# Metabolic Compensation for Profound Erythrocyte Adenylate Kinase Deficiency

## A HEREDITARY ENZYME DEFECT WITHOUT HEMOLYTIC ANEMIA

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ABSTRACT A child with hemolytic anemia was found to have severe erythrocyte adenylate kinase (AK) deficiency, but an equally enzyme-deficient sibling had no evidence of hemolysis. No residual enzyme activity was found in erythrocytes by spectrophotometric methods that could easily have detected 0.1% of normal activity. However, concentrated hemolysates were shown to have the capacity to generate small amounts of ATP and AMP from ADP after prolonged incubation. Hemolysates could also catalyze the transfer of labeled $\gamma$-phosphate from ATP to ADP. Intact erythrocytes were able to transfer phosphate from the $\gamma$-position of ATP to the $\beta$-position, albeit at a rate substantially slower than normal. They could also incorporate $^{14}$C-labeled adenine into ADP and ATP. Thus, a small amount of residual AK-like activity representing about 1/2,000 of the activity normally present could be documented in the deficient erythrocytes. The residual activity was not inhibited by *N*-ethylmaleimide, which completely abolishes the activity of the normal $AK_1$ isozyme of erythrocytes. The minute amount of residual activity in erythrocytes could represent a small amount of the $AK_2$ isozyme, which has not been thought to be present in erythrocytes, or the activity of erythrocyte guanylate kinase with AMP substituting as substrate for GMP.

Peripheral blood leukocytes, cultured skin fibroblasts, and transformed lymphoblasts from the deficient subject manifested about 17, 24, and 74%, respectively, of the activity of the concurrent controls. This residual activity is consistent with the existence of genetically independent AK isozyme, $AK_2$, which is known to exist in these tissues.

The cause of hemolysis in the proband was not identified. Possibilities include an unrelated enzyme deficiency or other erythrocyte enzyme defect and intraction of another unidentified defect with AK deficiency.

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## INTRODUCTION

Adenylate kinase (AK)[1] catalyzes the equilibrium between ATP, ADP, and AMP in the following reaction:

$$ATP + AMP \overset{AK}{\rightleftharpoons} 2\ ADP.$$

Among the most rare of known erythrocyte enzyme deficiencies, the clinical effects of a lack of AK have previously been unclear. We now report the highly instructive findings in a family in which only traces of AK activity could be detected in the erythrocytes of two siblings. This residual enzyme is shown not to be the normal erythrocyte AK and probably represents another normally present enzyme that exhibits AK-like activity.

*Case report.* This 8-yr-old black girl was in good health with a hemoglobin level of 10 to 11 g/dl until the age of 6 yr, when she developed dark urine in connection with a sore throat. The patient's parents and an older brother were in good health. There was no history of consanguinity.

On examination, the child was afebrile, manifested some cervical and inguinal lymphadenopathy, pharyngeal injection, a palpable liver 1 cm below the right costal margin, and a palpable spleen 2 cm below the left costal margin. The hemoglobin was 8.9 g/dl; he-

[1] *Abbreviations used in this paper:* AK, adenylate kinase; GK, guanylate kinase; Hb, hemoglobin; NEM, *N*-ethylmaleimide.

TABLE I
*Hematologic Findings*

| Subject | Leukocytes/μl | Erythrocytes/μl | Hemoglobin | Hematocrit | Reticulocyte count |
|---|---|---|---|---|---|
| | $\times 10^{-3}$ | $\times 10^{-6}$ | g/dl | % | |
| Proband | 8.4/6.7* | 2.74/2.87 | 8.4/9.6 | 28.1/30.7 | 5.2/10.9 |
| Brother | 4.6/4.9 | 4.04/4.21 | 11.9/12.8 | 35.9/39.7 | 0.8/0.9 |
| Mother | 4.6/7.3 | 4.57/4.05 | 12.8/12.2 | 39.8/37.0 | 0.2/2.5 |
| Father | 5.7 | 4.63 | 15.3 | 45.7 | 0.9 |

* Duplicate values, separated by a slash, were obtained on two separate blood samples, drawn several months apart.

matocrit, 27%; reticulocyte count, 2.7%. A mononucleosis spot test was positive and a heterophile antibody titer of 1:112 was recorded. Although she recovered from the acute episode in a few weeks, she has continued to pass dark urine periodically until now, 2 yr after the initial episode. Hemoglobin and hemosiderin were reported to be present in the urine. Hemoglobin electrophoresis was normal, no unstable hemoglobin could be demonstrated, the Coombs' test was negative, and a sucrose hemolysis test was normal on two occasions.

