EFFECT OF BLOOD STORAGE CONDITIONS ON THE ELECTROPHORETIC PATTERNS OF RED CELL HEMOLYSATES¹

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During the storage of acid-citrate-dextrose (ACD) blood in a cotton-plugged flask, the electrophoretic components of the red cell hemolysate undergo changes in concentration (1). Indirect evidence indicated that the decrease in the slowest moving boundary, designated as Component B, might be correlated with the viability of the red blood cell. The standard blood bank procedures now in use involve collection and storage of blood in glass bottles or in plastic bags (closed systems). Studies were undertaken to compare the changes in the concentration of Component B in hemolysates of blood stored under a variety of conditions.

METHODS

The general procedures followed for the collection of blood in ACD and for electrophoresis have been described (1). In the present experiments, the blood was cooled by collecting the sample in a container immersed in ice water, and the sample was immediately placed in the cold room at 4° C. The 50 and 100 ml. plastic bags (EX1226) used for storage of blood were kindly provided by the Cutter Laboratories. In one case, blood was drawn directly into a bag containing ACD (NIH Sol. B); in the remaining instances blood was collected in a 125 ml. Pyrex flask or in a standard glass collecting bottle, and aliquots were transferred to the plastic bag. Storage, centrifugation, washing, lysing, clarification and dialysis against the cacodylate buffer were all conducted in the cold room.

In the previous study (1) Drabkin's procedure (2), which was used for hemolyzing the washed red cells, was found to be time consuming and yielded a solution which was altered in color due to a dark pigment. Several methods for lysing red cells were studied, which included the use of freezing and thawing (3), water and toluene, CO₂ saturated water (4), and distilled water. The patterns of red cell hemolysates obtained by freezing and thawing red cells were abnormal and contained at least six components. The three remaining procedures yielded identical patterns. Since water alone was effi-

cient and simple to use, it was adopted as the lysing agent in the procedure used.

The samples of stored bloods were not disturbed except when aliquots were drawn. The plastic bags containing blood were each kept in an open wide-mouth bottle. Samples were stored in ice water (0° C.), on the shelf of the cold room (4° C.), or in a constant temperature bath maintained at 8° C. One sample was stirred by rotating an inclined cotton-plugged Pyrex flask continuously at 2 rpm.

After centrifugation of an aliquot, the plasma and buffy coat were removed, and the red cells were washed three times with 0.85 per cent NaCl. Lysis was accomplished by adding five volumes H₂O to one volume of red cells and shaking vigorously at intervals during a 30 minute period. The hemolysate was clarified by centrifugation for 30 minutes at 10,000 rpm in a Servall SS-1 centrifuge. The hemoglobin concentration was determined (5), and 5 ml. of a 1 per cent solution was prepared for analysis. This solution was dialyzed against 0.05 M sodium cacodylate-cacodylic acid buffer (pH 6.5) which was changed three times during an 18 to 24 hour period.

Electrophoresis proceeded for 90 minutes with closed electrodes. A photograph was taken by Longworth's scanning technique (6) immediately after the current was shut off (Figure 1,a). A second photograph was obtained after the refractive bar of the A boundary had cleared (Figure 1,b). In this paper, the latter pattern was used for analysis. It was observed that the component areas of plates *a* and *b* could not be compared unless they were developed under precisely the same conditions, since the planimeter values of the tracings for a given area varied considerably (as much as 20 per cent) if the density of the plates differed. However, the percentage area of a given component in terms of the total pattern is comparable, regardless of the densities of the photographic plates.

Previously, Component B was delineated by dropping a line at the minimum between boundaries (1). For reasons discussed below, this procedure appeared to be unsatisfactory. It was decided to measure the area designated as B/2, which is illustrated in Figure 2. It will be noted that the partitioning line is dropped from the peak of the B boundary to the base line, and the area of the trailing material is measured by planimetry.

Inosine, purchased from the Schwarz Laboratories, Inc., was dissolved in saline, autoclaved and added to ACD blood so that a final concentration of 2,500 micromoles per 100 ml. red cells was attained.

