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Electrophoretic Studies of Red Cell Extracts of Stored Blood

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Research Article

ELECTROPHORETIC STUDIES OF RED CELL EXTRACTS OF STORED BLOOD [1]

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It has been shown that red cells of blood collected in acid-citrate-dextrose (ACD) undergo changes in their dimensions, osmotic fragility, permeability and metabolism during storage at 4° C (1). Information concerning the effect of storage on the physical and chemical states of soluble red cell proteins, particularly hemoglobin, is not available. Such data may be of interest in view of the marked changes observed in the mobility and concentration of several proteins during the storage of plasma (2). This paper presents evidence from electrophoretic analyses that the concentration of components of extracts of red cells is changed during storage of blood.

METHODS

Healthy male medical students, about 21 years of age, and anemic hospitalized patients served as donors. Sterile precautions were taken in the collection, storage and sampling of the blood. As a rule, 50 ml. of blood was drawn from each individual before breakfast and collected in a 125-ml. cotton-plugged, sterile, Pyrex Erlenmeyer flask containing 12.5 ml. ACD (NIH Sol. B). Sterilized ACD-inosine or ACD-adenosine solutions were also used in the initial collection of blood. Stored blood was supplemented by calculated amounts of nucleoside in a sterilized saline solution. Phenergan® (phenothiazine, 10-(2-dimethylaminopropyl)-hydrochloride), dissolved in saline, was sterilized by filtration through an ultra-fine fritted Pyrex glass disc, and the required amounts were added to the sterile ACD solution. After removing a 5-ml. aliquot of blood for analysis, the remaining blood was stored at 4° C. It was necessary to withdraw 10-ml. aliquots for analysis if there was an excessive loss of red cells during the washing procedures or whenever the hematocrit values of the blood were low.

The blood sample was centrifuged, the plasma and the top cellular layer were removed; the cells were washed and lysed at room temperature and clarified by centrifugation in the refrigerator, according to Drabkin's procedure (3). The first step involved washing once with 0.9 per cent NaCl and three times with a 1.2 per cent NaCl-0.0025 M $AlCl_3$ mixture. Clarified solutions were examined under oil immersion with a phase microscope without observing stroma or stroma filaments. The hemoglobin concentration of the clarified red cell extract was determined (4) and an appropriate volume was diluted with distilled water to yield 5 ml. of a 1.2 or 1.4 per cent hemoglobin solution. This solution was dialyzed, in the cold, against 0.05 M sodium cacodylate-cacodylic acid buffer (pH 6.5) which was changed three times during a 24-hour period. Electrophoresis was done in a Klett Model of the Tiselius apparatus using a microcell of 2-ml. capacity. The temperature was maintained at 2.0 ± 0.01° C. Electrophoresis was allowed to proceed for 88 minutes with an open anode vessel and the boundaries were compensated (without interrupting the current) to the center of the cell; the run was continued for 30 additional minutes with a closed anode vessel. Photographs were taken by Longsworth's scanning technique (5) using CTC panchromatic plates and a Wratten No. 25 filter. These patterns were better defined than those obtained with the Philpot-Svensson cylindrical lens method. The ascending patterns were analyzed by dropping lines at the minima between boundaries (6), and where there was no well-defined minimum the same relative position of the partitioning line was maintained. The patterns of the descending limb were not analyzed because the boundaries were poorly separated.

The tests for osmotic fragility were similar to those recommended by Schales (7).

Adenosine and inosine were purchased from the Nutritional Biochemicals Corporation and from the Schwarz Laboratories, Inc., and the Phenergan® was obtained from Wyeth, Inc.

