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Research Article

CHANGES IN THE PHOSPHATE COMPOUNDS OF THE HUMAN RED BLOOD CELL DURING BLOOD BANK STORAGE *

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The normal human red blood cell, when withdrawn from the circulation, contains a substantial pool of water-soluble organic phosphate compounds, most of which are metabolic intermediates whose concentrations are maintained at constant levels by the glucose metabolism of the cell (1). If blood is stored under blood bank conditions in acid citrate dextrose (ACD) preservative, the organic phosphates of the red cell gradually disappear and inorganic phosphate accumulates. The decline of the organic phosphates coincides with a general deterioration of the cell, for example with a decrease in the glycolytic rate, an inability to concentrate potassium ions and a loss of viability measured by post-transfusion survival (2–4). Several observations have been made previously on the individual phosphate compounds during the storage of human blood in ACD (5–8). In the present study advantage is taken of the high resolution capacity of column chromatography on ion-exchange resins to obtain more complete quantitative information about the nature and changes of the phosphorylated intermediates of red cell metabolism.

METHODS

Units of blood were obtained from routine collections at the San Diego Blood Bank using glass bottles containing Formula B (National Institutes of Health) ACD, and were stored at 4° to 6° C. Seventy-five ml. aliquots were removed from the bottles aseptically at approximately weekly intervals for analysis of the red cells by ion-exchange chromatography. No evidence of bacterial contamination was found in the blood at the end of the storage period.

The aliquot of blood was centrifuged, the buffy coat removed and the packed red cells suspended in normal saline and recentrifuged. The washed cells were extracted with trichloroacetic acid and the acid was removed with ether. The extract was passed through a 1×12 cm. column of Dowex 1-X8 chloride (100 to 200 mesh) which was eluted at 2 ml. per minute with hydrochloric acid and ammonium chloride eluants as indicated in the figures. Fractions of approximately 20 ml. were collected and each was assayed for optical density at 260 mμ and for total phosphorus (9). Spectral measurements at the ultraviolet absorbing peaks indicated that most of the material was adenylate. The anthrone (10), carbazole (11) and orcinol (12) reactions were carried out on all fractions of the 0.01 N HCl and 0.1 N NH$_4$Cl elution sections to determine the content of sugar mono- and diphosphates. Inorganic phosphate was measured (13) in the 0.01 N HCl fractions. Further details of the procedures used for the isolation and extraction of the red cells, for ion-exchange chromatography and for the identification of the phosphate compounds are given elsewhere (1, 14).

RESULTS AND DISCUSSION

Analyses were carried out simultaneously on three units of blood from unselected donors at the blood bank (two male and one female, ages 21 to 32) with normal blood cell counts. The three samples of blood were assayed at 0, 8, 15, 22, 33, 44 and 62 days of storage. The results did not vary by more than 15 per cent and one was selected for presentation of the data. The 0, 15 and 62 day chromatographic patterns are shown in Figures 1 through 3. Figure 4 summarizes the changes observed in the phosphate compounds studied as a function of time.

The zero time assays revealed several marked differences between the cells collected in ACD in the present study and those red cells collected in heparin anticoagulant (1). Apparently a rapid displacement of the normal equilibrium followed shortly after the blood was mixed with the ACD solution. Actually "zero time" refers to two to three hours of *in vitro* life for the red cell, mostly at reduced temperature, including the period required for the washing and centrifugation of the

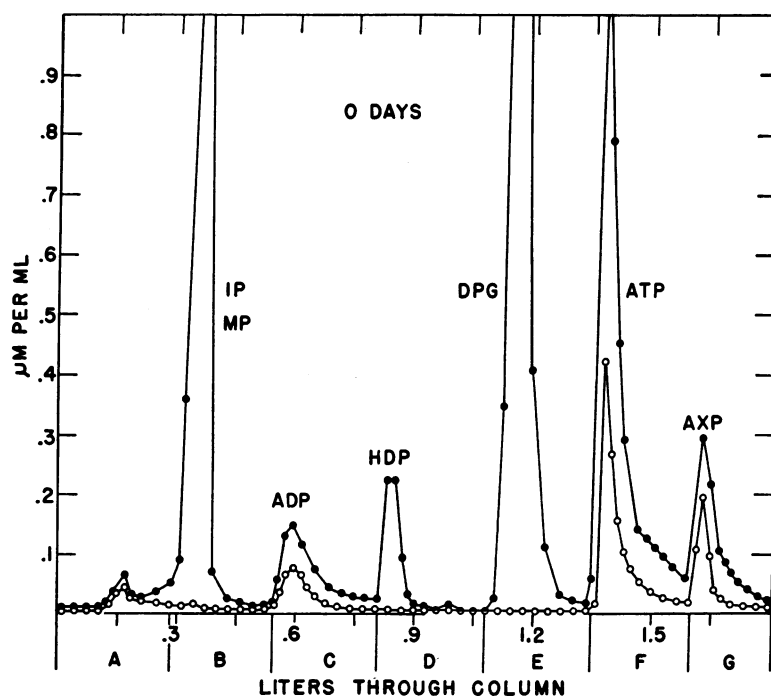† Present address: California Western University, San Diego, Calif.