The blood counts of the patient at the time of examination and that of her brother and both parents are presented in Table I. The brother was in good general health. His spleen was not palpable.

## METHODS

Perchloric acid extracts were prepared by adding 3 ml of freshly drawn blood directly from the syringe to 12 ml ice-cold 4% perchloric acid and neutralizing the supernatant after centrifugation (1). Venous blood for enzyme assays and metabolic studies was collected into tubes containing 1 mg EDTA/ml of blood. All samples were shipped and stored under refrigeration. Reagents and resins were obtained from commercial sources. Adenine-8-$^{14}$C, 546 mCi/mmol was obtained from ICN Pharmaceuticals, Inc., Irvine, CA. $\gamma$-$^{32}$P-ATP was synthesized as described previously (2). Radioactivity was estimated in a Beckman LS-245 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA), using Cerenkoff counting for $^{32}$P and ACS II (Amersham Corp., Arlington Heights, IL) for $^{14}$C.

Erythrocytes were rendered leukocyte free by filtration through $\alpha$-cellulose-microcrystalline cellulose columns (3). Leukocyte/platelet-free erythrocytes were prepared by recalcifying blood anticoagulated in EDTA by the addition of 0.15 ml 20 mM $CaCl_2$/ml of blood, defibrinating the blood with glass beads, and then passing it over the cellulose column. The erythrocytes were washed three times in at least 10 vol of 0.154 M NaCl solution. All studies were performed 2–4 d after the blood was drawn. Since the amount of blood available from the two AK-deficient children was very limited, some of the studies were carried out on the blood of the proband and others on her enzyme-deficient brother. Enzyme assays were performed and levels of intermediates were determined by previously described methods (1). Chromatographic separation of nucleotides was achieved using a Dowex-1 formate column eluted with a 2-liter gradient of 0–5 N formic acid/ammonium formate (1:4), pH 3.0 (4).

Skin fibroblast cultures were established and propagated in Eagle's minimal essential medium with 20% fetal calf serum by standard techniques. Peripheral blood lymphocytes were transformed with Epstein-Barr virus and propagated in RPMI 1640 with 10% fetal calf serum as previously described (5). Mononuclear leukocytes were harvested from the blood by the Ficoll-hypaque technique (6).

## RESULTS

*Enzyme assays.* The results of enzyme assays performed on the proband, her parents, and her brother are summarized in Table II. With the exception of AK, erythrocyte enzyme levels were normal, or, in the case of some of the age-dependent enzymes such as hexokinase, glucose-6-phosphate dehydrogenase, and glutamic oxaloacetic transaminase, increased in the erythrocytes of the proband. It is notable that the activity of pyruvate kinase was not increased, in spite of the young mean cell age. However, the thermal stability and results of screening kinetics were normal. The AK assay is normally performed with a 1:40,000 final cuvette dilution of hemolysate. Hemolysates prepared from the erythrocytes of the proband and her brother revealed no detectable enzyme activity, even when assayed at a final cuvette dilution of 1:400. Since <10% of normal activity is readily appreciated in the enzyme assay, the AK activity of these samples must have been well under 0.1% of normal. AK levels of leukocytes, cultured skin fibroblasts, and transformed lymphocytes were 17, 24, and 74% of concurrent normal controls, respectively.

To detect even lower levels of AK or AK-like activity, we measured the conversion of erythrocyte ADP to ATP and AMP by incubating stroma-free (1:4) water hemolysates made from the erythrocytes of the proband, her brother, and concurrent controls with 1.5 mM ADP, 100 mM Tris (pH 8.0), 0.5 mM EDTA, and

