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Inosine Effects on Plasma Inorganic Phosphate Movements in Fresh and Cold-Stored Blood.* (25070)

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Our purpose was to determine changes in phosphate movement in fresh and cold-stored blood in the presence of inosine, a substance currently used in clinical preservation of whole blood by cold storage. Influx of phosphate from plasma to the red cell has been studied several times in both fresh and cold-stored blood(1,2,3) but the efflux of phosphate from the cellular phase to plasma has been studied only in fresh blood(4,5,6). Inosine and other purine ribosides are metabolized by human erythrocytes(7,8,9) and this is associated with an increase in intracellular phosphorylated compounds. These and other intracellular metabolic changes doubtless influence the movements of phosphate between plasma and cells. We studied movements into and out of red cells by following changes in 1) plasma inorganic phosphate and 2) P^{32} in plasma and cells. To limit the number of variables, we added only anticoagulant and the reagents under study.

Methods. Blood from adult male donors was handled as described by Kahn and Acheson(10) except that glucose was omitted. At least 3 experiments were performed for each treatment. I. One hundred ml of whole blood either fresh or cold-stored was divided in half, and Sodium Radiophosphate (Abbott Labs) was added to one-half of the blood. Both samples of blood were incubated in constant temperature oven at 37°C for 75 minutes with intermittent gentle agitation. The 2 samples were then centrifuged at 0-4°C and the plasmas removed and saved. The cells were washed once with cold saline to remove most of trapped plasma. After centrifugation of the resuspended cells, the saline was discarded and the cells with P^{32} were added to the plasma without P^{32}, while the cells without P^{32} were added to the plasma with P^{32}. After the plasmas were exchanged, blood was added to glass-stoppered Erlenmeyer flasks containing 0.1 volume of either saline (control), or 40 mM inosine. The flasks were then shaken 3 hours at 37°C in a Dubnoff shaker. Samples were taken at different intervals and measurements of plasma radioactivity and inorganic phosphate were made. Zero time was the time the reconstituted blood was added to the flasks. II. In other experiments fresh blood was preincubated with P^{32} in the manner just described, but both samples (radioactive and non-radioactive) were stored in the cold room for 6 days before centrifugation, exchange of plasmas and 37°C incubation. A Beckman Model G pH meter measured pH of whole blood. Both fresh and cold-stored blood, containing inosine or saline, measured before, during and after the experiment, had pH's between 7.6 and 7.7. Hematocrits were measured in duplicate by the technic of Guest and Siler(11). When standard errors of the mean or probabilities were determined, the "t" test

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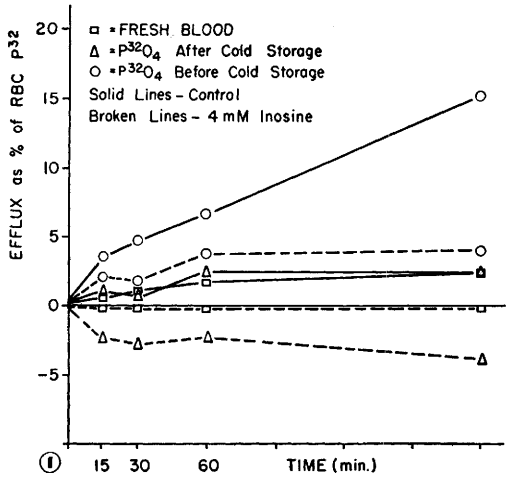

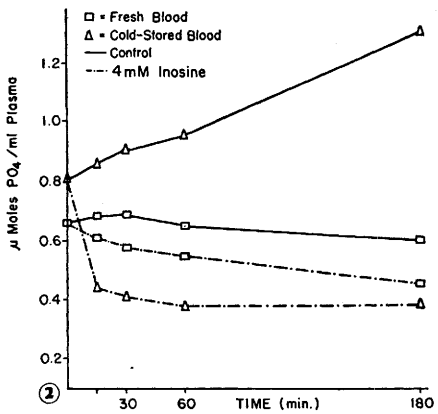

FIG. 1. Net efflux of P^{32} during incubation at 37°C.

