# A PROCEDURE FOR DECREASING THE LEVEL OF 2,3-BISPHOSPHOGLYCERATE IN RED CELLS IN VITRO

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SUMMARY: The physiological adaptation to anemia and other hypoxic states includes an increase in the level of 2,3-bisphosphoglycerate (2,3-DPG) in the red cell. We suggest that the high level of 2,3-DPG may have adverse effects in vivo. It has been found that red cells incubated with glycolate lose 2,3-DPG at a rapid rate relative to controls. ATP is stable. Net 2,3-DPG synthesis is observed after the glycolate is removed from the cells suggesting that they are not harmed. The effect appears to be specific for glycolate since lactate, glyoxylate, glycerate, acetate, and citrate were without effect. This procedure could be used to assess the effects of decreasing the 2,3-DPG level to normal in the erythrocytes of sickle cell and other anemias.

In anemia and other hypoxic states there is an increase in the level of 2,3-DPG (e.g. 1,2). Although this increase may be beneficial since the higher level of 2,3-DPG allows more efficient unloading of the oxygen from hemoglobin (3,4), it is possible that the abnormal level of 2,3-DPG may have adverse effects. For example, sickle cell hemoglobin gels more readily in the deoxy form (5) and the high level of 2,3-DPG found in the red cells in sickle cell disease will favor gelation. It is also possible that an abnormally high level of 2,3-DPG may contribute to the shortened life span of anemic cells since the internal pH and a variety of other properties (e.g. distribution of $Mg^{2+}$) will be altered by the presence of an excess of such a highly charged compound. We have found that it is possible to decrease the 2,3-DPG level of red cells in vitro by incubating the cells in the presence of sodium glycolate. This approach may make feasible the exploration of the possible beneficial effects of decreasing 2,3-DPG to more normal levels in treating sickle cell disease and other anemias and lead to an investigation of the therapeutic effects of glycolate feeding in such cases.

* Abbreviation: 2,3-bisphosphoglycerate, 2,3-DPG.

## METHODS

Human red cells were washed three times with 0.9% NaCl and the buffy coat was removed with suction. The cells were suspended in a medium which was pH 7.4 at 37° and contained: 50 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonate (HEPES)-$Na^+$, 106 mM NaCl, 5 mM KCl, 1 mM potassium phosphate, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM glucose.

Experimental conditions: Washed red cells were suspended in medium (hematocrit about 20%). The addition of sodium glycolate was balanced by the equimolar addition of NaCl to the controls. These additions caused a change of not more than 10% in the ionic strength of the medium. The pH was maintained at 7.40 ± 0.03 throughout the experiments by addition of NaOH as required. Samples were centrifuged at 4° and the medium removed. The cells were extracted with 5 volumes of 6% $CCl_3COOH$. Ether was used to remove the trichloracetic acid and the extracts were neutralized with triethanolamine base.

Assays: 2,3-DPG was assayed according to the method of Rose and Liebowitz (6) which makes use of the ability of glycolate-2-P to accelerate the hydrolysis of 2,3-DPG by phosphoglycerate mutase of muscle. ATP was assayed by the standard spectrophotometric procedure.

## RESULTS

When red blood cells were incubated in vitro in the presence of glucose and sodium glycolate, the 2,3-DPG level in the cells fell strikingly relative to the control (Fig. 1). After a lag of about 30 min the decrease proceeds at a constant rate. The lag is not due to a permeability barrier to glycolate, as we could show complete equilibration of glycolate-$^{14}C$ between the medium and the aqueous phase of the cells within 5 min. It is known that the 2,3-DPG level in the cell is sensitive to changes in pH (7-9) and therefore the pH was kept constant throughout. In the control under the conditions of these experiments, the rate of loss of 2,3-DPG was 0.1 μmol/hr/ml cells and ATP increased at nearly the same rate. The cellular ATP level did not fall in the presence of glycolate as might be expected if the effect of glycolate were to decrease the synthesis of glycerate-1,3-$P_2$. Fig. 1B shows two additional properties of the system. When the incubation was continued after washing away the glycolate, the net breakdown of 2,3-DPG slowed and then reversed indicating net synthesis of 2,3-DPG. Also shown is the response of the cells to a decrease in the glycolate level. After 2 hr in 34 mM glycolate (Fig. 1A), cells were placed in medium containing 16 mM glycolate. In the first subsequent 15 min period,

