PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 171, 24–33 (1982)

Phosphoenolypyruvate (PEP) Effects on Fresh and Stored Red Blood Cells[1,2] (41472)

PAUL R. SOHMER* AND RHONDA L. SCOTT†

*Division of Blood Research, and †Division of Combat Casualty Care, Letterman Army Institute of Research, Presido of San Francisco, California 94129

Abstract. The present studies were performed to evaluate the effects of phosphoenolpyruvate (PEP) on fresh and stored red blood cells as a possible adjunct to citrate phosphate dextrose (CPD) for preservation of blood. Red cell concentrates prepared from fresh blood drawn from human volunteers were incubated with CPD alone or CPD containing PEP. At 37° and an initial pH of less than 6.2, a significant increase in 2,3-DPG and maintenance of ATP were observed. A dose-dependent increase in 2,3-DPG was noted in blood incubated in CPD–PEP from 13 to 52 m*M*. 2,3-DPG did not change during incubation at either 4° or 25°. A significant increase in ATP was observed at 25°; ATP remained unchanged at 4° and 37°. Storage related depletion of 2,3-DPG and ATP was reversed by PEP incubation (26 m*M*) even when blood was stored for 42 days in CPD. In fact, 2,3-DPG levels two or three times greater than normal were regularly observed. The persistence, *in vivo*, of the PEP-induced increase in 2,3-DPG and P_{50} was demonstrated by 33% exchange transfusions in rats with homologous blood treated for 4 hr with 26 m*M* PEP. Unlike other preservation additives, incubation with PEP results in a dramatic increase in 2,3-DPG without depletion of ATP, a property which is maintained *in vivo*. Evidence shows that PEP should be considered as a potential adjunct to conventional blood preservation systems.

Tomoda *et al.* (1) and Hamasaki *et al.* (2–4) have demonstrated that the glycolytic intermediate phosphoenolpyruvate (PEP) accumulates inside human erythrocytes which have been incubated in acidified sucrose and citrate solutions containing PEP. Transport of PEP across the red cell membrane and its subsequent metabolism results in a significant increase in the intracellular concentration of 2,3-diphosphoglycerate (2,3-DPG) and adenosine triphosphate (ATP) during incubation in these media (1–4).

The present studies were designed to assess the utility of PEP as an adjunct to (a presently available blood preservation system) citrate phosphate dextrose (CPD). The importance of 2,3-DPG and ATP as *in vitro* indicators of erythrocyte function and viability suggests that a treatment which results in their simultaneous generation or maintenance would significantly improve our ability to store blood in the liquid state. We have evaluated the effects of varying concentrations of PEP on both fresh and stored red blood cells. Time, temperature, and pH of incubation were varied to determine optimum conditions for maximal generation of 2,3-DPG and maintenance of ATP.

Materials and Methods. *Fresh blood.* Fresh blood was drawn from healthy volunteers into syringes which had been prefilled with standard citrate phosphate dextrose (CPD) solution to achieve a final blood–anticoagulant mixture ratio of 7:1.[3,4]

[1] Presented in part at the American Federation of Clinical Research, Western Section Meeting, February 5, 1981, Carmel, Calif.

[2] The opinions or assertions contained herein are private views of the authors(s) and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense (AR 360–5).

[3] Human subjects participated in these studies after giving their free and informed voluntary consent. Investigators adhered to AR 70–25 and USAMRDC Reg 50–25 on the use of volunteers in research.

[4] The citation of trade names in this report (or paper) does not constitute an official endorsement or approval of the use of such items.

0037-9727/82/090024-10$01.00/0

Copyright © 1982 by the Society for Experimental Biology and Medicine.
All rights reserved.

Red cell concentrates (RCC) were prepared after centrifugation in a Sorvall RC-3 refrigerated centrifuge at 5000*g* for 5 min at 4°. Aliquots (4 ml) of the resultant RCC were incubated at 37° for 5 min in a shaking water bath (rpm = 140). Equal volumes of test solutions were added and samples removed for measurement of hemoglobin, 2,3-DPG, ATP, pH, and P_{50}. The aliquots were then incubated at 4°, 25°, or 37° for 4 hr. Samples were removed at intervals for the biochemical analyses noted above. Test solutions were prepared by dissolving phosphoenolpyruvate (tricyclohexylammonium salt obtained from Sigma Chemical Co., St. Louis, Mo.) in sterile CPD; the pH was adjusted to 5.7 with dropwise addition of 0.1 *M* NaOH or 0.1 *M* HCl.