FIG. 1. ION-EXCHANGE SEPARATION OF RED CELL PHOSPHATE COMPOUNDS;
ZERO DAYS' STORAGE

The red cell extract was passed through a 1 × 12 cm. column of Dowex
1-X8 chloride (100 to 200 mesh) which was eluted at 2 ml. per minute
with: A, 0.003 N HCl; B, 0.01 N HCl; C, 0.02 N HCl; D, 0.1 N NH₄Cl;
E, 0.2 N NH₄Cl; F, 0.5 N NH₄Cl; G, 1.0 N HCl. The data are plotted
as μMoles per milliliter of eluate and refer to: total phosphorus, ●; and
adenine (from the optical density of 260 mμ, $E = 15 \times 10^{3}$), ○. The peak
values at volumes of 361,378, 1,144, 1,162 and 1,181 ml. were, respectively,
1.1, 1.0, 2.3, 2.9, 2.1 and 1.3 μMoles phosphorus per ml. of eluate.

Abbreviations: IP, inorganic phosphate; MP, monophosphates (difference
between IP and total phosphorus in the A and B elution sections); ADP,
adenosine diphosphate; HDP, hexose diphosphate; DPG, 2,3-diphospho-
glycerate: ATP, adenosine triphosphate; AXP, unidentified nucleotide.

cells, and so would allow some opportunity for
metabolic changes.

The concentrations of both adenosine triphos-
phate (ATP) and 2,3-diphosphoglycerate (DPG)
were slightly lower than normal at zero time and
presumably their rapid initial breakdown ac-
counted for most of the extra inorganic phosphate
which was about three times higher than normal.

A striking immediate change was the disap-
pearance of fructose diphosphate. It had been
found that the hexose diphosphate fraction of the
normal human red cell consisted of a mixture of
glucose and fructose diphosphates in a ratio of
about two to one (1). This was remarkable in
that glucose diphosphate does not appear to be

present in other tissue in more than trace quan-
tities. That the glucose diphosphate was an ac-
tive intermediate of glycolysis in the human red
cell was demonstrated by its rapid turnover in
isotope labeling experiments with carbon[14] and
phosphorus[32] (15). In contrast to red cells col-
lected in heparin, the hexose diphosphate of the
ACD cells contained no fructose diphosphate at
zero time as shown by negative carbazole reac-
tion. The cells, however, had an approximately
normal amount of glucose diphosphate, identified
by chromatographic position and anthrone and
phosphorus ratios.

These relatively rapid changes in the red cell
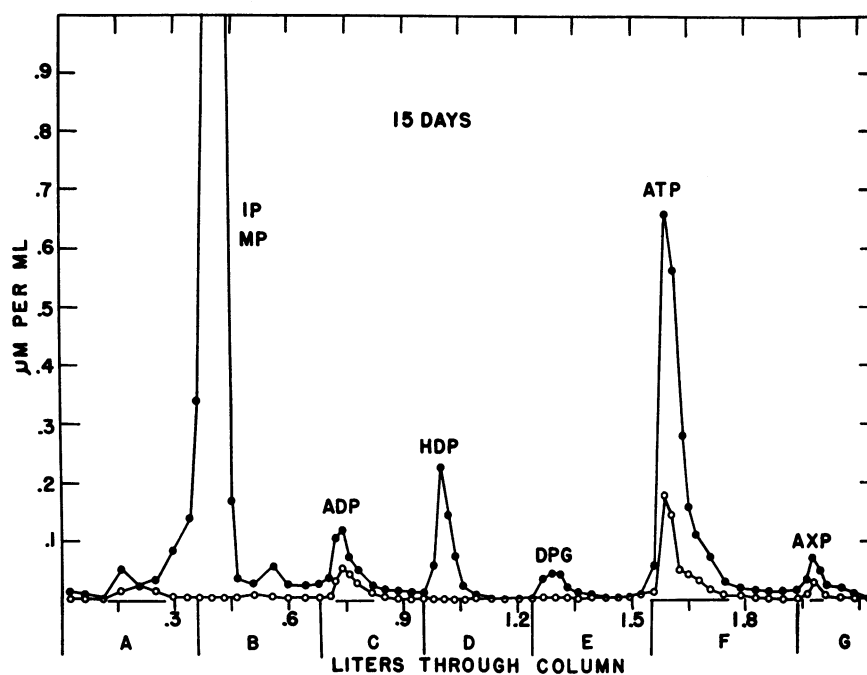which follow shortly after the addition of blood to