TABLE II
*Erythrocyte Enzyme Activities*

| Enzyme | Proband | Mother | Father | Brother | Activity* |
|---|---|---|---|---|---|
| | | | | | *IU/g Hb* |
| Hexokinase | 3.5 | 1.6 | — | 1.5/1.6 | 1.27±0.18 |
| Glucose phosphate isomerase | 67.7 | 52.1 | — | 67.1 | 60.80±11.0 |
| Phosphofructokinase | 8.0 | 8.5 | — | 7.8 | 9.05±1.89 |
| Aldolase | 4.2 | 3.1 | — | 3.5 | 3.19±0.86 |
| Triose phosphate isomerase | 2,825.8 | 1,573.5 | — | 1,627.2 | 2111±397 |
| Glyceraldehyde-P-dehydrogenase | 433.2 | 264.8 | — | 339.4 | 226±41.9 |
| Phosphoglycerate kinase | 447.5 | 373.5 | — | 460.6 | 320±36.1 |
| Diphosphoglycerate mutase | 4.4 | 6.0 | — | 7.0 | 4.78±0.65 |
| Monophosphoglyceromutase | 43.3 | 28.7 | — | 28.9 | 24.9±2.52 |
| Enolase | 9.4 | 4.5 | — | 4.9 | 5.39±0.83 |
| Pyruvate kinase | 13.6/12.0‡ | 11.9 | — | 10.0 | 15.0±1.99 |
| Lactate dehydrogenase | 296.0 | 186.8 | — | 171.1 | 200±2.09 |
| Glucose-6-P-dehydrogenase | 24.7 | 14.6 | — | 12.4/12.7 | 12.10±2.09 |
| 6-Phosphogluconic dehydrogenase | 15.7 | 10.1 | — | 9.5/10.3 | 8.78±0.78 |
| Glutathione reductase | 14.8 | 12.8 | — | 10.3 | 10.40±1.50 |
| Glutathione peroxidase | 48.1 | 36.1 | — | 34.9 | 31.71±2.97 |
| AK | 0.0 | 131.0/150.6 | 161.4 | 0.0 | 258±29.3 |
| Glutamate-oxaloacetate transaminase | 5.7 | 7.3 | — | 5.2/5.9 | 5.04±0.9 |
| α-Glycerophosphate dehydrogenase | 0.0 | 0.0 | — | 0.0 | 0.0 |
| Adenosine deaminase | 0.9 | 0.7 | — | 0.7 | 1.11±0.23 |
| Pyrimidine 5'-nucleotidase | 0.38 | 0.12 | — | 0.16 | 0.138±0.019 |

* ±SD.
‡ Duplicate values, separated by a slash, were obtained on two separate blood samples, drawn several months apart.

10 mM $MgCl_2$ for 4 h and by estimating the nucleotide levels in perchloric acid extracts prepared at intervals. The results of these studies are summarized in Table III. The results of such incubations cannot be expected to give quantitative results, since the levels of the nucleotides will be influenced by the activities of nonspecific phosphatases and of ATPase. However, the accumulation of ATP and of AMP strongly suggests that some AK activity was present in the hemolysate both from the proposita and her brother.

A more sensitive and more quantitative means of detecting the presence of AK activity is to measure the transfer of $\gamma$-$^{32}$P-labeled phosphate of ATP to AMP. The results of such a study are shown in Fig. 1. Chromatography of a perchloric acid extract of a 1:50 hemolysate incubated with 0.1 mM $\gamma$-$^{32}$P ATP, 4 mM AMP, 10 mM $MgCl_2$, and 100 mM Tris-HCl (pH 7.4) for 30 and 60 min, showed that radioactivity had been incorporated into the ADP peak, albeit more slowly than in the normal control. Carrier ADP was added to the perchloric acid extract before chromatography. The enzyme activity estimated from the incorporation of radioactivity into ADP after 30 min was 0.014 IU/g hemoglobin (Hb) and after 60 min was 0.022 IU/g Hb.

*Metabolic intermediates.* The levels of metabolic intermediates in the erythrocytes of the family members are summarized in Table IV. In spite of the presence of only a trace of AK activity, the levels of AMP

TABLE III
*Adenine Nucleotide Formation after Incubating Hemolysates with 1.5 mM ADP for 4 h*

| Min at 37°C | Subject | AMP | ATP |
|---|---|---|---|
| | | *μmol/g Hb* | |
| 0 | Control | 2.26 | 4.04 |
| | Proband | 1.44 | 5.16 |
| 120 | Control | 17.51 | 21.58 |
| | Proband | 12.58 | 16.37 |
| 240 | Control | 14.74 | 24.71 |
| | Proband | 15.42 | 15.81 |
| 0 | Control | 0.74 | 2.16 |
| | Brother | 0.56 | 1.83 |
| 120 | Control | 14.21 | 20.76 |
| | Brother | 9.45 | 6.33 |
| 240 | Control | 9.78 | 23.81 |
| | Brother | 12.60 | 6.54 |