The first experiment was designed to evaluate the effects of the concentration of PEP on P_{50} and intraerythrocytic 2,3-DPG and ATP. Test solutions were prepared to achieve final concentrations (test solution plus RCC) of 0, 13, 26, 52, and 78 m*M* PEP. After mixing and initial sampling, the RCC test solution mixtures were incubated at 37° for 4 hr. Samples for analysis of P_{50}, 2,3-DPG, and ATP were removed after 1 and 4 hr of incubation.

The second experiment was designed to evaluate the effects of incubation temperature of P_{50} and intraerythrocytic 2,3-DPG and ATP. Equal volumes of CPD or 52 m*M* PEP in CPD were added to red cell concentrates. The PEP and CPD–RCC mixtures were incubated at 4°, 25°, and 37° for 4 hr of incubation.

The third experiment was designed to evaluate the effects of pH on intraerythrocytic 2,3-DPG and ATP. Equal volumes of CPD or 52 m*M* PEP in CPD solution were added to red cell concentrates. The pH of the RCC solution mixtures was adjusted to 6.2, 6.6, and 7.2 (pH measures at 37°) by dropwise addition of 1 *M* NaOH or 0.1 *M* HCl. The RCC solution mixtures were incubated at 37° for 4 hr. Samples were removed for biochemical analysis after 1 and 4 hr of incubation.

In the fourth experiment, rats were subjected to a 33% exchange transfusion with either PEP-treated or control rat red blood cells to determine the *in vivo* persistence of the high 2,3-DPG and P_{50} induced by PEP incubation. Fresh rat blood was collected in CPD (1:7, V:V) from 16 retired breeder rats, 600–800 g. The donor rats were anesthetized with phenobarbital 50 mg/kg (Nembutal, Sodium, Abbott Lab., Chicago, Ill.) and bled via the abdominal aorta. Blood from two rats was pooled for each transfusion. Four pools were incubated with PEP for 4 hr as previously described (5) and four pools were held in the refrigerator at 4° for 4 hr. Following incubation or refrigeration, the red cells were washed twice in three vol of acid saline and resuspended in warm 4% albumin in 0.9% saline solution. The pH of the PEP-treated suspensions was 7.369 ± 002, the P_{50} was 56.6 ± 4.7, and the 2,3-DPG content was 31.45 ± 3.18 μm/g Hgb. The pH of the control suspension was 7.380 ± 0.011, the P_{50} was 38.6 ± 0.7, and the 2,3-DPG content was 2.324 ± 1.19 μm/g Hgb. Male Sprague–Dawley rats, 250–300 g, were used as recipients. The animals were anesthetized as above, and the right exterior carotid artery and jugular vein were catheterized with PE-50 polyethelene and silastic (0.04 in 1.0) catheters, respectively. One-third of the total blood volume was exchanged by withdrawal of 2 ml/100 g body weight through the carotid cannula and replacement with an equal volume of cell suspension through the jugular catheter. Following transfusion, the jugular catheter was removed, and the carotid cannula was tunnled subcutaneously to the back of the neck and exteriorized between the scapulae. The animals were then allowed to recover, and post-transfusion samples were drawn through the carotid cannula 6 hr following the transfusion.

Stored blood. A total of 450 ml of blood was drawn from each of three healthy volunteers and collected into McGaw Haemo-Pak units containing 63 ml of CPD. These units were stored as whole blood (Hct = 40 ± 5) under standard blood bank conditions at 4°. Aliquots (10 ml) were removed at weekly intervals and placed on ice. Baseline P_{50}, ATP, and 2,3-DPG levels were measured. The remaining sample from each unit was divided equally and incubated at

37°, pH 5.9–6.2, for 4 hr in equivalent volumes of CPD or PEP in CPD. The final concentrations of PEP in the experimental blood–solution mixtures was 26 m*M*. The P_{50}, ATP, and 2,3-DPG levels were measured immediately after mixing and after 1 and 4 hr of incubation in a shaking 37° water bath (rpm = 140) with either control (CPD) or experimental (PEP in CPD) solution.

Analytical procedures. P_{50} was measured by the biotonometry method described by Neville (5). Concentrations of ATP and 2,3-DPG were determined from protein-free filtrates prepared with 6% perchloric acid (0.5:2.0, blood:PCA) neutralized with potassium bicarbonate. Assays were performed using commercially available assay kits (ATP, Sigma technical bulletin 366-UV; 2,3-DPG, Sigma technical bulletin 35-UV). Glucose was measured in the protein-free filtrates using a hexokinase technique (7).

