Effect of Inorganic and Organic Phosphates on Formation of Hemoglobin-Phosphate Complexes as Determined by Electrophoresis.* (30514)

ALFRED CHANUTIN AND RICHARD R. CURNISH
Biochemical Laboratory, University of Virginia School of Medicine, Charlottesville

Recent studies(1,2) have suggested that hemoglobin forms complexes with phosphorylated compounds. It was shown by means of free boundary electrophoresis that a slow-moving Component (B) increased in concentration after the addition of a number of organic phosphate compounds, particularly 2,3-diphosphoglycerate (DPG) and tri- and dinucleotides, to hemolysates of aged erythrocytes or to a purified hemoglobin solution. The present paper describes the changes in Component B concentrations after adding inorganic phosphates and several previously unstudied phosphorylated compounds to hemolysates and purified hemoglobin.

Methods. The methods of preparing hemolysates from washed red cells, the procedures used for electrophoretic analysis in the Tiselius apparatus, and hemoglobin and total phosphate determinations have been described (1,2). To obtain hemolysates with low Component B levels, outdated bank blood was allowed to stand at room temperature for several days. The procedure used by Chernoff and Liu(3) for preparing purified hemoglobin from freshly drawn and aged red cells was modified by first removing the stroma of the hemolysate by high-speed centrifugation. The salt-free solutions were adjusted to 2% Hb with water, and each phosphate compound was added to yield a final concentration of 5 or 10 μmoles/g Hb. The solutions of each of these compounds were adjusted to about pH 6.5 before addition to either the hemolysate or the purified hemoglobin solutions. The mixtures remained at 4° overnight before dialysis against the cacodylate buffer (pH 6.5, $\Gamma/2$, 0.033) used for electrophoresis.

Sodium diphosphate and sodium triphosphate (95% pure) were provided by the Virginia-Carolina Chemical Co.; sodium tetrametaphosphate (95% pure) and sodium hexametaphosphate (98% pure) were donated by the Monsanto Co. Organic phosphate compounds were purchased from Calbiochem and Mann Research Laboratories.

Results. The effect of adding inorganic phosphates on the electrophoretic patterns of hemolysates or a purified hemoglobin solution is shown in Fig. 1. Monosodium phosphate had no effect on the pattern and was therefore omitted. After adding 10 μmoles of sodium diphosphate to the hemolysate, the pattern was identical with the control. Marked increases in Component B were observed in the hemolysate supplemented with 10 μmoles of tri-, tetra-, and hexaphosphates. The conformations of Component B in the tri- and tetraphosphate samples are different despite the same values for the percentage concentration. In the hexaphosphate sample, the peaks

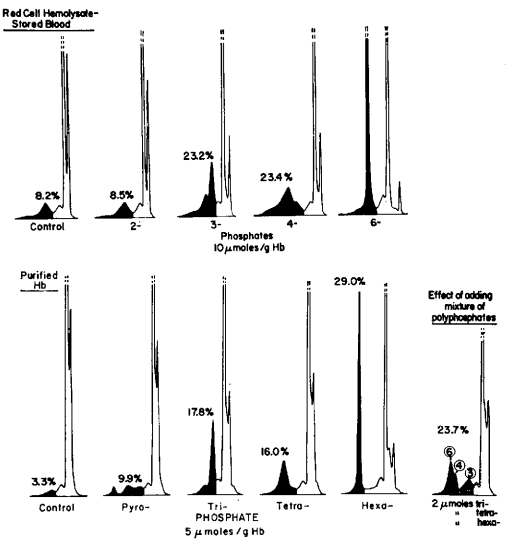

FIG. 1. Electrophoretic patterns of red cell hemolysates and of a purified hemoglobin solution which had been incubated with di-, tri-, tetra-, and hexaphosphates. The dark area represents Component B and the numbers represent the percentage of the total area. The encircled numbers show the positions of the peaks of the tri-, tetra-, and hexaphosphates.

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of both Components B and A were out of the field and thus could not be measured.

The purified hemoglobin solutions, supplemented with 5 μmoles of the various inorganic salts, yielded less complex patterns. In the presence of diphosphate, poorly resolved, slow-moving material was noted. Two Component B peaks were obtained with triphosphate, and a single broad peak was noted with tetraphosphate. The pattern for the hexametaphosphate was characterized by a tall, sharp, narrow peak for Component B.

To compare the mobilities of the components formed by tri-, tetra-, and hexaphosphates, 2-μmole quantities of each of these compounds were added to a purified hemoglobin solution (Fig. 1). The positions of the 3 new peaks corresponded to the mobilities of each of 2-μmole additions to the individual compounds added to aliquots of the hemoglobin solution. A progressive decrease in mobility was noted as the number of phosphates in the respective compounds increased. This observation is predictable since the increased number of negative phosphate charges will decrease the net positive charge of the hemoglobin-phosphate complex. The values for the percentages of the total area of the combined and the sum of the individual areas for Component B were 23.7 and 23.8, respectively; the value for the unsupplemented control was 3.2.

After adding 10 μmoles of pyridoxal-5-phosphate, riboflavin-5-phosphate, and ribose-1-phosphate to hemolysates of stored blood, the net increases in Component B were 17.8, 4.4, and 1.8%, respectively. Pyridoxal- and riboflavin-phosphates added to a purified hemoglobin solution in 5-μmole quantities caused respective net increases of 11.2 and 1.2%. Thiamine pyrophosphate, 0-carboxyphenyl phosphate, naphthyl acid phosphate, galactose-1-phosphate, thymidine-3′,5′-phosphate, mannose-1-phosphate, 2-phosphoenolpyruvate, and xylopyranosyl-1(dicyclohexylammonium phosphate), added to hemolysates in 10-μmole/g Hb quantities, had little or no effect on the Component B concentration.

Discussion. Since DPG and ATP are particularly effective in increasing Component B concentrations when added to hemolysates of red cells from stored blood, it is interesting to compare their effects with those of the polyphosphates studied in the present investigation. After a hemolysate with a Component B value of 3.7 was incubated with 10 μmoles of DPG or ATP, the Component B values showed net increases of 25.7 and 22.1%, respectively. These values are in contrast to net increases of 15.0 and 15.2% for the tri- and tetraphosphates (Fig. 1). However, 10 μmoles of hexametaphosphate caused a very marked increase in Component B which far exceeded any value previously obtained with 10-μmole supplements. After the addition of 5 μmoles hexaphosphate, the Component B values showed a net increase of 25.7% in contrast to values of 14.8 and 12.2 obtained with DPG and ATP, respectively.

The ability of hemoglobin to form complexes with such metabolites as DPG and ATP may indicate a step in the biochemical reactions of these phosphorylated compounds. It would be of some interest to study the role of the tri-, tetra-, and hexaphosphates in metabolic pathways.

Summary. Addition of tri-, tetra-, and hexaphosphates to hemolysates of aged red cells or to purified hemoglobin solutions increases the concentration of a slow-moving electrophoretic component (B). The hexametaphosphate causes a particularly large increase in the Component B concentration. Pyridoxal-5-phosphate was found to be effective in increasing the amount of the hemoglobin-organic phosphate complex.

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