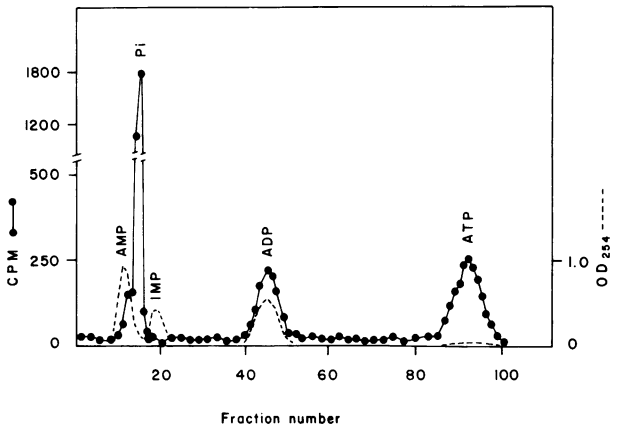


FIGURE 1 Dowex-1-formate chromatography of reaction products formed by incubating the proband's hemolysate with 0.1 mM $\gamma^{32}$P-ATP and 4 mM AMP for 60 min. Methods described in text.

and ADP in the erythrocytes were normal. However, the ATP level tended to be slightly lower than normal. The glutathione content of the erythrocytes of the proband were significantly increased on two separate occasions, but the glutathione level of the erythrocytes of the brother were normal. 2,3-Diphosphoglycerate and phosphoenolpyruvate levels of the erythrocytes of the proband and her brother were markedly increased.

*Erythrocyte adenine nucleotide metabolism.* Studies were performed to assess the interconvertability of AMP, ADP, and ATP in erythrocytes. Since the degradation of ADP is an AK-dependent step, it was thought possible that superior maintenance of ATP plus ADP levels might be observed in these cells. Three different types of investigations were carried out.

(*a*) The result of storage of erythrocytes on the AMP, ADP, and ATP level were assessed. AMP, ADP, and ATP estimations were carried out on leukocyte-free erythrocytes from blood that had been stored for 18 d at 4°C in EDTA. However, it is apparent (Table V) that the deficiency of AK activity did not prevent the loss of ATP and ADP during storage.

(*b*) The incorporation of labeled adenine into the adenine nucleotide pool was measured. When erythrocytes are incubated with adenine it is incorporated into AMP through the mediation of adenine phosphoribosyl transferase. The subsequent incorporation of adenine label into ADP and ATP is dependent upon the AK reaction.

A 45% suspension of leukocyte/platelet-free erythrocytes was incubated for 60 min with 0.5 $\mu$Ci [$^{14}$C]adenine in a solution containing 136 mM triethanolamine-HCl (pH 7.8), 154 mM NaCl, 0.3 mM glucose, 9 mM $K_2HPO_4/KH_2PO_4$ (pH 7.6), 0.9 mM pyruvate, 0.9 mM adenine, and 9 mM inosine. A perchloric acid extract was chromatographed on Dowex-1-formate. As expected, AMP was heavily labeled. Label had also been incorporated, to a much lesser extent, into ADP and ATP. The extent of labeling of these nucleotides was ~40% of normal in the AK-deficient cells (Fig. 2).