Results. *Fresh blood.* The effects of varying the concentration of PEP on intraerythrocytic 2,3-DPG are presented in Fig. 1. A progressive fall in 2,3-DPG occurred in CPD alone such that levels 25% of the initial value were observed after incubation for 4 hr. Increasing the concentration of PEP from 13 to 52 m*M* resulted in increased generation of 2,3-DPG. Postincubation 2,3-DPG levels obtained with 78 m*M* PEP did not differ from those obtained with a 52 m*M* solution. The effect of varying the concentration of PEP on pH, P_{50}, and ATP are presented in Table I. A progressive fall in ATP occurred in CPD alone; an increment in ATP was observed with all concentrations of PEP. Increments in P_{50} paralleled the rise in 2,3-DPG. An initial increase in P_{50} was observed following addition of CPD alone. This could be attributed to an alkaline Bohr effect due to the low pH of the incubation mixture (8). However, the P_{50} increments observed with PEP incubation were significantly greater ($P < 0.05$) than those achieved with CPD alone, although the pH was the same in both CPD and PEP incubations. A moderate degree of hemolysis was observed when PEP was added to exceed a final concentration of 26 m*M*.

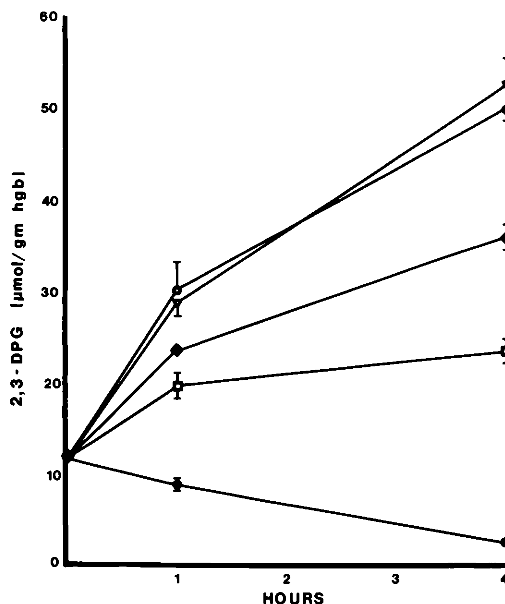

FIG. 1. Effect of varying PEP concentration on red cell 2,3-DPG production. Each value represents mean ± SEM (n = 3) symbols without vertical bars indicate the standard errors were smaller than the symbol used. ▼, 78 m*M*; ○, 52 m*M*; ◆, 26 m*M*; □, 13 m*M*; ●, 0 m*M*.

The effect of incubation temperature on intraerythrocytic 2,3-DPG is presented in Fig. 2. A 26 m*M* concentration of PEP in CPD was chosen because of hemolysis observed with higher concentrations. A dramatic increase in 2,3-DPG was observed at 37°. When measured at 4 hr, 2,3-DPG was three times greater than initial value. 2,3-DPG was maintained without significant change at 4° in both PEP–CPD and CPD alone. When the blood–PEP in CPD mixture was incubated at 25°, 2,3-DPG was also maintained at a level which approximated the initial value, whereas incubation at 25° with CPD alone resulted in a slight reduction in 2,3-DPG concentration. The effects of incubation temperature on ATP are presented in Fig. 3. ATP was maintained close to its initial value in PEP in CPD (and CPD alone) at 4° after incubation for 4 hr. However, a small but significant increase ($P < 0.05$) in ATP was observed at 25°. ATP was maintained close to its initial value at 37°.

The effect incubation pH on intraerythrocytic 2,3-DPG and ATP is presented in Table II. A fall in 2,3-DPG concentration

TABLE I. Effect of Varying PEP Concentration on Red Cells

		1 hr				
	Initial	0 mM	13 mM	26 mM	52 mM	78 mM
ATP[1,2] (μmol/g Hgb)	3.72 ± .16	3.55 ± .14	4.09 ± .51	4.35 ± .58	4.50 ± .69	4.06 ± .24
pH[1,2]	7,065 ± .038	6.25 ± .008[b,a]	6.287 ± .013[a]	6.196 ± .024[b]	6.088 ± .034[c]	6.057 ± .019[c]
P_{50}[1,2]	25.4 ± .61	34.55 ± 1.65	39.08 ± .87	38.79 ± 1.03	38.04 ± .60	37.47 ± 1.63

Table 1. *(cont'd).*

		4 hr				
	Initial	0 mM	13 mM	26 mM	52 mM	78 mM
ATP[1,2] (μmol/g Hgb)	3.72 ± .16	2.81 ± .05[b]	3.81 ± .21[a]	4.09 ± .28[a]	4.31 ± .08[a]	3.99 ± .74[a]
pH[1,2]	7,065 ± .038	6.347 ± .019[a]	6.376 ± .024[a]	6.321 ± .031[a]	6.203 ± .041[b]	6.126 ± .010[c]
P_{50}[1,2]	25.4 ± .61	30.89 ± 99[b]	42.93 ± .74[a]	44.24 ± 1.06[a]	46.95 ± 1.30[a]	45.07 ± .23[a]

[1] Each value represents mean ± SEM, $n = 3$.

