ROLE OF INORGANIC PHOSPHATE IN STIMULATING THE GLUCOSE UTILIZATION OF HUMAN RED BLOOD CELLS.*

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Increased phosphate (P_i) is known to stimulate the metabolism of glucose in a variety of cells (Kvamme, 1958; Wu and Racker, 1959; Holzer and Grunicke, 1961; and Sterk and Zarkaria, 1963). It has been proposed that the mode of action of P_i is as a substrate for glyceraldehyde-P dehydrogenase (Wu and Racker, 1959; Lynen, et al., 1959) or as an activator of P-fructokinase (Passonneau and Lowry, 1962). Recent experiments from this laboratory (Rose and O'Connell, 1964) have established that the rate of the hexokinase reaction determines the steady state rate of glucose utilization in human red blood cells, and that this rate is governed by the concentration of glucose 6-P (G-6-P), a strong inhibitor of hexokinase, as well as by the ATP concentration of the cell. In an effort to determine the primary site of action of P_i on the rate of glucose metabolism in the red cell, the effect of increased P_i on the concentrations of cellular ATP and G-6-P was examined, with the expectation that stimulation of glyceraldehyde-P dehydrogenase would affect the hexokinase through an increased ATP whereas stimulation of P-fructokinase would operate through a decreased G-6-P. As is seen in Figure 1 the 2-or 3-fold increased rate of glucose utilization that results from additions of

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P_i occurs without appreciable change in the cellular concentrations of ATP or G-6-P. In these experiments, conducted for a 60 minute period, the rate of glucose utilization and the concentrations of cellular P_i, ATP, and G-6-P were the same at 30 minutes, indicating that a steady state had been established in these factors. Hence a direct effect of P_i on the hexokinase reaction was sought.

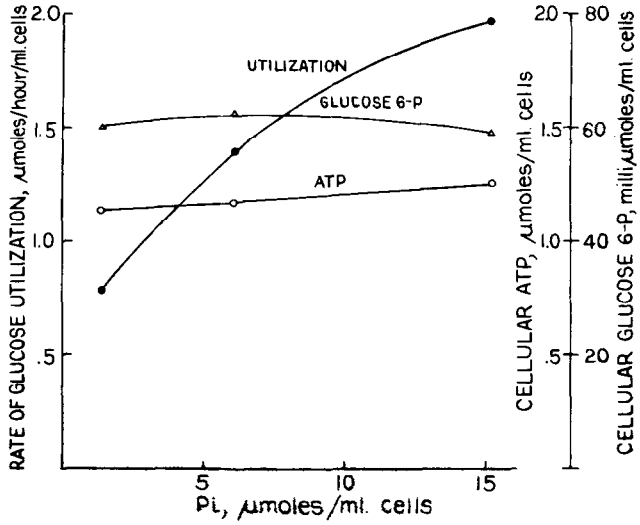

Figure 1. Velocity of glucose utilization, cellular ATP and G-6-P vs. intracellular P_i. Human red cells (.25 ml/ml of incubation) were shaken in Tris-Cl (.05 M, pH 7.3) and Na^+, K^+, and Cl^- to a total of .3 M ion concentration including P_i at 3, 20, or 50 mM. Glucose-6-C^{14} (7.5 mM, 25,000 cpm/μmole) was present and the amount utilized after 60' at 37° was determined by analyzing the acid extract for counts retained by an anion exchange resin (Dowex 1, acetate). ATP was determined in the extract by coupling yeast hexokinase (Sigma) and G-6-P dehydrogenase (Boehringer) with TPN reduction. G-6-P was determined fluorometrically with the G-6-P dehydrogenase-TPN system. Cellular P_i was determined in parallel incubations in which cells were centrifuged and washed rapidly with 20 volumes of cold saline before addition of acid (Christensen and Jones, 1962).

In the presence of adequate Mg^{++}, up to 20 mM P_i was without appreciable effect on the initial rate of the red cell hexokinase reaction as measured in the G-6-P dehydrogenase-coupled system. On the other hand, when the enzyme was assayed in the presence of

inhibitory concentrations of G-6-P by an isotopic assay, P_i was found to overcome the inhibition completely. As seen in Figure 2, the effect of 15 mM P_i is to raise the apparent inhibition constant of G-6-P from 35 μM to 150 μM under the conditions of the experiment.