RESULTS

Conditions for electrophoresis of red cell extracts

Aliquots of a red cell extract were analyzed by electrophoresis in cacodylate buffer solutions (pH 6.5) with molar concentrations of 0.1, 0.075, 0.05, and 0.025. Under these experimental conditions, a single boundary is obtained with the 0.1 M buffer. The pattern obtained with 0.075 M buffer shows a well-defined small and a large component

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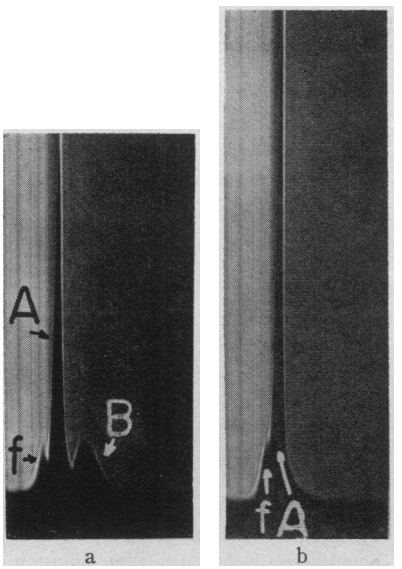

FIG. 1. ELECTROPHORETIC PATTERNS OF RED CELL EXTRACTS

(a) Freshly drawn blood.
(b) ACD blood stored 50 days.

and a small shoulder on the leading edge of the front component. Three well-defined boundaries (f, A and B) are observed in the 0.05 M buffer (Figure 1, a). The pattern of the 0.025 M buffer aliquot was difficult to analyze because of excessive sharpening of the boundaries. Aliquots of a red cell extract were adjusted to 1.25 per cent total hemoglobin and were analyzed at pH 6.4, 6.5 and 6.6 without showing significant differences in the distribution of the components. In addition, solutions varying in concentration from 0.75 to 1.75 per cent hemoglobin were analyzed at pH 6.5 and the results were similar to those observed with the 1.25 per cent solution. As a result of these preliminary experiments, the red cell extracts were routinely analyzed in a 0.05 M cacodylate buffer at pH 6.5 between 1.25 and 1.5 gm. per cent hemoglobin. Under these conditions, a steady electrophoretic state, as defined by Hoch (8) and by Nichol (9), is obtained.

ACD controls

The patterns of red cell extracts of freshly drawn ACD bloods from male students were analyzed by the Philpot-Svensson method. The concentration for component B was determined as per cent of the total area for the three components. The average per cent concentration of component B for 12 subjects was 24.5 with minimum and maximum values of 22.0 and 26.7, respectively. The Longsworth scanning procedure gives values which are 6 to 10 per cent greater than by the Philpot-Svensson method.

Effect of storage

ACD and ACD plus Phenergan®, inosine and adenosine. The effects of Phenergan®, inosine and adenosine on the concentration of component B and on the degree of hemolysis in 0.6 and 0.85 per cent NaCl were determined on a single sample of blood in order to evaluate their relative effects (Figures 2, 4, 6, 7). A unit of blood (480 ml.), obtained from a healthy young man (E. S.), was collected in 120 ml. ACD (NIH Sol. B) and aliquots were supplemented with the test materials.

The values for the percentage distribution of boundaries f, A and B are subject to errors inherent in the Tiselius-Kabat line-dropping procedure (6) and in the dissymmetry of the boundaries. Inspection of pattern b in Figure 1 indicates that component B is not present. Nevertheless, the line-dropping procedure makes it necessary to designate about 10 per cent of the total pattern as component B, when in reality most, if not all, of this area represents the trailing foot of component A. In presenting results for changes in terms of per cent of the original component B concentration, values of 30 to 40 per cent may be

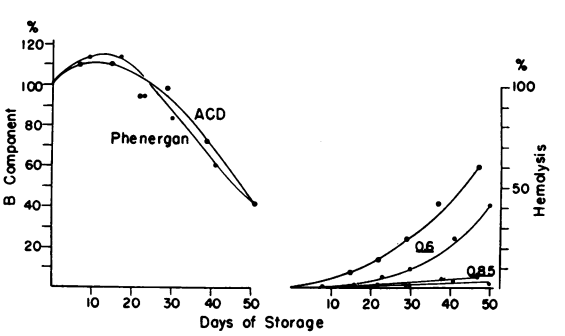

FIG. 2. EFFECT OF STORAGE OF ACD BLOOD SUPPLEMENTED WITH PHENERGAN® (0.4 MM PER L. BLOOD) ON COMPONENT B CONCENTRATION AND ON OSMOTIC FRAGILITY

The total hemoglobin concentration of the red cell extracts in this and subsequent experiments was about 1.2 per cent. The figures 0.6 and 0.85 in this and other figures refer to the percentage concentration of NaCl.