FIG. 2. ION-EXCHANGE SEPARATION OF RED CELL PHOSPHATE COMPOUNDS;
15 DAYS' STORAGE

See Figure 1. The peak values at volumes of 388, 410 and 433 ml. were, respectively, 1.1, 2.2 and 2.7 μMoles phosphorus per ml. of eluate.
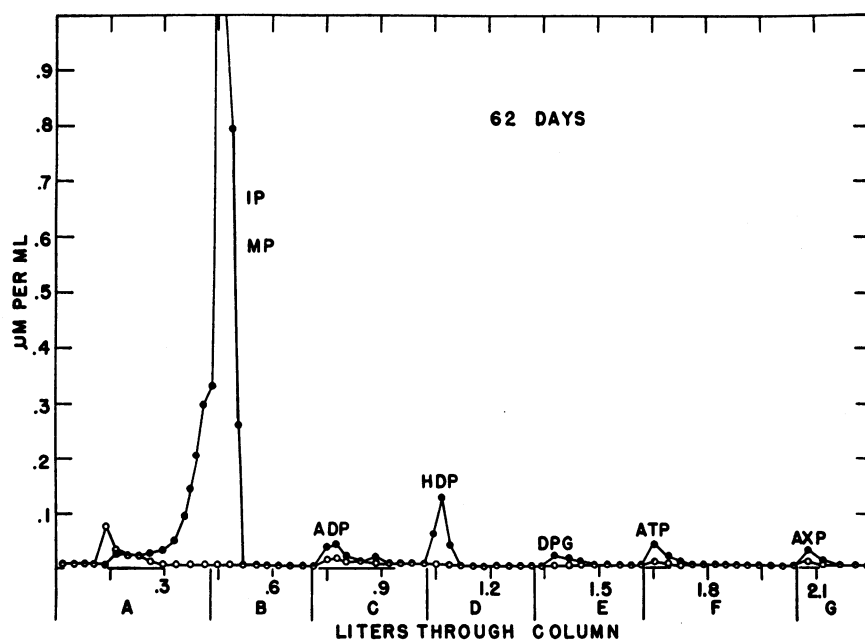
FIG. 3. ION-EXCHANGE SEPARATION OF RED CELL PHOSPHATE COMPOUNDS;
62 DAYS' STORAGE

See Figure 1. The peak values at volumes of 452 and 472 ml. were, respectively, 1.5 and 3.0 μMoles phosphorus per ml. of eluate.
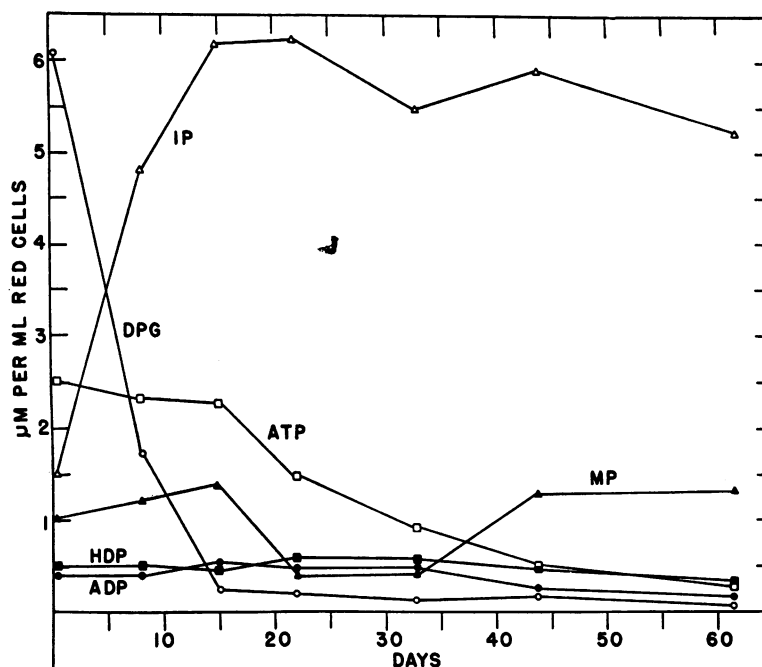
Fig. 4. Summary: Changes of Red Cell Phosphate Compounds during
Storage in ACD at 4° to 6° C.

