

Glucose- and Mannose-1,6-P₂ as Activators of Phosphofructokinase in
Red Blood Cells¹

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SUMMARY

Glucose-1,6-P₂ and mannose-1,6-P₂ are concluded to be important activators from "reconstruction" experiments showing that the other known effectors of phosphofructokinase poise it at ~ 0.1% of its V_{max}, compared with the in vivo rate of ~ 1%. These activators may explain the relative insensitivity of red cell glycolysis to fructose-1,6-P₂. Glucose-1,6-P₂ is elevated more than two-fold in pyruvate kinase deficient cells but not in cells from patients with alkalosis although both have increased levels of the inhibitor, glycerate-2,3-P₂.

Human red blood cell glycolysis operates within the rate range of 0.015-0.03 μmoles of glucose consumed/min/ml cells, i.e., 1-2 μmoles/hr at 37°. When the phosphofructokinase (PFK)² step is bypassed, as with methylene blue, glucose consumption can reach 0.08 μmoles/min as the glc-6-P level goes to zero (1,2). Normally the pentose-P pathway represents only ~ 5% of the glucose consumed, and PFK, present at ~ 2.5 units/ml, at 37°, is the primary director of glc-6-P metabolism and is therefore regulated at ~ 1% of its maximum velocity in the steady state. When studied in vitro at their physiological levels, glycerate-2,3-P₂ (glt-2,3-P₂)³ and ATP depressed red cell PFK to ~ 0.1% V_{max}. The present study attempts to evaluate the factors that raise the PFK activity to its physiological range.

MATERIALS AND METHODS

The PFK activating fraction of a red cell acid extract was usually prepared from blood stored in the cold which had accumulated fru-P₂ (3). The

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² PFK: phosphofructokinase

³ glt-2,3-P₂: glycerate-2,3-P₂; glt-1,3-P₂: glycerate-1,3-P₂

washed cells in 30% suspension were incubated for 30 min at 37° in 0.9% NaCl with 1 mM pyruvate to consume the fru-P₂ (3). The recovered cells were extracted with 2 vol 1 N HClO₄ and the supernatant was immediately neutralized with KOH. The procedure of Bartlett (4) for ion exchange separation of red cell constituents was used. All fractions were neutralized with tetramethylammonium hydroxide to avoid activating effects of K⁺ or NH₄⁺. Only the fraction eluting with 0.04 N HCl in the region of fru-P₂ had the properties of activator shown in the crude extract.

Glc-P₂ (Sigma) was purified on Dowex 1 Cl⁻ as above. Man-P₂ was prepared by the procedure of Pasternak (5). Ribose-1,5-P₂ was prepared from the monophosphate by phosphoglucomutase reaction using glc-P₂ as phosphate donor and glc-6-P dehydrogenase to pull the reaction to completion. The products were purified as for glc-P₂ above.

Glc-P₂ and man-P₂ were assayed following acid hydrolysis (1 M HCl, 30 min at 100°) as the respective sugar-6-Ph with glc-6-P dehydrogenase followed by P-glucose isomerase and P-mannose isomerase. Glt-2,3-P₂ was assayed by the method of Z. Rose and Liebowitz (6). Other cell components and glyceraldehyde-P dehydrogenase were assayed by standard spectrophotometric methods.

The standard assay for PFK included 50 mM Tris-Cl pH 8.0, 1 mM ATP, 2 mM MgCl₂, 1 mM dithiothreitol, 0.15 mM DPNH, 1 mM fru-6-P 0.5 unit glycerol phosphate dehydrogenase, 2.4 units triose phosphate isomerase, 0.8-1 unit aldolase in a volume of 1 ml. All assays were at 25°.

PFK was prepared following Layzer et al. (7) without the heat step. All other enzymes were obtained commercially.

For Table I washed human red blood cells were suspended at 30% in glucose-salts medium: NaCl (77 mM), KCl (5 mM), NaHCO₃ (60 mM), Na₂HPO₄ (5 mM) and glucose (4 mM) equilibrated with 15% CO₂-85% air to give pH 7.4. Samples were taken as noted for determination of intermediates and glucose utilized by radioactivity retained by Dowex 1 acetate columns (1).

Table I

Control of PFK by FDP in Red Cells

In experiment 1 cells were first incubated for 60' at 37° in the glucose salts medium with the noted additions prior to the addition of 6-¹⁴C glucose. After another hour, samples were taken to determine ¹⁴C utilized, glucose specific activity, and amounts intermediates. In experiment 2 the cells were first incubated in 0.9% NaCl at 37° for 10' with the noted additions. After centrifugation and one wash with 4 vol saline the cells were suspended in 6-¹⁴C glucose salts medium supplemented with 2.5 mM pyruvate. Samples were taken at 60' for determinations including glyceraldehyde-P dehydrogenase (GAPDH) activity.