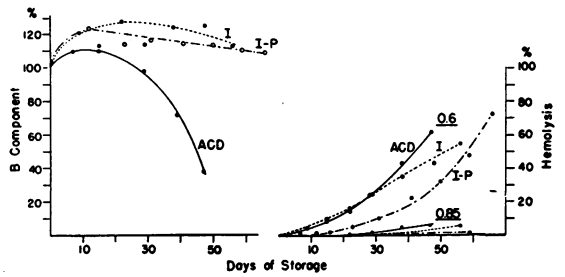

FIG. 3. EFFECT OF STORAGE OF ACD BLOOD SUPPLEMENTED WITH INOSINE (I) AND INOSINE AND PHENERGAN® (I–P)

Inosine (2,500 μM per 100 ml. RBC) and Phenergan® (0.4 mM per L. blood) were added immediately after collection of blood (E. S.).

encountered for patterns having practically none of this material present.

Phenergan®. The observation of Schales (7) that Phenergan® retarded the rate of increase of osmotic fragility of red cells in 0.6 per cent NaCl of ACD stored blood is confirmed, as shown in Figure 2. The changes in the concentrations of component B in stored ACD and ACD-Phenergan® blood are about the same. There appears to be no relationship between the osmotic fragility and the distribution of the components of the red cell extracts in these experiments.

A study of the effect of another phenothiazine derivative, chloropromazine, was discontinued because of its hemolytic properties.

Inosine. Donohue, Finch, and Gabrio (10) implicated inosine, the product of adenosine deamination, as the agent which is effective in erythrocyte preservation, and Gabrio, Donohue, Huennekens, and Finch (11) demonstrated that this nucleoside prolonged the viability of the red cell. Data plotted in Figure 3 show the changes in the concentration of the component B in (a) ACD control, (b) inosine and (c) inosine-Phenergan®-supplemented aliquots of a single blood during storage. The concentration of component B remains elevated for more than 60 days in the inosine-containing aliquots. In contrast, the values for this component in the ACD aliquot increase during the first few weeks and subsequently decrease at a comparatively rapid rate. The osmotic fragility of the inosine-Phenergan® red cells in 0.6 per cent NaCl is much lower than that of the inosine and ACD samples. The values for the per cent hemolysis after 47 days of storage of aliquots a, b, and c are 61, 43 and 28, respectively.

Inasmuch as the addition of inosine plus Phenergan® to ACD blood had a profound effect on maintaining a high component B concentration and low osmotic fragility during storage, additional data were obtained with samples of blood from three different donors (Figure 4). Considerable individual variations are noted in these experiments. The component B concentrations remained elevated during periods of about 40 to 60 days. It will be noted that the extent of hemolysis in 0.6 per cent NaCl is related to the time that the concentration of component B remains elevated.

Experiments were designed to determine the influence of varying concentrations of inosine on the electrophoretic patterns of stored blood. In Figure 5, results are shown for three individual ACD bloods supplemented with 1) 2,500 μ moles, 2) 1,250 μ moles, and 3) 625 μ moles inosine per 100 ml. of red cells. The data for bloods 2 and 3 indicate that the rate of decrease in component B concentration is greater than that of ACD blood (Figure 2). After storage for 48 days, 2,500 μ moles and 1,250 μ moles of inosine per 100 ml. of red cells were added to bloods 3 and 2, respectively. The results are striking since the concentration of component B of blood 3 is elevated to the control level and remains high during the period of observation. The value for blood 2 is also elevated from about 40 to 85 per cent but