The data are taken from ion-exchange chromatographic separations at
0, 8, 15, 22, 33, 44 and 62 days of storage and are plotted as micromoles of
phosphorus per milliliter of red cells.

the ACD solution might represent a readjustment of the metabolic equilibrium pattern to the lowered pH rather than reflecting any damage to the metabolic system.

After 62 days of storage, more than 90 per cent of the trichloroacetic acid extractable organic phosphorus had disappeared from the red cell; of special interest was the variable rate of change of the individual compounds.

The precipitous fall in the concentration of DPG during the first 15 days indicated a rapid change of some part of the metabolic machinery. The ATP content decreased slowly during the same period and then fell more rapidly after the DPG had reached a very low level. If the formation of 1,3-diphosphoglycerate were to slow down because of some defect higher in the chain of reactions, then the synthesis of ATP by the transfer of phosphorus from 1,3-diphosphoglycerate to adenosine diphosphate (ADP) would be decreased as would also ATP generation from phosphopyruvate derived from the monophosphoglycerate part of the 1,3-diphosphoglycerate. If monophospho-

glycerate is no longer feeding into the phosphoglycericmutase enzyme reaction, a mechanism could be postulated for activating DPG phosphatase to produce 2-monophosphoglycerate and phosphopyruvate and consequent ATP formation, as long as the supply of DPG held out. Assuming a constant rate of controlled metabolic utilization of ATP by the red cell, its net loss would be accelerated when synthesis from DPG stops.

The sudden loss of fructose diphosphate at the beginning of the storage is difficult to evaluate. Maintenance of a stable level of glucose diphosphate throughout most of the storage might result from the absence of an active mechanism for degrading this compound. It should be pointed out that enzymes outside of the main glycolytic pathway must be brought into action to obtain any net synthesis or breakdown of either glucose diphosphate or DPG.

It would be helpful to know more about the concentrations of glucose-6-phosphate and fructose-6-phosphate during the storage period. The organic phosphate in the weakly acidic ester area

(0.01 N HCl elution section) of the chromatograph was variable and relatively high throughout the storage. This section would include the glucose, fructose and ribose monophosphates. Although positive sugar color tests were obtained it is difficult to characterize and quantify these substances without the use of larger aliquots of red cells and formate rechromatography. Further experiments must be performed before anything definite can be said about the components of this fraction.

The intracellular inorganic phosphate rose rapidly to very high levels and at 15 days was probably derived chiefly from the decomposition of the DPG. The concentration of inorganic phosphate inside of the cell leveled off after 15 days even though considerable breakdown of organic phosphate continued, suggesting that the intracellular saturation point had been reached.

The phosphorus-containing material which was eluted with 1.0 N HCl, and labeled AXP (adenine spectrum) in the figures, included, in the zero and eight day analyses, some ATP which had not been completely removed from the column with 0.5 N NH₄Cl. However, there was also present at these times and at the later assays a nucleotide-like substance which has not been identified. Recent studies indicate that a small amount of guanosine triphosphate might be found in this area (16, 17).

It would be premature to interpret the observed changes in terms of any specific metabolic block. Present knowledge of carbohydrate metabolism is very extensive in respect to the nature of the individual enzymatic reactions but is exceedingly limited as to how these reactions are integrated into the delicately controlled metabolic processes within the living cell. Eventually the changes of the metabolic intermediates in such abnormal states' as that of blood storage should prove to be a sensitive indicator of the metabolic pathology. However, much more must be learned about what determines the intermediate concentrations in the normal steady state and how they will change under various experimental modifications.

SUMMARY

Phosphorylated metabolic intermediates of the red cell have been assayed by column chromatography on ion-exchange resin at intervals during 62 days of storage of human blood in acid citrate dextrose (ACD) at 4° C. There was a rapid loss of fructose diphosphate and a reduction of adenosine triphosphate and 2,3-diphosphoglycerate on mixing blood with ACD solution with a corresponding increase in intracellular inorganic phosphate. Almost all of the 2,3-diphosphoglycerate disappeared from the red cell during the first two weeks. The adenosine triphosphate concentration fell slowly during the same period and then more rapidly with a much slower loss of adenosine diphosphate. The glucose diphosphate level remained fairly constant throughout the incubation. Intracellular inorganic phosphate rose to a very high level at two weeks with no further rise. Some factors which might influence these changes have been discussed.

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