[2] Analysis of variance was performed within each time period. If a F test indicated a significant difference ($P < 0.05$) existed, Duncans multiple range test was performed. Means with same superscripts are not significantly different ($P < 0.05$). Means with no superscripts indicate an insignificant F statistic was calculated.

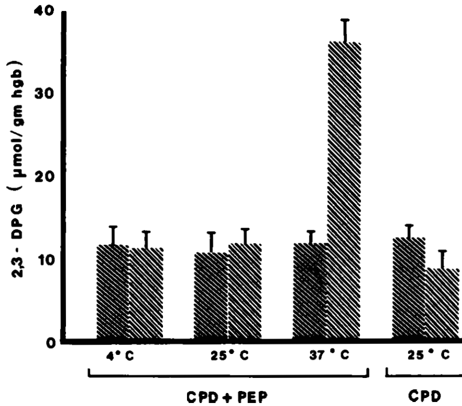

FIG. 2. Effect of temperature of incubation on red cell 2,3-DPG. At each temperature tested, the left bar represents initial mean 2,3-DPG; the right bar represents 2,3-DPG measured after 4 hr of incubation. Vertical bars represent SEM ($n = 4$). A paired t test was used to determine if the 2,3-DPG concentrations were different before and after incubation at each temperature. There was a significant ($P < 0.001$) increase when cells were incubated at 37° in the presence of PEP. There was a significant ($P < 0.001$) decrease when cells were incubated at 25° in CPD alone.

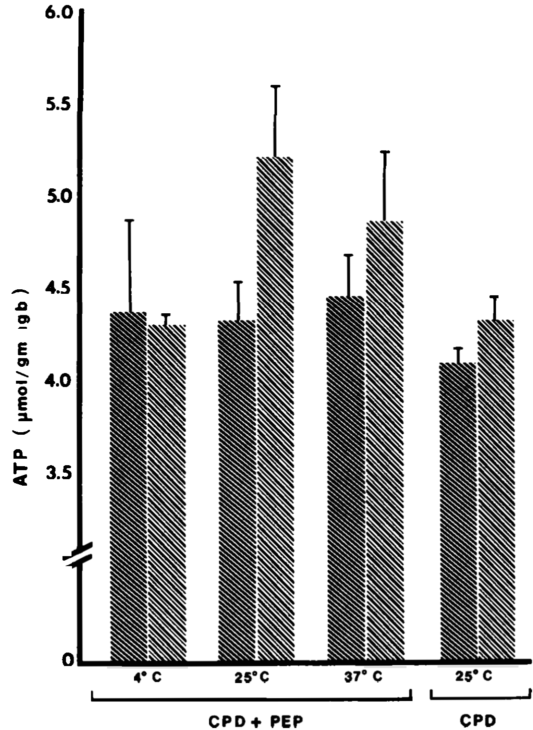

FIG. 3. Effect of temperature of incubation on red cell ATP. At each temperature examined, the left bar represents initial mean ATP, the right bar represents ATP after 4 hr of incubation. Vertical bars represent the SEM ($n = 4$). A paired t list was used to determine if the ATP concentration after incubation differed from the initial value. There was a significant increase ($P < 0.05$) in ATP concentration only when cells were incubated in the presence of PEP at 25°.

which was similar to that observed with CPD alone occurred at both pH 6.7 and 7.2 in red cells which had been incubated in PEP in CPD. However, incubation at pH 6.2 resulted in a 100% increase in 2,3-DPG. ATP concentration increased or was maintained regardless of the pH of incubation with PEP in CPD whereas, with CPD alone it was maintained at pH 6.7 and 7.2, but decreased at pH 6.2.

Stored blood. The effect of storage and subsequent incubation with either CPD or PEP in CPD on P_{50} is presented in Table III. As expected, P_{50} decrease progressively relative to the length of time in storage in CPD from Day 14 to Day 42. On all days studied, post-storage incubation with CPD alone resulted in an initial increase in P_{50} which can be attributed to the reduced pH of the incubation medium and an alkaline Bohr effect. Incubation with PEP in CPD resulted in P_{50} values which were significantly greater than that achieved with CPD alone. This effect was noted on all days studied.

The effect of storage and subsequent incubation with either CPD or PEP in CPD on red cell 2,3-DPG is presented in Table IV. As expected, red cell 2,3-DPG decreased progressively during storage in CPD. Post-storage incubation with CPD alone resulted in a further decrement in 2,3-DPG on all days studied. However, incubation with PEP in CPD resulted in a significant increase in 2,3-DPG; levels two to three times greater than those found in fresh red blood cells before storage were observed even after 42 days of *in vitro* storage.