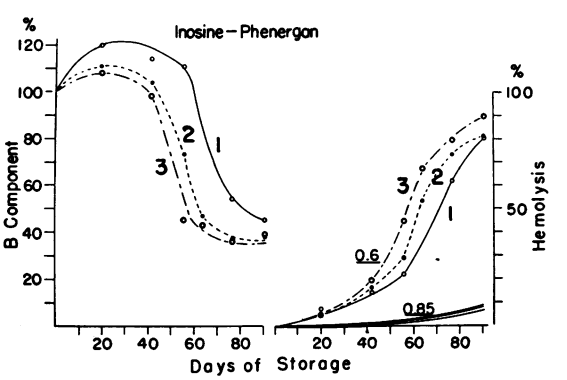

FIG. 4. EFFECT OF STORAGE OF ACD SUPPLEMENTED WITH INOSINE PLUS PHENERGAN®

Curves 1, 2 and 3 represent data for bloods from three healthy male donors. Concentrations of inosine and Phenergan® are the same as those in the previous experiment (Figure 3).

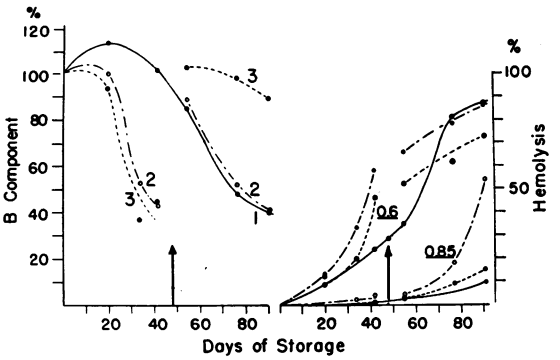

FIG. 5. EFFECT OF STORAGE OF ACD BLOOD SUPPLEMENTED WITH VARYING AMOUNTS OF INOSINE

Inosine was added to three different bloods when drawn. Sample 1, 2,500 μ moles, sample 2, 1,250 μ moles, and sample 3, 625 μ moles per 100 ml. RBC. After samples 2 and 3 were stored for 48 days, 1,250 and 2,500 μ moles inosine per 100 ml. RBC were added to the respective bloods.

subsequently decreases at the same rate observed for blood 1.

The values for the per cent hemolysis in 0.6 per cent NaCl of red cells of blood 1 are markedly increased after about 50 days of storage and practically all the cells are hemolyzed after 90 days. The osmotic fragilities of the red cells of bloods 2 and 3 are similar but are appreciably greater than those of blood 1, during the first 40 days of storage. The additional supplement of 2,500 μ moles of inosine to blood 3 prevents the marked increase in osmotic fragility. On the other hand, addition of a smaller amount of inosine to blood 2 has a temporary effect. The increased values for the per cent hemolysis of red cells of blood 2 in isotonic saline after the second addition of inosine probably reflect the lack of optimal amounts of nucleoside, which is undoubtedly concerned with the integrity of the stroma.

Adenosine. Component B concentration of ACD blood supplemented with adenosine remains elevated during a 60-day period of observation (Figure 6). It will also be noted that these red cells are more readily hemolyzed than those of the control ACD blood. The addition of adenosine or inosine to ACD blood stored for 22 days is responsible for maintaining a prolonged, elevated component B concentration and for a decreased osmotic fragility.

Effect of temperature. Three aliquots of the ACD blood were stored at 4°, 20° and 37° C. The results in Figure 7 show that the storage temperature has a pronounced effect on the concentration of component B and on the osmotic fragility. After 10 hours of storage at 37° C, about 60 per cent of the original concentration of component B disappears. A similar but not as marked a change is observed after storage at 20° C. Osmotic fragility is increased within 48 hours after storage at 37° C; a corresponding value is observed at the end of 8 days of storage at 20°.