FIG. 2. Plasma inorganic phosphate during incubation at 37°C.

was used(12). The method of counting P^{32} was that described by Kahn(3) and the method of determining inorganic phosphate was the Martin and Doty(13) modification of method of Berenblum and Chain(14).

Results. A: When fresh or cold-stored cells are loaded with P^{32} and resuspended in plasma initially free of P^{32} according to method I, an intracellular P^{32} concentration at least 10 times higher than that of the plasma is obtained. With further incubation at 37°C there is a measurable loss of P^{32} from cells to plasma. Both in fresh blood and in cold-stored blood this loss occurs within the first hour of incubation and is unchanged after 2 further hours incubation (Fig. 1). The loss amounted to less than 5% of radioactivity of cells (at hematocrits between 25 and 35). This result is similar to that previously found(4,5). In contrast, red blood cells which had P^{32} present during the 6-day cold-storage period (method II) always lost more than 15% of red cell P^{32} to the plasma (Fig. 1). Inosine, 4 mM, decreased the net loss in all experiments; when method I was used, this change was enough to cause a net gain of P^{32} by cells from the plasma (Fig. 1).

B: The movement of P^{32} from plasma into cells was consistent with findings already reported in fresh blood(1,2,3). In fresh blood with no inosine present 64 ± 1.2% of plasma P^{32} left the plasma during 3 hours of incubation and in cold-stored blood 32.6 ± 1.1% moved from plasma to cells. Inosine in fresh blood had no effect on loss of P^{32} from plasma in fresh blood, but in cold-stored blood it decreased this loss. The latter is in agreement with Kahn's result(3).

C: To evaluate the role of hemolysis, hemolysate was added to blood and distribution of P^{32} (added as orthophosphate) between the inorganic phase (butanol-benzene fraction) and the organic residue in plasma, was measured. After 3 hours of incubation, hemolysis did not affect this distribution in control flasks or with added glucose (20 mM). In the presence of inosine, however, hemolysis caused a rapid incorporation of P^{32} into organic phosphate in the plasma. Using the method of Kaplan and Greenberg(15) we found this to be primarily glycerophosphate. When there was more than the equivalent of .01 ml of packed red cells hemolyzed/ml of plasma, 80% of P^{32} of plasma was incorporated as plasma organic phosphate in less than 15 minutes at room temperature.

D: In fresh blood there is 10% fall in plasma inorganic phosphate during 3 hour incubation, whereas cold-stored blood had a 70% rise in plasma inorganic phosphate. Added inosine, strikingly decreased amount of plasma inorganic phosphate in cold-stored blood, where there was noticeable hemolysis. In fresh blood the decrease was smaller (Fig. 2).

E: In 13 experiments with fresh and cold-

TABLE I. Effects of Inosine on Intracellular Inorganic Phosphate (μMoles/ml Packed RBC) $\pm$ S.E.M.

Treatment	Fresh blood		Cold-stored blood	
	Before incubation	After 3 hr incubation	Before incubation	After 3 hr incubation
None	1.01 $\pm$ .09	1.86 $\pm$ .23	.40 $\pm$ .037	2.02 $\pm$ .229
1 mM inosine		.96 $\pm$ .337		1.11 $\pm$ .10
4 mM "		.46 $\pm$ .10		.44 $\pm$ .09

stored bloods, treated in the same way as previous experiments, except they were not subjected to preliminary incubation, incubation at 37° for 3 hours increased intracellular inorganic phosphate. Inosine at 4 mM final concentration decreased intracellular inorganic phosphate below the initial value in both fresh and cold-stored bloods (Table I).

Discussion. Inosine decreased net loss of P^{32} from plasma of cold-stored blood in Kahn's work(3) and in ours. In both cases cold-stored blood probably contained close to 1% hemolysis. This amount of hemolysis together with inosine can cause a measurable formation of plasma organic phosphate (section C). It appears that inosine decreases the net loss of plasma P^{32} by forming organic phosphate compounds in plasma containing this P^{32}, *i.e.*, by diminishing the inorganic P^{32} available for movement into the cells.

The marked drop in plasma inorganic phosphate in cold-stored bloods when inosine is added (Fig. 2) is probably due to formation of plasma organic phosphate. This fall was not as marked in fresh blood where there was little hemolysis.