The effect of storage and subsequent incubation with either CPD or PEP in CPD on red cell ATP is presented in Table V. Post-storage incubation with CPD alone resulted in a reduction in intraerythrocytic ATP.

TABLE II. EFFECT OF pH OF INCUBATION ON RED BLOOD CELLS INCUBATED WITH PEP

	Fresh blood	Post-4-hr incubation					
		CPD			CPD + PEP		
pH[1]		6.2	6.7	7.2	6.2	6.7	7.2
2,3-DPG[2,3] (μmol/g Hgb)	13.03 ± 1.70[c]	4.69 ± 0.73[a]	5.07 ± 1.20[a]	8.76 ± 0.01[b]	26.22 ± 0.83[d]	8.13 ± 0.71[b]	11.71 ± 0.82[c]
ATP[2,3] (μmol/g Hgb)	4.00 ± 0.10[b,c]	3.00 ± 0.00[a]	3.80 ± 0.20[b]	4.35 ± 0.05[c,d]	4.45 ± 0.05[d]	3.95 ± 0.15[b,c]	4.75 ± 0.15[d]

[1] pH.
[2] Each value represents mean ± SEM, $n = 4$.
[3] Analysis of variance was performed initially; Duncan's multiple range test was performed to determine which means differed. Means with same superscripts are not significantly different ($P < 0.05$).

However, incubation with PEP in CPD resulted in a slight increment or maintenance of ATP on all days studied.

The *in vivo* effects of 33% exchange transfusion with PEP treated or control rat red cells are presented in Table VI. Although only one-third of the blood was replaced, there was a significant increase in P_{50} following transfusion. This result indicates (a) the cells are viable and are not cleared by 6 hr post-transfusion and (b) that there is no immediate catabolism of the supranormal 2,3-DPG levels following transfusion.

Discussion. Since Chanutin and Curnish (9) and Benesch and Benesch (10) first observed that the interaction of 2,3-DPG with hemoglobin reduces hemoglobin-oxygen affinity, the importance of 2,3-DPG as a mediator of oxygen delivery has been confirmed both *in vitro* and *in vivo* (11). A functional red cell defect reflected by an increase in hemoglobin-oxygen affinity has been directly correlated with depletion of 2,3-DPG in all conventional liquid blood preservation systems. A variety of additives have been employed to maintain red cell 2,3-DPG and hemoglobin function during prolonged liquid blood storage. In general, these additives have stimulated increased generation of 2,3-DPG at the expense of the ATP (12–14) whose contribution to the maintenance of red cell membrane integrity (15, 16), deformability (17), and post-transfusion viability (18, 19) is well accepted. Thus, hemoglobin function may be preserved at the expense of red cell viability. Conversely, additives such as adenine, which stimulate generation of ATP do so at the expense of 2,3-DPG (20); thus, red cell viability may be preserved at the expense of function.

In the present studies, we have confirmed the earlier reports by Tomoda *et al.* (1) and Hamasaki *et al.* (2–4), regarding incubation of human red blood cells in a citrate solution which contains PEP. In addition, we have demonstrated that the incubation of red blood cells stored for long periods in CPD with PEP results in the generation of 2,3-DPG and the simultaneous generation of ATP. Unlike other preservation additives, PEP may facilitate the mainte-

TABLE III. EFFECT OF INCUBATION WITH PEP ON P_{50} OF RED CELLS STORED IN CPD

		P_{50} (mm Hg)			
		1 hr[1,2]		4 hr[1,2]	
Day	Initial	CPD	PEP	CPD	PEP
0	25.52 ± 1.03	33.24 ± 2.41	40.95 ± 2.49	34.16 ± 3.44	48.63* ± 1.43
7	24.45 ± 3.26	33.36 ± 3.34	39.67 ± 2.56	35.64 ± 3.8	48.74* ± 2.31
14	20.31 ± 2.30	28.39 ± 1.67	39.44* ± 1.40	33.80 ± .12	49.05* ± .73
21	17.85 ± .65	26.04 ± 1.10	38.96* ± .75	34.01 ± 2.18	52.25* ± 1.53
28	18.07 ± .36	25.74 ± .04	39.82* ± .62	34.47 ± .52	56.77* ± 2.67
35	18.75 ± .71	28.00 ± .98	41.22* ± 1.29	36.82 ± 2.19	60.62* ± 2.67
42	17.89 ± 1.16	24.37 ± 1.35	38.52* ± 2.84	29.90 ± 2.05	53.03* ± 6.66

[1] Each value represents mean ± SEM; $n = 3$.
[2] On each sampling day, comparisons were made between cells incubated in CPD alone and CPD + PEP at each time period; an asterisk (*) indicates a significant difference was found using a paired t test ($P < 0.05$).

nance of *both* hemoglobin function and red cell viability during or after (rejuvenation) prolonged blood storage. However, the results of our efforts to optimize the PEP treatment clearly demonstrate that its effectiveness is dependent on the maintenance of incubation conditions which are generally considered to be detrimental to human red blood cells.