Bloods from anemic patients. The data in Figure 8 show the changes in component B concentrations during storage of 13 bloods from patients with anemia of primary and secondary origin. The rapid decrease in values for red cell extracts from patients 1 to 5 is unusual and striking. The data for patients 6 to 8 fall within the range observed for control ACD blood during the first 30 days of storage. The values for cases 9 and 10 remain at a constant level during the period of observation. An unusually high concentration (36 per cent) of component B values for the erythrocytes of patients 11, 12 and 13 is

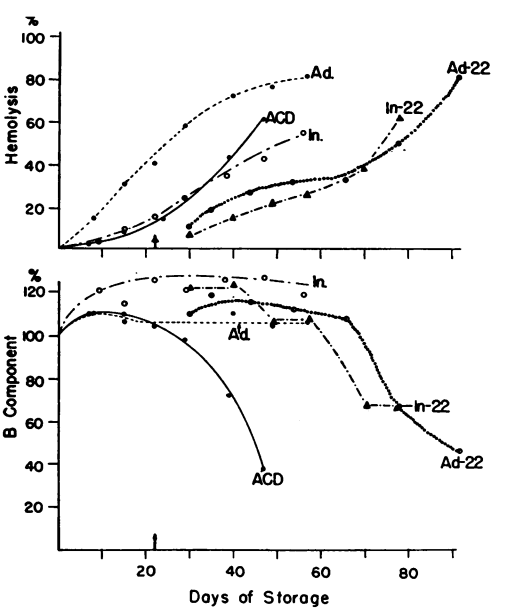

FIG. 6. EFFECT OF STORAGE OF ACD BLOOD SUPPLEMENTED WITH ADENOSINE (Ad) AND WITH INOSINE (In)

At 0 days, a suspension of adenosine (2,500 μ moles per 100 ml. RBC) was added to the blood (E. S.). After storage of ACD blood for 22 days, adenosine (2,500 μ moles) was added and incubated for 1 hour at 37° C. Inosine was added to blood under the same conditions.

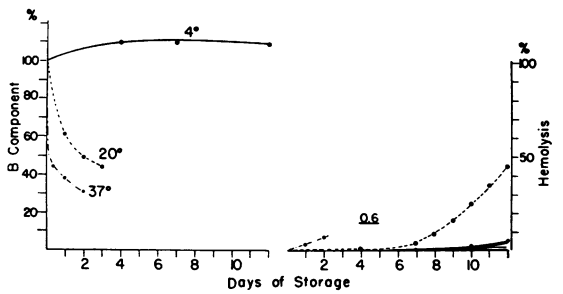

FIG. 7. EFFECT OF STORAGE OF ACD BLOOD (E. S.) AT 4°, 20° AND 37° C

striking. This is particularly true for the case of lymphocytic leukemia in which the initial concentration of component B (17.7 per cent) was the lowest value observed in these experiments.

The osmotic fragilities of the red cell in 0.6 per cent NaCl from these anemic patients tend to be consistently below the values observed for red cells from healthy individuals. The lack of any relationship between the behavior of component B and osmotic fragility is apparent in this group.

DISCUSSION

Evidence for the heterogeneity of hemoglobin has been presented by a number of investigators. Geiger (12) first demonstrated the presence of two different hemoglobins in the red cells of a number of species. He found that the hemoglobins could be separated most effectively if a red cell extract was subjected to cataphoresis at a low ionic strength. Additional evidence was provided by solubility studies (13), by electrophoresis (14–16), by oxygen capacity measurements (17, 18), by chromatographic separation (19–21), and by stability to alkaline denaturation (22–27). Schapira, Dreyfus, and Kruh (28) were able to prove the existence of at least two hemoglobins after treating extracts of Fe^{59}-labelled red cells of a single blood by alkali denaturation, electrophoresis, paper chromatography and alumina chromatography. In addition, globin, prepared from hemoglobins of several species, was found to consist of two components (29, 30). Data presented in this paper show the presence of at least two pigmented boundaries or components in red cell extracts which are assumed to be hemoglobins.