TABLE IV
*Metabolic Intermediates*

| Metabolic intermediates | Proband | Mother | Father | Brother | Normal |
|---|---|---|---|---|---|
| | | | *nmol/g Hb* | | |
| ATP | 2,730/3,410* | 3,260/3,230 | 2,570 | 2,630/4,250 | 3,530±300 |
| ADP | 624/932 | 614 | 380 | 591/956 | 635±105 |
| AMP | 78/106 | 70 | 47 | 77/197 | 62±10 |
| 2,3-Diphosphoglycerate | 21,700/26,660 | 14,640 | — | 23,320 | 12,270±1,870 |
| Reduced glutathione | 11,140/10,650 | 6,320 | — | 6,000 | 6,570±1,040 |
| Pyruvate | 340.60‡ | — | — | — | 355.3±143.3‡ |
| Lactate | 6,325.30‡ | — | — | — | 6,213.3±1,406.7‡ |
| Glucose 6-P | 129.20 | — | — | — | 82±22 |
| Fructose 6-P | 141.90 | — | — | — | 27±5.8 |
| Glyceraldehyde 3-P | Negative | — | — | — | Negative |
| Dihydroxyacetone-P | 13.02 | — | — | — | 27.6±8.2 |
| Fructose-diphosphate | 7.80 | — | — | — | 5.6±1.8 |
| 3-Phosphoglyceric acid | 274.10 | — | — | — | 132±15.0 |
| 2-Phosphoglyceric acid | 26.49/92.8 | — | — | 46.16 | 21.5±7.35 |
| Phospho(enol)pyruvate | 116.50/210.1 | — | — | 92.3 | 35.9±6.47 |

* Duplicate values, separated by a slash, were obtained on two separate blood samples, drawn several months apart.
‡ Millimolar in whole blood.

TABLE V

*Adenine Nucleotides in Fresh Blood and Blood Stored at 4°C for 18 d*

| Subject | Freshly drawn blood | | | 18-d-old blood | | |
|---|---|---|---|---|---|---|
| | ATP | ADP | AMP | ATP | ADP | AMP |
| | | | *nmol/g Hb* | | | |
| Proband | 2,920 | 930 | 116 | 730 | 420 | 490 |
| Mother | 3,870 | 683 | 68 | 520 | 540 | 54 |
| Father | 2,930 | 470 | 103 | 520 | 410 | 290 |
| Brother | 4,670 | 888 | 116 | 1,380 | 480 | 340 |
| Control | | | | 790 | 400 | 190 |
| Normal values | 3,530±300 | 635±105 | 62±10 | — | — | — |

(*c*) The ability of inorganic phosphate to be incorporated into the beta position of ATP was assessed. Inorganic phosphate is incorporated into the gamma (terminal) position of ATP in the phosphoglycerate kinase step of metabolism. Labeled phosphorus is normally rapidly randomized between the gamma and beta position by AK through the reactions shown schematically in Fig. 3. To determine how rapidly such randomization occurred in the erythrocytes of the patient, a 45% suspension of leukocyte-free erythrocytes from the proband and from a control subject were incubated for 10 min with 10 $\mu$Ci of $^{32}PO_4^{-3}$ in 150 mM triethanolamine-HCl (pH 7.8), 154 mM NaCl, and 0.3 mM glucose. A perchloric acid extract was prepared and the ATP was isolated by Dowex-1 formate chromatography. Estimation of the radioactivity of the $\beta$- and $\gamma$-phosphate of ATP was achieved by lyophilizing the chromatographically isolated ATP peak, reconstituting to 10 ml, adjusting the pH to 8.2, and incubating with 10 U/ml hexokinase, 9 mM $MgCl_2$, and 90 mM glucose. The radioactivity of the ADP formed was measured after rechromatography (7). The results of this experiment are shown in Table VI.

*The nature of the residual enzyme.* A 1:20 stroma-free hemolysate was prepared in water from leukocyte-free erythrocytes from the proband and from a

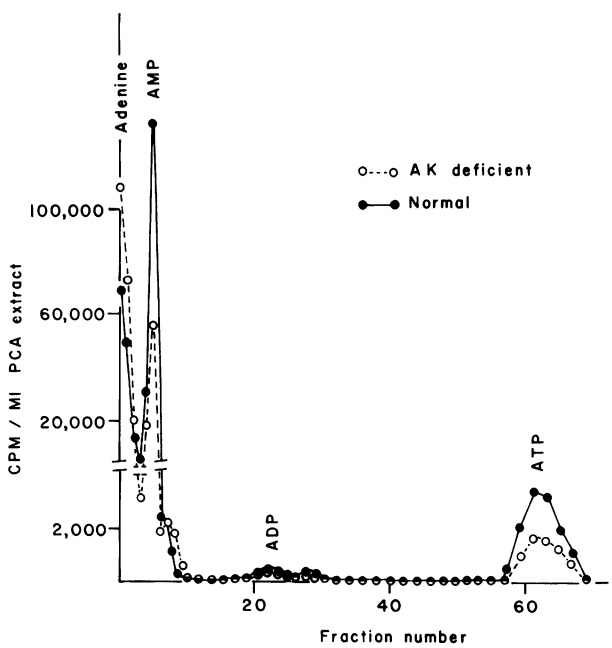


FIGURE 2 Dowex-1-formate chromatography of the reaction products formed by incubating erythrocytes from the proband (O) and a normal control (●) with [$^{14}$C]adenine for 60 min. Methods described in text.