Tomoda *et al.* (1) and Hamasaki *et al.* (2–4) have demonstrated that PEP accumulates inside human erythrocytes which have been incubated in acidified sucrose and citrate solutions (pH = 4.5–6.5). Transport is apparently mediated via a membrane-bound anion transport system. In the presence of solutes to which the red cell membrane is impermeable (sucrose and citrate) the Donnan equilibrium is maintained such that PEP can accumulate within the red cell against the concentration gradient (2). The present studies confirm the pH dependency of the PEP transport. We have observed that the optimum pH range is 5.9 to 6.2 using standard CPD solution as the PEP solvent. Above this range, PEP has no effect on incubated red blood cells.

The effect of PEP on P_{50} is negligible at 4°. This suggests that the action of PEP is mediated by an enzymatic mechanism since at temperatures which are closer to the optimum for glycolytic activity a dramatic increment in P_{50} is observed. In our work with species other than human (21) we have observed that the reduction in hemoglobin-oxygen affinity associated with PEP

TABLE IV. EFFECT OF INCUBATION WITH PEP ON 2,3-DPG CONTENT OF RED CELLS STORED IN CPD

		2,3-DPG (μmol/g Hgb)			
		Post-CPD[1,2]		Post-PEP[1,2]	
Day	Initial[1,2]	1 hr	4 hr	1 hr	4 hr
0	10.42 ± 1.13[b]	8.00 ± 1.32[a,b]	3.90 ± 2.38[a]	24.97 ± 4.57[c]	42.85 ± .15[d]
7	10.67 ± 2.17[b]	8.47 ± 3.52[a,b]	5.23 ± 1.99[a]	20.83 ± 3.29[c]	48.13 ± 6.93[d]
14	7.22 ± 0.84[a]	3.50 ± 1.31[a]	2.87 ± 0.94[a]	16.00 ± 3.16[b]	41.70 ± 1.56[c]
21	2.48 ± 1.66[a]	1.67 ± 0.44[a]	0.63 ± 0.38[a]	16.50 ± 0.47[b]	38.30 ± 1.97[c]
28	0.84 ± 0.35[a]	0.50 ± 0.85[a]	0.37 ± 0.12[a]	14.80 ± 0.75[b]	33.10 ± 1.97[e]
35	0.22 ± 0.10[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]	12.07 ± 0.20[b]	33.97 ± 1.39[c]
42	0.33 ± 0.20[a]	0.83 ± 0.42[a]	0.10 ± 0.12[a]	11.53 ± 0.54[b]	31.10 ± 3.07[c]

[1] Each value represents mean ± SEM; $n = 3$.
[2] On each sampling day, analysis of variance was performed initially; Duncan's multiple range test was then performed to determine which means varied. Means within rows with same superscripts are not significantly different.

TABLE V. EFFECT INCUBATION WITH PEP ON ATP OF RED CELLS STORED IN CPD

		ATP (μmol/g Hgb)			
		Post-CPD[1,2]		Post-PEP[1,2]	
Day	Initial[1,2]	1 hr	4 hr	1 hr	4 hr
0	4.76 ± 0.25[b]	4.47 ± 0.30[b]	3.53 ± 0.24[a]	5.60 ± 0.49[b]	4.80 ± .33[b]
7	4.25 ± 0.16[c]	3.20 ± 0.44[b]	2.17 ± 0.12[a]	4.23 ± 0.09[c]	3.67 ± 0.64[b,c]
14	3.77 ± 0.38[b,c]	3.00 ± 0.35[b]	1.57 ± 0.18[a]	3.90 ± 0.27[c]	3.80 ± 0.25[b,c]
21	2.70 ± 0.25[b,c]	2.03 ± 0.36[b]	1.23 ± 0.20[a]	3.60 ± 0.30[d]	3.13 ± 0.23[c]
28	2.28 ± 0.19[b]	1.83 ± 0.24[a,b]	1.20 ± 0.15[a]	3.30 ± 0.40[c]	3.17 ± 0.23[c]
35	2.32 ± 0.30[a,b]	1.87 ± 0.18[a]	1.43 ± 0.39[a]	3.73 ± 0.43[c]	3.20 ± 0.36[b,c]
42	1.80 ± 0.12[b]	1.57 ± 0.19[b]	0.97 ± .09[a]	2.87 ± 0.15[c]	2.67 ± 0.23[c]

[1] Each value represents mean ± SEM; $n = 3$.
[2] On each sampling day, analysis of variance was performed initially; Duncan's multiple range test was then performed to determine which means varied. Means within rows with same superscripts are not significantly different.

incubation is limited to those species whose hemoglobin is sensitive to 2,3-DPG, although in some cases, the PEP effect is limited by species specific differences in membrane transport. Furthermore, PEP fails to react directly with stroma-free hemoglobin (P. J. Scannon, unpublished data).