The identity of each of the three boundaries observed in the electrophoretic patterns of red cell extracts is not known. The fast moving boundary (component f) can be observed as a colorless material and probably represents the concentration gradient of the cacodylic acid mixture. Experiments to test this were conducted by comparing the areas of component f after electrophoresing aliquots of a red cell hemolysate in 0.05 M buffer into 0.047, 0.050 and 0.053 molar cacodylate buffers (pH 6.5) in the ascending limb. The areas of this boundary were smaller in the 0.047 and greater in the 0.053 molar solutions, which indicates that this boundary is due to the cacodylate ion. Components A and B are deeply pigmented boundaries which are assumed to be hemoglobins.

Derrien and Reynaud (14) observed heterogeneity in 0.1 M cacodylate (pH 6.5) if electrophoresis was continued for a sufficiently long period. In the present experiments, a single boundary was obtained for red cell extracts when electrophoresis was done at pH 6.5 in 0.1 M cacodylate buffer for two hours and heterogeneity was encountered in 0.075 M and lower concentrations. Shavit and Breuer (16) believe that hemoglobin dissociates into several different elementary units at low ionic strengths. During the storage of

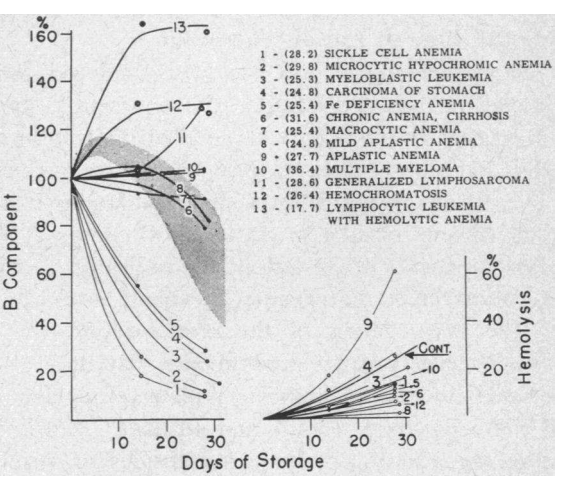

FIG. 8. EFFECT OF STORAGE OF BLOOD (ACD) OF PATIENTS WITH ANEMIA

The patterns for these bloods were obtained with the Philpot-Svensson cylindrical lens method. The numbers in parentheses represent the 0 day concentration of component B; these values are designated as 100 per cent. The stippled area represents the range of values for component B concentration of 12 bloods from healthy young men.

blood, electrophoretic analyses of red cell extracts under identical conditions show that the component B concentration decreases and component A concentration increases. After addition of nucleosides to stored blood, the component B concentration is appreciably increased. It must be concluded that these effects are not due to changes in ionic concentrations.

The degree of hemolysis of red cells of ACD blood washed with 1.2 per cent NaCl-0.0025 M $AlCl_3$ solution increased progressively with the time of storage. The wash solutions and the red cell extracts of bloods stored for 20 and 50 days were studied by electrophoresis. It was observed that the wash solutions contained components f and A while the respective red cell extracts contained components f, A and B. To explain these results it may be assumed that the oldest red cells are hemolyzed during washing and the mobility of component B has increased to that of component A.

Crystallized hemoglobin was prepared by the two methods (3, 31) described by Drabkin. In both cases, only components A and f were observed in the electrophoretic patterns of these crystals. The absence of component B may be accounted for by assuming that this material was altered during crystallization so that its net charge was the same as that of component A.