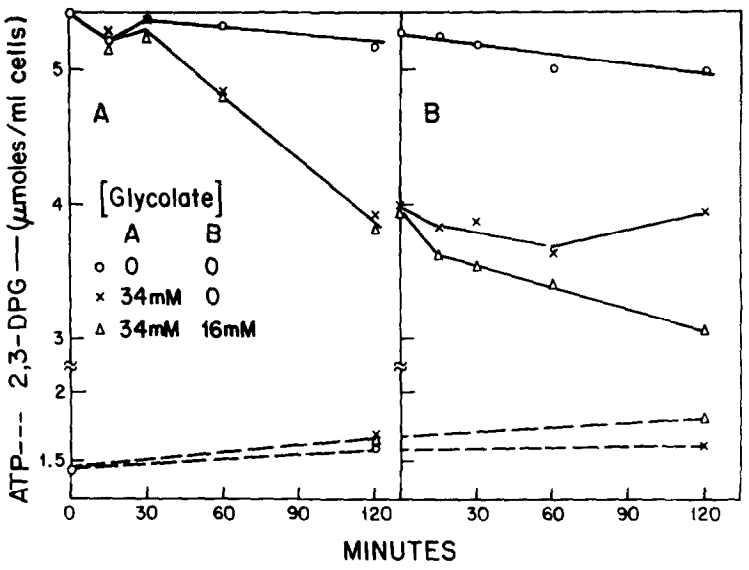


Fig. 1. Effects of glycolate on red cells incubated in vitro. A: Analysis of samples taken during the first 2 hr of incubation. The remaining cells in each incubation were then packed, washed with fresh medium, and the incubation continued with media modified as indicated. B: Subsequent analyses.

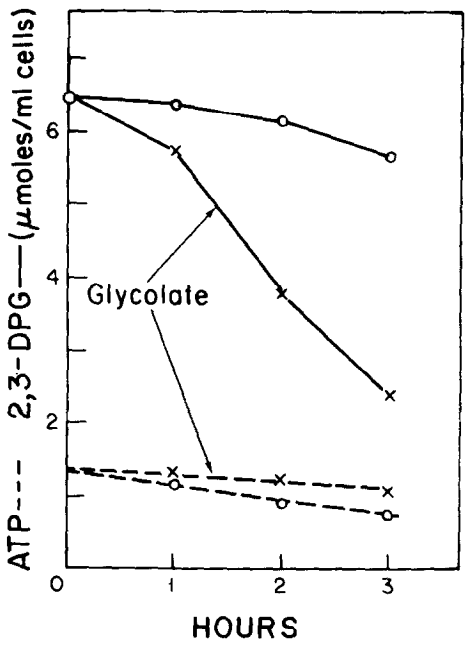


Fig. 2. Effect of glycolate on red cells incubated without glucose. Glycolate: none, ⊙; 42 mM, X.

the rate of loss of 2,3-DPG resembles that observed in the earlier period. This was followed by a new slower steady rate of loss that is compatible with the decreased level of glycolate. These observations indicate that the effect of glycolate is reversible.

Glycolate effectively induces the rapid loss of 2,3-DPG in the absence of glucose (Fig. 2). The ATP level is maintained almost unchanged throughout the 3-hr incubation period and is more stable than in the control. The control rate of disappearance of 2,3-DPG in the absence of glucose may be considered to indicate the normal rate of that reaction in the cell and, since the concentration of 2,3-DPG is usually steady, this also represents the normal rate of synthesis of 2,3-DPG. The observed rate of loss of 2,3-DPG in the absence of glucose is noted to increase with time. This may be attributed to the activation of 2,3-DPG phosphatase activity by the inorganic phosphate (10) produced from the hydrolysis of both ATP and 2,3-DPG. Therefore it is the initial rate of 2,3-DPG disappearance that may be indicative of the normal rates of synthesis and hydrolysis in the cell. Since glycolate can elicit a rate of 2,3-DPG breakdown so much faster than the normal rate of 2,3-DPG synthesis as estimated in this manner, it must be concluded that glycolate is functioning largely by an acceleration of the rate of 2,3-DPG hydrolysis. This does not eliminate the possibility of a direct effect on 2,3-DPG synthesis as well.