At the pH of incubation required for PEP transport, it is unlikely that 2,3-DPG is generated by the upper portion of the glycolytic pathway since phosphofructokinase, the rate-limiting enzyme, is certainly inhibited. We observed no difference in final glucose concentration following incubation in CPD or CPD plus PEP (data not shown). The mechanism of accumulation of 2,3-DPG observed in red blood cells which have been treated with PEP may be comparable to that observed in pyruvate kinase deficiency where a defect occurring late in glycolytic pathway results in an abnormal accumulation of PEP, 3-phosphoglycerate, and 2,3-DPG (22). In patients who suffer from this hereditary abnormality of red cell glycolysis, 2,3-DPG levels may be two to three times greater than normal and P_{50} may be significantly elevated (23, 24). The coincident maintenance of ATP which was observed in the present studies, however, suggests that pyruvate kinase function persists and that some PEP is dephosphorylated to produce pyruvate. This is confirmed by the attendant increments of pyruvate noted by Tomoda *et al.* (1) in their initial experiments.

PEP is a high-energy intermediate of the glycolytic pathway. As an intermediate compound, it is usually rapidly dephosphorylated in the presence of magnesium and potassium ions to form pyruvate. This reaction is catalyzed by pyruvate kinase and results in production of ATP. This is

TABLE VI. EFFECT OF EXCHANGE TRANSFUSION ON *In Vivo* 2,3-DPG P_{50} IN RATS

	Control[1,2]	PEP-treated[1,2]
Pretransfusion 2,3-DPG (μm/g hgb)	21.25 ± 1.60	22.04 ± 0.87
Pretransfusion P_{50} (Torr)	39.64 ± 1.24	37.41 ± 1.03
Post-transfusion 2,3-DPG (μm/g Hgb)	21.81 ± 1.34	27.58 ± 1.44*
Post-transfusion P_{50} (Torr)	39.28 ± 2.55	46.0 ± 2.37*
Post-transfusion pH	7.39 ± .03	7.37 ± .02

[1] Each value represents mean ± SEM of samples from four rats.
[2] Paired t test was used to compare pre- and post-transfusion 2,3-DPG content and P_{50} of blood from transfused rats. Asterisk (*) indicates a significant difference ($P < 0.05$).

one of two sites in the glycolytic pathway from which energy, in the form of ATP, is made available.

PEP has two possible metabolic fates: generation of pyruvate with concommitant phosphorylation of ADP to produce ATP or hydration via reversal of the normal activity of enolase. It is our hypothesis that during incubation at 37°, the latter mechanism predominates and results in the accumulation of added substrate for the generation of 2,3-DPG. At 25°, however, the former mechanism apparently prevails as ATP accumulates in excess of 2,3-DPG. This observation is consistent with the work of Hagart *et al.* (24) which demonstrated the relative insensitivity of pyruvate kinase to temperature reduction within the range of 20° to 37°. Insofar as it is inappropriate to extrapolate the results of studies performed at physiologic pH to a grossly perturbed system, the metabolic action of PEP under the conditions described herein is presently under investigation in this laboratory.

The high P_{50}s reported here are somewhat misleading. As evidenced by the P_{50} of cells incubated in CPD alone, the markedly reduced pH of incubation increased P_{50} independently of any effect of PEP. However, studies performed on rat red blood cells which were incubated with PEP, washed, and the pH adjusted to 7.40 ± 0.05, demonstrate that an increase in P_{50} occurs which is directly related to the PEP-induced generation of 2,3-DPG. Indeed, this effect persists *in vivo* at least 6 hr after transfusion (Table VI).

The effect of PEP incubation on erythrocytes has been evaluated under a variety of conditions. The evidence suggests that use of this technique for the rejuvenation of stored red blood cells may be limited by the conditions required to initiate and sustain its effect. However, unlike other preservative additives, incubation with PEP results in a dramatic increase in 2,3-DPG without depletion of ATP. We have demonstrated that this effect is maintained *in vitro* in an animal model. If it is possible to correct the deleterious conditions required for PEP action before storage under normal conditions, PEP has potential as an adjunct to conventional blood preservation systems.