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FIG. 1. ELECTROPHORETIC PATTERNS OF A RED CELL HEMOLYSATE (a) IMMEDIATELY AFTER SHUTTING OFF THE CURRENT AND (b) AFTER DIFFUSION

RESULTS

Detailed changes during storage

Patterns of red cell hemolysates of a blood sample stored for varying periods are shown in Figure 2. The changes in a) the B/2 concentrations, b) the shapes of the envelopes of Components A and B, and c) the mobilities of these boundaries are readily observed. A marked trailing of the B/2 area is seen in the 0 and 26 day old samples. Subsequently, the range of mobility of the B/2 material decreases and reaches a limiting value after 35 days of storage. During the first month, the percentage of B/2 in relation to the total pattern area remains fairly constant, but the height of Component B increases and the skewness decreases due to the sharpening of the boundary. After this time the B/2 concentration decreases. Examination of the patterns shows a pronounced increase in the mobilities of the three components during the first 46 days of storage. After storage for 56 and 76 days, it will be noted that the boundary for Component A is broadened and makes a contribution to the B/2 area.

An interpretation of the changes in the patterns during storage cannot be made until the individual components are separated and studied. The increased mobilities must be due to alteration in the

net charges of the hemoglobins. There appears to be no doubt that any specified area represents a mixture of materials. Despite these difficulties,

FIG. 2. ELECTROPHORETIC PATTERNS OF RED CELL HEMOLYSATES OF A BLOOD STORED FOR 76 DAYS AT 4° C. IN A COTTON-PLUGGED FLASK

The positions of the peaks for the f, A and B boundaries and for the trailing limit of the B/2 area at zero days are drawn through the base lines of each pattern. The arrow shows the position of the trailing limit of B/2 area in each pattern. The black areas represent B/2. The changes in mobility for the A and B boundaries are designated by the stippled and the lined areas.

FIG. 3. CHANGES IN B/2 CONCENTRATION OF EIGHT SAMPLES OF BLOOD STORED AT 4° C. IN COTTON-PLUGGED FLASKS

a quantitative measure of B/2 area appears to serve as the best indicator for judging the overall changes occurring during storage of red cells.

Controls (Figure 3)

Blood samples drawn from five healthy young men were stored in ACD in cotton-plugged Pyrex flasks and the B/2 concentration was determined at intervals. Three samples of blood, drawn from one individual at six month intervals, yielded identical results. In another individual, two blood samples drawn at an interval of six months showed differences in the rate of decrease of the B/2 component. It is apparent that there are two families of curves in this series. A set of curves is also

presented in which the results are shown as percentage concentrations of the zero day B/2 value; data were presented in this manner in a previous paper (1).

The data for the family of five curves in Figure 3 were analyzed by the method of least squares between 0 and 30 days and between 31 and 60 days of storage. The regression lines for these two periods appear to show that the B/2 concentration remains constant for at least a month of storage and that subsequently this material decreases at a steady rate. The extrapolated value of B/2 at zero concentration is 73 days. At this time, the material present in the B/2 area, which is undoubtedly a mixture of altered materials, decreases at a slower rate.

Duplicate analyses of a red cell hemolysate can be reproduced with an error of plus or minus 3 per cent. In Figure 4, the range represents a deviation of 3 per cent from the maximum and minimum values.

FIG. 4. A SCATTER DIAGRAM SHOWING THE REGRESSION LINES AND THE RANGE FOR THE B/2 CONCENTRATIONS OF FIVE STORED BLOODS

The stippled area is used as a standard for comparing the data presented in Figures 5 through 9.

Effect of anaerobic storage (Figures 5 and 6)

The changes in the B/2 concentration of blood stored in tightly stoppered tubes which contained no air or under mineral oil are compared with blood stored in a cotton-plugged flask. The rapid decrease in B/2 concentration during the first 10 days of anaerobic storage is striking; subsequently the values decrease at a slower rate.

Units of blood were drawn into standard glass

collecting bottles from four healthy individuals and a single sample was drawn for analysis from each bottle at varying periods of storage. It should be emphasized that a static air space is present in these bottles. The results obtained with these bloods are similar to those observed under strictly anaerobic conditions.