Additions (mM)		V_{PFK}	Glc-6-P	ATP	Fru-P ₂
		μmole/hr/ml	mM	mM	μM
1. D-Arabitol (50) + Pyruvate (5)		1.42	.072	2.30	2.4
D-Ribitol (50)		1.82	.061	2.36	114.
	GAPDH (%)				
2. None	100	1.56	-	1.90	2.5
Iodoacetate (.05)	14.5	1.69	-	1.90	12.5
" (.1)	4.6	2.04	-	1.70	193.

RESULTS AND DISCUSSION

When either rabbit muscle or human red cell PFK were assayed under conditions of fru-6-P, ATP, ADP, AMP, glt-2,3-P₂, KCl, Mg²⁺, and pH 7.4 similar to those of the human red cell, the activity was only 0.1% of the maximum velocity of the enzyme even when assayed at concentrations of enzyme similar to those in the cell. Since the glycolytic flux of the red cell is in the range of 1% of V_{max} for PFK, it seemed that important activators must be present in the cell. An acid extract of washed red cells, neutralized and treated with charcoal to

remove 260 nm absorbing compounds was found to increase the PFK rate at least 20-fold when added back at its original concentration. The activator(s) were not removed by cation exchange resin (Dowex 50⁺) but could be precipitated by Ba²⁺ from aqueous solution and absorbed by passage through a short column of Dowex 1 Cl⁻. Elution with progressively stronger HCl indicated the presence of activator(s) only in the region after ADP and before glt-2,3-P₂ or ATP where glc-P₂, man-P₂ and fru-P₂ are eluted (4,8). Consistent with glycosyl-bisphosphates as the activators were the following properties: The activator was stable to treatment with NaBH₄ under conditions that destroyed fru-P₂. Incubation at pH 11 for 4 hrs at 25° failed to destroy the activator. When heated at 60° in 1N HCl the activation was decreased to about 15% in 10 min, as determined after reisolation on the Dowex 1 Cl⁻ column by elution with 40 mM HCl. Under similar conditions both glc-P₂ and man-P₂ were rapidly hydrolysed so that only 28% and 40% remained in each case. After 30 min at 60°, all activator was lost and only 2 and 5%, respectively of the sugar bisphosphates were detected by enzymatic assay (see Methods section). These properties therefore suggest that the activator is a phosphate ester of a glycosyl phosphate.

Bartlett reported the presence of glucose and mannose bisphosphates in human red blood at substantial concentrations, 0.1-0.2 mM each (8). Glc-P₂ was subsequently reported to activate brain and muscle PFK (9,10). When these compounds in the amounts present in the Dowex 1 fraction, were added to red cell PFK inhibited by ATP and glt-2,3-P₂, they caused activation comparable to the cell fraction, as shown in Figure 1.

When assayed as in Figure 1 as activators, the following compounds were without effect: D-threose-2,4-P₂ (200 μM), L-threose-2,4-P₂ (250 μM), galactose-1,6-P₂ (12 μM) and ribulose-1,5-P₂ (200). Man-P₂ was somewhat less effective than glc-P₂, Figure 2. D-Ribose-1,5-P₂ was found to be an extremely effective activator, about 8x more active than glc-1,6-P₂, Figure 2. However the cell activator cannot be ribose-1,5-P₂ for the following reasons: It is much more

Table II

Correlation of Glt-2,3-P₂, Glc-P₂, and Man-P₂ in Red Cells

Metabolic State	Amount/ml cells			
	Serum pH	2,3-DPG μmoles	Glc-P ₂ nmoles	Man-P ₂ nmoles
Normal	-	3.79	80	102
"	-	3.56	81	61
"	-	3.49	93	81
"	-	4.38	84	50
Post operative acidosis	7.19	1.74	89	91
Alkalosis, liver disease	7.48	5.61	89	74
Alkalosis, respiratory	7.51	4.87	44	75
Pyruvate Kinase Def. (JY)	-	7.65	243	120
" (LY)	-	7.02	210	109

acid stable; i.e., compared with ribose-1,5-P₂ which was 50% destroyed as an activator in 0.15 mM HCl at 35° in 5 min, the cell activator took 30 min under the same conditions; orcinol reacting material was not present in the isolated activator fraction. Of particular significance is the observation that the level of activators was not much different in outdated blood or in cells incubated overnight in the absence of glucose. These properties agree with those of glucose and mannose bisphosphates as reported by Bartlett (8).