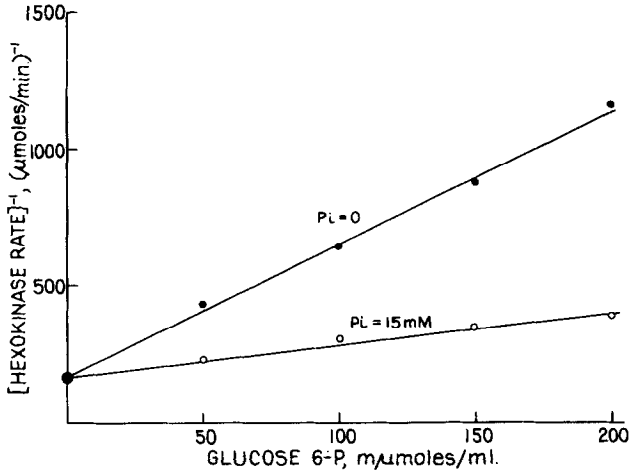

Figure 2. Plot of (rate of hexokinase)$^{-1}$ vs. G-6-P concentration at 0 or .015 M P_i. Glucose-6-C^{14}, 0.3 mM is incubated at 32° with varying amounts of G-6-P and an amount of red cell hexokinase which converts 0.006 μmoles of glucose to G-6-P per minute in a medium containing 5 mM ATP, 10 mM $MgCl_2$, and triethanolamine-Cl^- buffer, 0.10 M, pH 8.0. The amount of product formed was determined by counts held on the anion exchange resin.

To determine whether this was the mechanism of action of P_i in the intact cell, the apparent inhibition constant of G-6-P for glucose utilization was determined at two levels of P_i. In such experiments (Rose and O'Connell, 1964), increasing amounts of methylene blue are added to separate cell incubations to produce decreasing steady state concentrations of G-6-P. The effect of methylene blue is to stimulate the removal of G-6-P via the G-6-P dehydrogenase reaction. Figure 3 shows that over the series of methylene blue concentrations used, the rate of glucose utilization by the cells had the expected reciprocal relation to G-6-P level and that the apparent inhibition constant for G-6-P was increased by P_i from 10 to 19 mμmoles per ml. of packed cells.

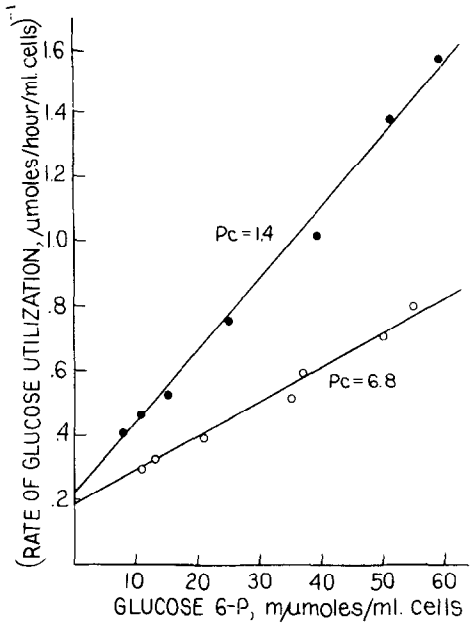

Figure 3. Effect of P_i on glucose utilization at varying P_i. Washed red cells were incubated as in Figure 1 with either 3 or 20 mM P_i except that glucose-1-C^{14} at 7.5 mM and 22,700 cpm/μmole was present as well as methylene blue at 0, .2, .7, 1.5, 4, 10, or 25 μM in a Warburg flask with a alkali center well. The $C^{14}O_2$ and C^{14}-anions that were formed in 60' at 37° were determined. The reciprocal of the sum of these is compared with the G-6-P found in the acid extract. The cellular P_i concentrations, P_c, were 1.4 and 6.8 μmoles/ml of packed cells and did not vary greatly with the methylene blue content.

Discussion. The profound effect of P_i on the rate of glucose utilization without altering the G-6-P level of the cell significantly must result from an exact coordination of the effects of P_i on the hexokinase and P-fructokinase rates. This has the property of making P_i an effector of the rate of ATP production through the Embden Meyerhof pathway without altering the rates of other metabolic events, such as glycogen synthesis and the phosphogluconate pathway, which might depend on G-6-P concentration. Furthermore, the stability of the G-6-P level means that P_i-stimulated P-fructokinase is not required to proceed at an increased rate with a decreased steady state concentration of its substrate, fructose

6-P, as would be required if the sole mechanism for the P_i stimulation were its effect on P-fructokinase. The ability of P_i to modulate the effectiveness of G-6-P as an inhibitor of hexokinase is also seen with both the particulate and soluble enzymes of ascites tumor cells and perhaps other mammalian cells and may explain, in part, the incongruous relation between glucose metabolism rates and G-6-P level in some materials (Özand, Narahara, and Cori, 1962; and Helmreich and Eisen, 1959). Experiments are in progress to test the possibility that this effect of P_i may be important in the postulated role of P_i in the Pasteur effect.

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