As a working hypothesis, it is believed that changes in the concentration of component B during storage of blood may serve as an indication of red blood cell viability. Gabrio and her co-workers (11, 32–34) have shown that the addition of adenosine or inosine to ACD blood causes a reversal of the "storage lesion," as indicated by the regeneration of the organic phosphate esters and by the preservation of the erythrocytes. It is shown in the present experiments that these nucleosides have been found to replete component B of red cells stored for 21 and 48 days. Furthermore, the addition of these nucleosides to freshly drawn blood is responsible for an elevation and a prolonged maintenance of component B concentration. According to Gabrio, Stevens, and Finch (35), red cells stored at 37° in ACD maintain viability for one or two days. The rapid decrease in component B concentration during the first 24 hours at 20° and 37° parallels the *in vivo* studies. It is reported that sickle cells survive from 15 to 60 days after transfusion into a normal individual (36). The defect in the sickle cell is reflected by a rapid decrease of component B in blood cells of patient 1 (Figure 8). An excellent correlation exists between the post-transfusion survival of red cells, as recorded in the literature, with the changes observed for component B of red cells. *In vivo* survival studies and electrophoretic analyses on the same red cells must be conducted before a definite correlation is established.

Sack, Gibson, and Buckley (37) have shown that there is a fair correlation between osmotic resistance in 0.6 per cent NaCl and *in vivo* survival of red cells. It would undoubtedly be important to understand the role of stroma integrity in red cell preservation. Schales (7) found that Phenergan®, a phenothiazine derivative, decreased the rate of development of the increased fragility of red cells during storage of ACD blood. Chaplin, Crawford, Cutbush, and Mollison (38) demonstrated that a related phenothiazine compound (RP 3300) had a similar effect but did not increase the ability of the red cell to survive after transfusion. The deterioration and increased permeability of red cells during storage has been attributed to the loss of cholinesterase, which could presumably be prevented by Phenergan® (39). In the present study, Phenergan® depressed the osmotic fragility of stored erythrocytes without affecting the rate of disappearance of component B of stored ACD blood. In the presence of Phenergan® and inosine, both osmotic resistance and component B concentration of stored red cells are maintained for an appreciable time at levels approximating freshly drawn blood. After this blood is stored for a long period, the concentration of component B is maintained at a high level but the osmotic fragility is markedly increased in 0.6 per cent NaCl. If it is assumed that the viability of the stored red cell is dependent on maintaining the integrity of its physical, chemical and metabolic characteristics, it is possible that Phenergan® and inosine may be useful in blood preservation.

SUMMARY

Aqueous red cell extracts prepared from human blood were analyzed by electrophoresis. Three boundaries (f, A and B), listed in the order of

decreasing mobility, were observed in a cacodylate buffer at 0.05 M concentration and pH 6.5. The concentration gradient of the buffer salt is responsible for the f boundary while components A and B appear to be hemoglobins. Component B represents about 25 per cent of the total pattern area of red cell extracts from freshly drawn blood of healthy males.

During storage of ACD and ACD plus Phenergan® bloods, the component B concentrations decrease at the same rate after about 30 days. Osmotic fragility in 0.6 per cent NaCl is lower in the Phenergan®-supplemented blood.

The addition of adenosine (2,500 μ moles per 100 ml. RBC), inosine (2,500 μ moles per 100 ml. RBC) or inosine plus Phenergan® (0.4 mM per liter of blood) is responsible for maintaining component B concentrations at elevated levels during 50 to 60 days of storage. The osmotic fragility was lowest in the presence of inosine plus Phenergan®.

Small amounts of inosine (1,250 and 625 μ moles per 100 ml. RBC) added to ACD blood appear to increase the rate of disappearance of component B during storage.

Addition of adenosine or inosine to ACD blood, stored for 21 or 48 days, causes the repletion of component B.

A marked decrease in component B concentration was observed within the first 24 hours of storage of ACD blood at 20° and 37° C.

Component B concentrations of red cells from patients with anemia showed wide differences during storage.

The component B concentration may serve as an indicator for judging the viability of red cells. The importance of osmotic resistance as a factor in red cell preservation cannot be assessed.

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ANNOUNCEMENT OF MEETING

The 49th Annual Meeting of the American Society for Clinical Investigation will be held in Atlantic City, New Jersey, on Monday, May 6, 1957, with headquarters at the Chalfonte-Haddon Hall. The scientific session will begin at 9 A.M. at the Steel Pier Theater.