The authors wish to thank N. Stevenson, D. Trail, J. Greene, L. Wettstein, and F. Sorensen for their skillful technical and secretarial assistance.

---

1. Tomoda A, Hamasaki N, Minakami S. Transport of phosphoenolpyruvate through red cell membrane in acidified isotonic sucrose medium. Biochem Biophys Res Commun 66:1127, 1975.
2. Hamasaki N, Tomoda A, Harasaki H, Minakami S. Accumulation of phosphoenolpyruvate in red cells incubated with the phosphate ester in an acidified isotonic sucrose medium. J Biochem 81:1505, 1977.
3. Hamasaki N, Hardjono IS, Minakami S. Transport of phosphoenolpyruvate through the erythrocyte membrane. Biochem J 170:39, 1978.
4. Hamasaki N, Ideguchi H, Ikehana Y. Regeneration of 2,3-biphosphoglycerate and ATP in stored erythrocytes by phosphoenolpyruvate: A new preservative for blood storage. Transfusion 21:391, 1981.
5. Sohmer PR, Scott RL. Rejuvenation of stored blood with phosphoenolpyruvate. Clin Res 29:42A, 1981.
6. Neville, JF. Hemoglobin oxygen affinity measurement using biotonometry. J Appl Physiol 37:967, 1974.
7. Bergmeyer H. Methods of Enzymatic Analysis, 2nd ed. New York, Academic Press, p1196, 1974.
8. Bohr C, Hasselbalch K, Krogh A. Ueber einen in biologischer Beziehung wichtigen Einfluss, den die Kohlensaurespannung des Blutes auf dessen Sauerstoffbinding Ubt. Skand Arch Physiol 16:402, 1904.
9. Chanutin A, Gurnish RR. Effect of organic and inorganic phosphates on the oxygen equilibrium of human erythrocytes. Arch Biochem Biophys 121:96, 1967.
10. Benesch R, Benesch RE. The effect of organic phosphates from the human erythrocyte on the allosteric properties of hemoglobin. Biochem Biophys Res Commun 26:162, 1967.
11. Sohmer PR, Dawson RB. The significance of 2,3-DPG in red blood cell transfusions. Clin Lab Sci 11:107, 1979.
12. Brake JM, Deindoerfer FH. Preservation of red blood cell 2,3-diphosphoglycerate in stored blood containing dihydroxyacetone. Transfusion 13:84, 1973.
13. Wood L, Beutler E. The effect of ascorbate on maintenance of 2,3-DPG in stored red cells, Brit J Haematol 25:611, 1973.
14. Moore GL, Ledford ME. Extended blood storage

using optional additive systems containing ascorbate-2-phosphate. Submitted for publication.

15. Weed RT, LaCelle PC. ATP dependence of erythrocyte membrane deformability. In Greenwalt TJ. (Ed.), Red Cell Membrane Structure and Function, Philadelphia, Lippincott, 1969, p. 318.
16. Weed RT, LaCelle PC, Merrill EW. Metabolic dependence of red cell deformability. J Clin Invest 48:795, 1969.
17. LaCelle PC. Alterations of deformability of the erythrocyte in stored blood. Transfusion 9:238, 1969.
18. Nakao K, Wada T, Kamiyama T. A direct relationship between adenosine triphosphate and *in vivo* viability of erythrocytes. Nature (London) 194:877, 1962.
19. Dern RJ, Brewer GJ, Wirokowski JJ. Studies on the preservation of human blood. II. The relationship of erythrocyte adenosine triphosphate levels and other *in vivo* measures to red cell storageability. J Lab Clin Med 69:968, 1967.
20. Sugita Y, Simon ER. The mechanism of action of adenine in red cell preservation. J Clin Invest 44:629, 1965.
21. Scott RL, Sohmer PR. Comparative aspects of the effect of phosphoenolpyruvate on mammalian erythrocyte metabolism. Clin Res 29:42A, 1981.
22. Minakami S, Yoshikawa H. Studies on erythrocyte glycolysis. II. Free energy charges and rate limiting steps in erythrocyte glycolysis. J Biochem 59:139, 1966.
23. Tanaka KR, Paglia DE. Pyruvate kinase deficiency. Semin Hematol 8:367, 1966.
24. Oski FA, Marshall BA, Cohem PJ. Exercise with anemia: The role of the left shifted or right shifted oxygen hemoglobin equilibrium curve. Ann Intern Med 74:44, 1971.
25. Hasart E, Jacobasch G, Rapoport S. Der Einfluss der Temperature auf die Regulation der glycolyse in Erthrozyten des Menchen und der Ratte. Acta Biol Med German 24:725, 1970.

Received September 2, 1981. P.S.E.B.M. 1982, Vol. 171.