Control by fru-P₂. Fru-P₂, a strong activator of PFK, is known to be present in freshly drawn human red cells in very low concentration, < 5 μM (11,12). Previous studies have shown that the steps between fru-P₂ and enolpyruvate-P are in virtual equilibrium (13). Therefore one may raise the fru-P₂ and test its effect on glycolytic flux either by suitably perturbing the equilibrium, i.e., by raising the intracellular DPNH/DPN or by disrupting the

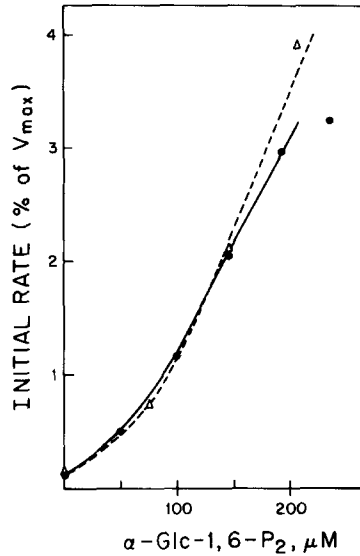


Fig. 1. Activation of PFK by red cell factor and of glc- P_2 plus man- P_2 . Each cuvette contained (mM): Tes, pH 7.5 (50), KCl (100), $MgCl_2$ (2.5), dithiothreitol (1), ATP (2.2), ADP (0.14), AMP (0.008), P_i (0.6), glt-2,3- P_2 (6), DPNH (0.1), fru-6-P (0.06), the coupling enzymes aldolase, triose-P isomerase, α -glycerol-P dehydrogenase and P glucose isomerase in excess. Either Dowex Cl^- fraction (●) or the same amount of glc- P_2 plus man- P_2 (Δ) were added successively as noted after addition of 32 m units PFK. Man- P_2 /glc- P_2 = 1.05 for all points.

equilibrium by slowing one of the enzymes (Table I). The first approach made use of an unpublished finding that ribitol causes the reduction of pyruvate added to red cells and is not metabolized further. Arabitol does not cause the reduction of pyruvate and therefore is included as a control for nonspecific effects. In the second approach iodoacetate was added at the indicated concentration to inactivate glyceraldehyde-P dehydrogenase to varying degrees. About 85% of the enzyme can be inactivated with little increase in fru- P_2 and about 95% of the activity lost without appreciable fall in ATP or decrease in glucose utilization. Thus the enzyme activity is normally about 10-fold greater than the limiting glycolytic rate, in agreement with the most recent analysis from this laboratory (13) and contrary to subsequent conclusions based on properties of the isolated enzyme (14). The increase in fru- P_2 shown in both experiments has only a slightly stimulatory effect on PFK, probably attenuated by the glc- P_2

Correlation of glc- P_2 and glt-2,3- P_2 levels in red cells. Since glucose

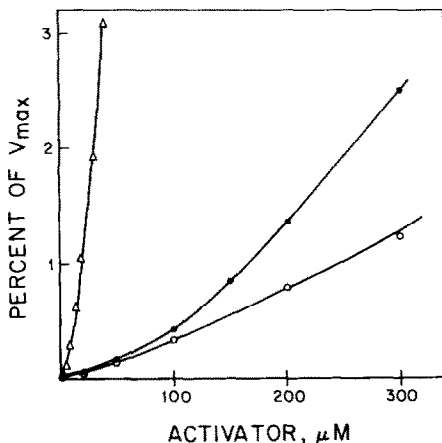


Fig. 2. Independent effect of activators. Conditions differed from Fig. 1 as follows: $MgCl_2$ (6.5 mM), fru-6-P (0.3 mM), and the noted additions, ribose-1,5-P₂ (Δ), glc-P₂ (\bullet), and man-P₂ (O).

and mannose bisphosphates are characteristically high in mammalian erythrocytes (8) which are also high in glt-2,3-P₂ it was of interest to determine whether these activators and this inhibitor of PFK change in parallel, *in vivo*, thus acting to maintain a balance in the control of PFK. Two conditions known to elevate glt-P₂ levels in red cells are blood pH (15) and pyruvate kinase deficiency (16). As seen in Table II, the content of hexose bisphosphates does not seem to change with glt-2,3-P₂ as it responds to blood pH. On the other hand there was a marked increase in glc-P₂ levels in pyruvate kinase deficient cells. The origin of this increase in glc-P₂ is very likely the elevated glt-1,3-P₂ of these cells (13) since as shown by Alpers (17) glt-1,3-P₂ can phosphorylate muscle phosphoglucomutase and by Tempkine (18) a comparable enzymatic reaction exists in red blood cells.

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