The changes in the B/2 concentrations of blood samples stored in plastic bags (anaerobic) were determined. In all cases the values remained within the control range for about 10 days and decreased appreciably during the next 5 days. In one experiment, 50 ml. of blood was collected in a 100 ml. bag and 50 ml. sterile air was injected so that the sample was under "aerobic" conditions during storage. In this experiment the B/2 con-

FIG. 5. CHANGES IN THE B/2 CONCENTRATIONS OF BLOOD STORED IN GLASS-STOPPERED TUBES (NO AIR SPACE), UNDER MINERAL OIL, AND IN STANDARD GLASS COLLECTION BOTTLES

centration decreased in the same manner observed for the blood stored in the stoppered tubes. The rates of decrease after 20 days of storage are similar for four different blood samples.

Effect of storage under oxygen, nitrogen and carbon dioxide (Figure 7)

Aliquots of two blood samples were stored under conditions in which each of the three gases flowed continuously above the surface of the blood. In one group the gas was at atmospheric pressure and in the other under a slightly positive pressure (5 cm. H₂O). In both instances, the changes in the B/2 concentration were the same during storage under nitrogen or oxygen and fell within the range of values observed for blood stored in cotton-stoppered flasks. Exposure to carbon dioxide caused a rapid and marked decrease in the B/2

FIG. 6. EFFECT OF STORAGE IN PLASTIC BAGS ON THE B/2 CONCENTRATION

The anaerobic blood samples were stored in 50 ml. plastic bags; the aerobic samples were stored in a 100 ml. bag containing 50 ml. blood and 50 ml. air.

centration during the first few days; the values reach a lower level in the blood stored under positive pressure.

The pH values of the hemolysates were determined immediately after each red cell sample was lysed and clarified. The pH values for bloods exposed to CO₂ at atmospheric and slightly in-

FIG. 7. EFFECT OF STORAGE UNDER OXYGEN, NITROGEN AND CARBON DIOXIDE ON THE B/2 CONCENTRATION

The gases were bubbled through water and through a sterile cotton plug before entering the flask containing the blood.

FIG. 8. EFFECT OF CONTINUOUS STIRRING ON THE B/2 CONCENTRATION

Blood in an inclined cotton-plugged flask rotated at 2 rpm.

creased pressures were 6.8 and 6.4, respectively. The oxygen and nitrogen exposed samples were at about pH 7.4.

Effect of continuous stirring (Figure 8)

Gentle stirring, accomplished by slow rotation in a cotton-plugged Pyrex flask, does not influence the B/2 concentration during the first month of storage. After this time, the rate of decrease of this material is appreciably greater than that of the control.

FIG. 9. EFFECT OF STORING ALIQUOTS OF THE SAME BLOOD SAMPLE AT 0, 4 AND 8° C. ON THE B/2 CONCENTRATION

Effect of temperature

Data for aliquots of a single blood sample, stored in plastic bags or in Pyrex flasks at 0, 4 and 8° C., are shown in Figure 9. No significant difference is observed for the changes in the respective 0 and 4° C. blood samples stored in plastic bags or in cotton-plugged Pyrex flasks. Identical results were obtained for a different blood sample stored at 0 and 4° in Pyrex flasks. It is remarkable that the B/2 concentrations are decreased earlier and fall to a lower level when stored under anaerobic and aerobic conditions at 8° C. These results indicate that a slight elevation in temperature has a profound influence on the composition of red cells as judged by electrophoresis.

FIG. 10. EFFECT OF THE ADDITION OF INOSINE AT 0 DAY AND AFTER AN INITIAL 21 DAY PERIOD OF STORAGE IN PLASTIC BAGS ON THE B/2 CONCENTRATION

The break in the curve after adding inosine after 21 days storage represents the period of incubation.

Effect of inosine

In a previous paper (1), it was shown that the addition of inosine to blood stored in Pyrex flasks is responsible for maintaining the Component B concentration at elevated levels for a prolonged period. In view of the more rapid decrease in the B/2 concentration during storage in plastic bags, experiments were conducted to determine the effect of inosine under these conditions. The plot in Figure 10 shows that the addition of inosine at 0 days is responsible for maintaining the B/2 concentrations at the 0 day level for about 60 days. After adding inosine on the twenty-first day of storage, at which time the B/2 value is markedly depressed, a partial regeneration of this material is noted.