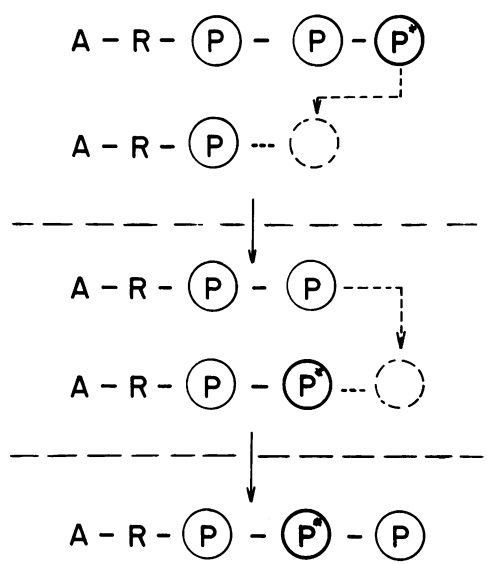


FIGURE 3 The mechanism by which the $\gamma$-(terminal) phosphate of ATP is transferred to the $\beta$-position in the AK reaction.

TABLE VI
*β- and γ-Phosphate Label in ATP after Incubating Erythrocytes with Inorganic $^{32}P$ for 10 min*

| | γ-label | Percentage of total | β-label | Percentage of total |
|---|---|---|---|---|
| | *cpm* | | *cpm* | |
| Patient | 37,545 | 93.89 | 2,442 | 6.11 |
| Control | 39,297 | 69.77 | 17,024 | 30.23 |

The β-position represents the radioactivity found in ADP and the γ-position is the difference of the radioactivity of the original ATP peak isolated and the ADP formed from it. Method is described in text.

normal control. Hemolysates were treated for 10 min at room temperature with and without 5.25 mM *N*-ethylmaleimide (NEM) by mixing 2.0 ml hemolysate, 0.5 ml 1 M Tris-HCl (pH 7.4), 1 ml 0.1 M $MgCl_2$, 0.25 ml 0.1 M NEM or water, and water to give a final volume of 4.755 ml. The control hemolysates were then diluted 1:2,500, maintaining the same concentration of buffer and salts to obtain an activity comparable to that of the deficient sample. Then 0.2 ml 100 mM neutralized AMP, 0.025 ml 20 mM neutralized ATP, and 0.02 ml γ-$^{32}$P-ATP were added to 4.755 ml of each system. The final systems contained (0.1 mM) γ-$^{32}$P-ATP, (4 mM) AMP, (10 mM) $MgCl_2$, and (0.1 M) Tris-HCl (pH 7.4), with and without 5 mM NEM. Incubation for 60 min at 37°C was terminated by perchloric acid precipitation and chromatographic separation of the reaction products was performed on Dowex-1-formate. AK activity was estimated by measuring the radioactivity of the ADP peak formed. The enzyme activity manifested by untreated hemolysates and NEM-treated hemolysates is presented in Table VII. NEM completely inhibited the enzyme activity of the normal hemolysate, but did not affect radioactive ADP formation in the hemolysate from the proband. This indicates that the residual enzyme present in the mutant cells does not represent a trace of the normal $AK_1$ isozyme of adenylate kinase.

TABLE VII
*Transfer of γ-$^{32}P$ from ATP to AMP after 60-min Incubation*

| Erythrocytes | Untreated | Treated with NEM |
|---|---|---|
| AK-Deficient | 0.022 | 0.026 |
| Control | 37.89 | Not detected |

Hemolysates from control and AK-deficient erythrocytes were preincubated with and without NEM. Activity is expressed as micromoles of labeled ADP formed per minute per gram of Hb. Assay conditions were as described in text.