Other compounds were assessed for their ability to decrease the 2,3-DPG levels in red cells and, concomitantly, for any effects on the ATP levels in the cells (Table I). If the effect of glycolate on 2,3-DPG breakdown were due to an alteration of the DPN:DPNH ratio brought about by reaction (1),

$$\text{glycolate} + \text{DPN} \xrightleftharpoons{\text{lactate dehydrogenase}} \text{glyoxylate} + \text{DPNH} \qquad (1)$$

lactate itself should produce similar effects and glyoxylate might produce the opposite effect. Neither glyoxylate nor lactate causes a loss of 2,3-DPG, however. Glycerate, which is chemically similar to glycolate, has no effect on the level of 2,3-DPG. Acetate and citrate are also without effects. The results suggest that high levels of glycolate would be required for saturation (see Table I, exp. 1).

TABLE I

The Specificity of the Effect of Glycolate on the 2,3-DPG Level of Red Cells

| Exp. | Compound | | 2,3-DPG % of Control | ATP % of Control | Length of Incubation |
|---|---|---|---|---|---|
| 1 | Glyoxylate | 6.7 mM | 122 | | 4 hours |
| | | 16.7 mM | 128 | | |
| | Glycolate | 6.7 mM | 58 | | |
| | | 16.7 mM | 33 | | |
| 2 | Lactate | 30 mM | 99 | 92 | 3 hours |
| | Glycolate | 30 mM | 25 | 136 | |
| 3 | Glycerate | 42 mM | 108 | 91 | 2 hours |
| | Glycolate | 42 mM | 54 | 145 | |
| 4 | Acetate | 42 mM | 100 | 102 | 3 hours |
| | Citrate | 42 mM | 96 | 104 | |
| | Glycolate | 42 mM | 44 | 111 | |

In all cases the controls showed negligible changes of 2,3-DPG and ATP during the incubation periods.

## DISCUSSION

The decrease of 2,3-DPG in red cells incubated with glycolate is probably due to an acceleration of the rate of hydrolysis. The red cell has one enzyme, best designated bisphosphoglycerate synthase, responsible for the synthesis and hydrolysis of 2,3-DPG (11-15) according to the following reactions:

$$\text{Synthase:} \quad \text{glycerate-1,3-P}_2 + \text{3-PGA} \longrightarrow \text{2,3-DPG} + \text{3-PGA} \qquad (2)$$

$$\text{Phosphatase:} \quad \text{2,3-DPG} \longrightarrow \text{PGA} + \text{P}_i \qquad (3)$$

The phosphatase activity is stimulated by a variety of anions (10). The physiological effector is probably $P_i$, which gives half maximal stimulation ($K_a$) at 0.7 mM. The best activator of the phosphatase is glycolate-2-P ($K_a$ 0.04 mM),

which has a maximal velocity 100 times that with $P_i$ (10). Manyai and Varady (16) showed that red cell 2,3-DPG decreases when cells are incubated with bisulfite, another phosphatase activator; bisulfite has a $K_a$ of 1.7 mM and a maximal velocity 4 times that obtained with $P_i$ (10).

It is unlikely that glycolate-2-P could be introduced directly into cells since the cell membranes are usually not permeable to phosphorylated compounds. It was shown by Kayne (17) that pyruvate kinase of muscle could phosphorylate glycolate and thus it appeared possible that the effector might be produced intracellularly in red cells incubated with glycolate. It could be expected that glycolate, like acetate and lactate, would be readily permeable. The lag in the response to glycolate could be due to the need to synthesize glycolate-2-P and the persistence of the effect after removal of the glycolate from the medium (Fig. 1) could indicate the rate of disappearance of glycolate-2-P itself. The results in Table I indicate that the effect of glycolate is specific. The effects of glycolate on red cells appear to be readily reversible (Fig. 1). Therefore the system may have merit as a means of investigating possible physiological advantages of decreasing 2,3-DPG levels *in vivo*. We are continuing the study of the mode of action of glycolate in the red cell.

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