DISCUSSION

The changes in the concentration of the slow moving Component B/2 of red cell hemolysates during storage of ACD blood in a cotton-stoppered flask differ slightly from those reported (1). The differences are chiefly manifested in the constancy of its concentration during the early stages of storage and the subsequent steady rate in its decrease. These data which appear to be more reliable result from the following improved techniques: *a*) collection of blood, *b*) hemolysis, *c*) electrophoresis, *d*) photography, and *e*) analysis of the pattern.

It was determined that blood should be cooled immediately after it was drawn in order to avoid the changes in the electrophoretic pattern that are readily observed when samples are allowed to remain at room temperature for short periods of time. A unit of blood (550 ml.) in a standard glass collecting bottle was warmed to 37° C., and the time required for the blood to equilibrate to 4° C. in the cold room was determined under various conditions: It required five hours when the bottle stood undisturbed, three hours when the blood was mixed at frequent intervals, and one-fourth hour if the blood was mixed while the bottle was immersed in ice cold water. It is suggested that rapid cooling of blood is essential if reliable electrophoretic data for red cell hemolysates are to be obtained.

It has been noted (1) that the Component B concentration increased during the early stages of storage of blood in a cotton-plugged Pyrex flask and decreased after about 30 days. According to the data presented in this investigation for blood stored under these same conditions, the concentration of B/2 remained fairly constant for about a month and then decreased at a steady rate for about 30 days. However, it was found that the B/2 concentration decreased within a relatively short time in bloods stored under anaerobic conditions in stoppered tubes, under mineral oil, or in plastic bags. The same results were obtained in bloods stored statically in the presence of an air space in either a plastic bag or a standard glass collecting bottle. It was surprising that blood, stored under a stream of nitrogen, gave the same results for B/2 concentration that were obtained when stored under a continuous flow of

O₂ or in the flask stoppered with a cotton plug. These results were in marked contrast to the almost immediate decrease in the B/2 concentration of blood stored under a continuous flow of CO₂. In view of these findings, it is assumed that the difference in the results with blood samples stored under closed and open systems are associated with the partial pressure of CO₂.

In a previous paper (1), it was suggested that the Component B concentration might serve for judging the viability of red cells. If this assumption is valid, the viability of red cells stored in a closed system would be decreased more rapidly than in an open system. Data are not yet available for correlating the electrophoretic analyses and the viability of transfused red cells.

SUMMARY

1. Standardized procedures for preparing red cell hemolysates for electrophoretic analysis and for analysis of patterns are described. Blood is cooled during collection and all procedures are conducted in the cold.

2. Detailed changes in the electrophoretic patterns of red cell hemolysates of stored blood are described. The shapes, concentrations, and mobilities of Components A and B change continually during storage. Measurement of an area designated as B/2, which represents about one-half of Component B, was used as an index of the changes occurring in the pattern during blood storage.

3. When blood is stored in a cotton-plugged flask, the B/2 component concentration remains constant for about a month and then decreases at a steady rate. The B/2 concentration decreases after about a week when blood is stored *a*) in stoppered tubes, *b*) under mineral oil, *c*) in plastic bags with and without an air space, and *d*) in standard glass collection bottles. The B/2 material begins to decrease almost immediately during exposure to CO₂.

4. The results obtained after storage of blood at 0 and 4° C. are the same. At 8° C., the B/2 concentration decreases rapidly.

5. During storage of blood under a continuous stream of O₂ or N₂, the change in B/2 concentration is similar to that observed in the cotton-plugged flask.

6. Continuous gentle stirring of blood during storage does not affect the B/2 component during the first month, but subsequently the rate of disappearance is marked.

7. Addition of inosine to blood immediately after collection maintains the B/2 concentration at a constant level during a two month storage period in a plastic bag. Addition of inosine to blood stored in a plastic bag for 21 days causes an appreciable rise in B/2 concentration.

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