## DISCUSSION

Two families manifesting hereditary nonspherocytic hemolytic anemia with a partial deficiency of this enzyme have been reported previously (8–12). In each case, the defect in the affected patients was only a partial one. In the family reported by Szeinberg et al. (8, 9), interpretation of the findings was confounded by the fact that glucose-6-P-dehydrogenase deficiency co-existed in the family. There was some uncertainty whether the hemolysis that was observed was due to AK deficiency or to a deficiency of glucose-6-P-dehydrogenase, although it was recently suggested that hemolysis was most prominent when both defects were present (9). Boivin et al. (10) reported the case of a 14-yr-old boy, noted to be anemic since the age of 3 mo. This patient's erythrocytes were found to have between 1 and 13% of normal AK activity. At the time of examination, the hemoglobin level was normal, there was a reticulocytosis of 3.5%, and he manifested a thrombocytopenia of 31,000/$mm^3$, slight splenomegaly, and mental retardation.

In evaluating the clinical effect of an enzyme deficiency, one must beware of the bias introduced by the population under study. If the target population for the detection of deficiencies of erythrocyte enzymes is that class of patients who have hemolytic anemia, clearly all patients initially detected with enzyme deficiencies will be found to have hemolysis. This may have been the case with the rare enzyme defect under consideration here. Each index case with AK deficiency that has been found, including our own, has had hemolytic anemia. However, the family study which we were able to perform proved to be particularly telling: The brother of the proband manifested as severe an erythrocyte AK deficiency as his sister, but was hematologically normal. Thus, AK deficiency of the erythrocytes must be assigned, at least in some cases, to that group of erythrocyte enzyme deficiencies we have previously designated as nondiseases (13).

The lack of clinical manifestations in the face of very severe deficiency of AK is quite surprising. AK is normally over 200 times as active as hexokinase, the first enzyme of glycolysis. It forms a metabolic bridge between AMP, on the one hand, and ATP and ADP, on the other. The pool of adenine in AMP, ADP, and ATP turns over as the adenine moiety is irreversibly deaminated in the adenosine deaminase and adenylate deaminase (14) reactions. The pool of adenine is replenished by AMP synthesized in the adenine phosphoribosyl transferase reaction. AK permits AMP formed in this reaction to become part of the vital ADP-ATP pool of the erythrocyte. Since no other pathway for phosphorylation of AMP to ADP is generally recognized, AK might be considered to be an indis-

pensable erythrocyte enzyme. Yet the fact that the brother of the proband in our family was hematologically normal indicates that erythrocytes can easily tolerate a profound deficiency of AK activity. Although the residual AK activity could barely be detected, and can be estimated as being only ~1/2,000 of normal, this minute residual activity was apparently sufficient to allow the erythrocytes to circulate with minimally decreased ATP levels and without impaired survival.

When such a low amount of residual enzyme is present, one must always be concerned that leukocyte or platelet enzyme is being measured, rather than erythrocyte enzyme. In our study, virtually complete removal of these formed elements was assured by the passage of defibrinated blood through $\alpha$-cellulose-microcrystalline cellulose (3). Moreover, the labeling of the $\beta$-phosphate of erythrocyte ATP can be achieved only through the existence of intracellular AK activity. Erythrocytes incubated with inorganic $^{32}PO_4^{-3}$ initially incorporate label into the $\gamma$-position of ATP. This label is then transferred to the $\beta$ position in the AK reaction (Fig. 3). In normal cells that are pulse labeled with $^{32}PO_4^{-3}$, the activity of the $\beta$- and $\gamma$-phosphate thus quickly becomes equal (15). In our studies, labeling was allowed to continue, so that the extent of labeling of the $\gamma$-position was somewhat higher than that of the $\beta$-phosphate, even in the normal control. The AK-deficient erythrocytes incorporated only ~1/6 as much label into the $\beta$-position as the normal cells, but the fact that the $\beta$-position was labeled at all confirms that intracellularly effective AK activity was present. Finally, the fact that ATP plus ADP disappears when blood is stored confirms that an intracellularly functioning AK activity exists in the erythrocyte.