To study the contribution of cellular phosphate to changes in plasma inorganic phosphate, we used cells containing high concentrations of P^{32}. Using cells loaded with P^{32} immediately before the test incubation, we found no difference between fresh and cold-stored blood. The most striking result was the larger gain of P^{32} by plasma when P^{32} had been present for 6 days during cold-storage (Fig. 1). We propose that there are at least 2 sources of P^{32} (or phosphate) to move out of the cell. One would be a small compartment, less than 5% of red cell P^{32}, which is rapidly turning over; this we propose is cellular inorganic phosphate. The larger compartment would be the cellular organic pool of phosphates. Brief exposure of cells to P^{32} would label the smaller compartment and most of this P^{32} would enter the cellular organic pool. After this loading period, the relatively small loss to plasma of recently acquired P^{32} suggests that the anabolic processes place the phosphate in compartments from which little of it escapes to the inorganic phase. P^{32} present for 6 days, even at cold-room temperature, would be distributed more evenly through the organic compartments, including the principal compartments subject to catabolism. Accordingly, the increase of P^{32} outflow when Method II is used would represent more uniform labeling of phosphate emerging from the organic pool.

Since, in presence of hemolysis, inosine promotes formation of plasma organic phosphate, we would expect inosine added to cold-stored blood to cause a greater net loss of P^{32} from cells because of the P^{32} retention by plasma compounds. Just the opposite is seen, *i.e.*, inosine decreases the net appearance of P^{32} from the cells into the plasma. This is true in fresh as well as cold-stored blood. Since inosine reduces flow of P^{32} into the cells in cold-stored blood, the observed effect of inosine on plasma P^{32} must underestimate retention of phosphate by the cells. Retention of cellular phosphate is consistent with the idea that purine ribosides are excellent substrates for human red cells(8). Cold-stored cells would show a greater effect with inosine because they lack substrate and therefore use more of the inosine. In the small cellular compartment, which we believe to be inorganic phosphate rapidly exchanging with plasma, almost all the phosphate would form ribose phosphate. Subsequent formation of other phosphorylated intermediates would divert the phosphate from leaving the cell. This expectation is confirmed by results of section

E where it is shown that inosine (which has previously been demonstrated to increase cellular organic phosphate(1)) sharply decreases cellular inorganic phosphate.

Summary. Movements of $P^{32}O_4$ in and out of fresh and cold-stored human erythrocytes were measured in presence and absence of inosine. I. Small amounts of hemolysis when inosine was present caused a decrease in plasma inorganic phosphate and incorporation of P^{32} into plasma organic phosphate. This formation of plasma organic phosphate decreased movement of P^{32} into the cell. II. When P^{32} was originally present in the red cell, inosine decreased the net movement from cells to plasma. We attribute this effect to utilization of inosine as substrate in the cell. III. Cells containing P^{32} during 6-day cold-storage lost 3 to 4 times as much P^{32} as cells having P^{32} for a short time. Movements from cells to plasma are described as the result of at least 2 phosphate compartments within the cell.

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Rate of Acetic Acid Production in the Rumen Determined by Isotope Dilution.* (25071)

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The fact that fermentation of carbohydrates in the rumen produces large quantities of short-chain fatty acids which are important sources of energy for the ruminant animal(1) has led to *in vitro*(2), isolated rumen(3), perfused rumen(4), and *in vivo*(1,5) technics of estimating the rates of production of these acids. To obtain better estimates of acetate production in the practically-fed ruminant, an isotope dilution method has been used in the work reported here.

Methods. A mature sheep fitted with a rumen cannula was maintained on an all-clover hay ration with minerals and water free-choice. *Via* a rumen cannula, 2.267 mc of sodium acetate-1-C^{14} was given 20 minutes after withdrawal of the morning hay. Samples of rumen liquor were then withdrawn through the cannula from the ventral-sac of the rumen near the ventral curvature, at approximately half-hour intervals over a 12-hour period. Immediately following withdrawal, each sample was strained through cheesecloth, the pH was lowered to approximately 1.5 with 10% H_2SO_4, and the sample was quick-frozen in dry ice and stored at 0°F until analyzed. Acetic, propionic, butyric, and the higher fatty acids were separated quantitatively by

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