waller and Löhr's two cases had K_m (PEP) values in crude hemolysates two to four times higher than normals, but with less than 8% of the usual measurable activity, in contrast to our observations of 10-fold higher K_m associated with borderline normal or elevated activity rates when assayed with 1.5 mM PEP as substrate. Boivin and Galand, working with PK partially purified by diethylaminoethyl (DEAE)

[2] Personal communication.

[3] Personal communication.

extraction and ammonium sulfate precipitation, found K_m (PEP) five times *lower* than normals in red cells having some 20% of the usual activity levels at full enzyme saturation, indicating that in their case, enhanced catalytic efficiency might be possible at low PEP concentrations.

Such diverse observations make it difficult to incriminate either a control-gene or structural-gene mutation alone as the sole genetic determinant of PK–deficiency hemolytic anemia. It would seem more appropriate to postulate that the syndrome may result from a mutant regulator or mutant structural gene, or both in combination, with a greater probability being associated with control-gene defects.

Certainly such conjectures must be considered speculative, not only because alternative explanations exist, but also because some of the published information remains unconfirmed and was reported originally with incomplete specification of assay conditions. Nonetheless, two blocks of reliable data now indicate that familial hemolytic anemia, conforming in virtually all respects to the PK-deficient type, may be associated with either a quantitative or a qualitative enzyme defect, lending a priori support to a basic pathogenetic role for the enzymopathy per se in the induction of premature hemolysis.

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