Interpretation of the significance of the minute quantity of residual erythrocyte activity is complicated by the fact that three genetic loci coding for AK activity are known to exist. $AK_1$ and $AK_3$ have been assigned to different regions of chromosome 9, whereas the gene for $AK_2$ has been localized to chromosome 1 (16). The levels of AK activity we observed in peripheral blood leukocytes and in cultured skin fibroblasts and the levels of the lymphoblastoid line from our patient are consistent with the content of the $AK_2$ enzyme of these tissues (17, 18). In our studies, we would not have detected $AK_3$ activity, since this enzyme does not appear to utilize ATP as a phosphate donor (17). Of the known AK genes, only $AK_1$ has been found to be expressed in erythrocytes (17, 19), but the sensitivity of techniques used was insufficient to detect traces of the other AK isozymes. Our finding that the residual enzyme in the erythrocytes of the proband was resistant to inhibition by NEM indicates that it is not $AK_1$. The residual enzyme may well be guanylate kinase (GK), which has been found previously to be present in erythrocytes (20). GK is defined as the enzyme that catalyzes the equilibrium

$$ATP + GMP \overset{GK}{\rightleftharpoons} GDP + ADP.$$

This enzyme has been reported to have an activity of 1.2 U/g Hb at 30°C (20), an activity of ~0.8% of that of AK. Moreover, the purified enzyme was also found to use AMP as as phosphate receptor in the place of guanosine 5'-monophosphate (GMP), albeit at ~1.5% of the rate exhibited with GMP. Thus, the AK activity of GK in erythrocytes might be ~0.01% of normal AK activity, a level that is of the same order of magnitude as the estimates of residual AK activity we were able to make. The activity could also represent a low level of expression of the $AK_2$ gene, which is regarded as not being expressed in erythrocytes (19, 21).

The virtual absence of AK activity from the erythrocytes of the brother of the proband without any evidence of hemolysis clearly demonstrates that the erythrocyte can circumvent severe AK deficiency without serious consequences. Our investigations leave in doubt the reason for hemolysis in the proband. Although we are inclined toward the view that hemolysis in this patient is quite unrelated to her AK deficiency, representing an acquired hemolytic anemia of unknown origin, other possibilities do exist. For example, it may be that severe AK deficiency in combination with some other metabolic property of the erythrocyte may lead to shortened erythrocyte life span, whereas AK deficiency alone does not do so. This type of explanation for hemolysis was proferred in the family reported by Szeinberg et al. (8, 9), in which it appeared that only family members who had inherited both glucose-6-P-dehydrogenase deficiency and AK deficiency manifested hemolytic anemia. The proband in our family had an erythrocyte pyruvate kinase activity slightly lower than average, despite a young erythrocyte population in which one might expect pyruvate kinase activity to be increased (Table II). The elevated level of 2,3-diphosphoglycerate and of phosphoenolpyruvate in the erythrocytes of the proband is consistent with the existence of a functional deficiency in pyruvate kinase. However, the brother, who did not have hemolysis, also had increased levels of erythrocyte 2,3-diphosphoglycerate, and phosphoenolpyruvate. Moreover, the level of enzyme activity in the erythrocytes of the proband was not as low as is usually observed in pyruvate kinase-deficient patients and screening kinetics and thermal stability were normal. The enzyme activity of the erythrocytes of the mother were within the normal range. The significance of the

elevated glutathione levels, observed in the proband on two occasions but not in the other family members, remains unexplained.

Our observations reemphasize the hazard in assuming that a cause-and-effect relationship exists between an enzyme defect and the existence of a hemolytic anemia. The metabolism of the erythrocyte is remarkably flexible, and the opportunities for by-passing an enzyme deficiency, even a very severe one, are remarkably great. One might argue that in previously described cases of AK deficiency a cause-and-effect relationship between the enzyme deficiency and hemolysis might have existed because of some undefined kinetic abnormality of the residual enzyme. However, it seems highly improbable that such an explanation would be correct. It would require that a partial defect of the enzyme would have more severe clinical consequence than absence of the enzyme. It is possible that an additional defect, such as glucose-6-P-dehydrogenase deficiency must be present for AK deficiency to cause hemolytic anemia (9), but the mechanism of such an interaction is obscure. It may be more reasonable to conclude that AK deficiency—like partial or total deficiencies of certain other erythrocyte enzymes, including 6-phosphogluconate dehydrogenase, glutathione reductase, glutathione peroxidase, acetyl cholinesterase, catalase, and lactate dehydrogenase—represents defects without detectable clinical consequences (22).

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