THE EFFECT OF PRE-TREATMENT OF CELLS AND THE PRESENCE OF ANTIBIOTICS ON THE SHEEP CELL HEMOLYTIC SYSTEM

by

LAWRENCE ALLEN CHITWOOD

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INTRODUCTION

The sheep red blood cell hemolytic system consists of three reactive components: hemolysin (antibody), sheep erythrocytes (antigen), and guinea pig complement. This indicator system is used in most complement-fixation tests. Numerous variables will affect the final titer of this hemolytic system. Salt concentration, total volume, hydrogen ion concentration, presence of certain organic and inorganic substances, activity of complement, fragility of the erythrocyte, total number of erythrocytes, temperature and time of incubation, and the relative amount of antibody are perhaps the most important factors. With so many factors, it is difficult to state what the optimum condition is with respect to any one of the factors, since it may differ with a variation of the other factors. The purpose of this study was to determine what effect antibiotics and pre-treatment of cells with hypertonic solutions had on the hemolytic system while holding other variables constant.

The treatment of certain diseases which may be in part diagnosed by the complement-fixation test entails the use of antibiotics. A small amount of antibiotic would be introduced into the hemolytic system through the patient's serum. The first part of this study was to determine what effect an antibiotic might have in the hemolytic system.

Various solutions have been recommended for the collection of blood. The second part of this study was to determine what effect pre-treatment of erythrocytes with certain organic and inorganic compounds would have on the hemolytic system.

REVIEW OF LITERATURE

Introduction

Buchner's discovery in 1893 that a blood serum of one animal species was able to hemolyze the erthrocytes of another species initiated the beginning work in this field of serology. This reaction is still not fully understood. Unlike other antigen-antibody reactions, this effect cannot be demonstrated without a component of normal serum which is termed "complement" or "alexin". This type of hemolytic activity is restricted to the erythrocytes and to a few species of the Gram negative enteric bacteria.

This reaction has not only been useful as a means of demonstrating a particular type of lytic reaction in the laboratory, but also as an indicator of antigen-antibody combinations when no visible lysis occurs. From a diagnostic viewpoint, the sheep cell hemolytic system is used estensively in the complement fixation test.

Complement

Buchner (1893) reported that the blood serum of one animal species was able to hemolyze the erthrocytes of another species, but this hemolytic activity was destroyed by heating the serum at 55° C. for one-half hour. This substance which is found in the serum has been named "complement" or "alexin". Complement has been found to be not a single entity but a complex of several substances. Guinea pig complement can be separated by dialysis against water into an insoluble fraction or midpiece and a soluble fraction or endpiece.

Muir and Browning (1904) demonstrated that an antibody passes through

a Berkefeld filter unchanged, but if mixed with complement and filtered at 37° C., the antibody passes through the filter and the complement is retained which indicates that complement and antibody do not combine in the absence of the antigen. It has been shown that complement must combine with sensitized corpuscles before it can hemolyse them (Eagle, 1929a).

At the present time there are four separate components recognized. They have been given the numerical designation of C'1, C'2, C'3, and C'4.

The water soluble portion is C'2, and the insoluble fraction is C'1. When complement is treated with yeast, the third component (C'3) is destroyed (Raffel, 1953). The fourth component (C'4) is destroyed when treated with ammonia (Raffel, 1953).

C'1 and C'2 are very sensitive to heat, whereas C'3 and C'4 are relatively stable to heat. When complement is fractionated by water dialysis into C'1 and C'2 components, C'3 and C'4 are distributed in both fractions. The majority of the C'3 activity remains with C'1 in the insoluble fraction and the bulk of C'4 is found in the supernatant of C'2.

Properties of Complement. A temperature of 56° C. will destroy the activity of complement within one-half hour. Hydrogen ion concentrations of pH 4.8 and 8.8 will produce an irreversible change of complement (Eagle, 1929b). The activity of complement is greatest in solutions in the neighborhood of pH 7.5 (Mayer, et al., 1946). Topley (1915) reported that complement is inactive in solutions free from inorganic ions. Lundberg (1922) indicated that ultra-violet radiation would destroy complement. Pancreatic extracts have been shown to destroy the activity of complement (Wormall, et al., 1925).

Preservation of Complement. Complement will lose its hemolytic activity when kept on ice from four to ten days. Frozen serum will show little change

in activity after 14 days, and a temperature of -12° C. will maintain complement for a month (Browning and Mackie, 1913-14).

Various methods have been described in the literature for the preservation of complement. The addition of ten percent sodium acetate and four percent boric acid to the serum will result in very little change in complement activity over a period of four weeks. Sodium sulfate and boric acid have also been used in the preservation of complement. The addition of electroytes to form a hypertonic serum inactivates complement but preserves the latent activity. Subsequent dilution with water will restore the activity. Other methods in use involve the dehydration from the frozen state and lyophile methods.

Aged complement, not more than 22 days old can be reactivated by hydrogen sulfide, while 8-day old complement can be reactivated by either hydrogen sulfide or ascorbic acid (Ecker, et al., 1938a). The reversibility of the inhibition of serum complement by certain neutral salts has been studied. The effect of sodium and potassium chlorides, nitrates and bromides appear to be completely reversible on dilution after twenty-four hours' action (Gordon and Thompson, 1933). Serum treated in vitro with optimum quantities of ascorbic acid show a distinct rise of complementing activity and increased stability (Ecker, et al., 1938b).

The Mammalian Erythrocyte

The mammalian erythrocyte is a balloon-like structure which consists of a cell membrane enclosing the hemoglobin, salts, and other substances in solution. The membrane contains 80 percent protein (stromatin) with an isoelectric point of pH 5.5 and 20 percent lipoidal material, mainly lecithin and cholesterol (Ponder, 1937). This membrane is normally permeable to water

and the anions but is not permeable to the cations such as sodium and potassium under normal in vivo conditions. Frick and Morse (1925) estimated the layer which exhibits the semi-permeability is about 0.003 microns thick. This thin layer, however, forms only a portion of the cell envelope, the rest being much thicker. Ponder (1934) indicated that the membrane as a whole has a liquid crystal type structure.

Fragility of the Erythrocyte. The fragility of the red blood cell is affected by at least four factors: (1) the water content, (2) the osmotic pressure of its interior, (3) the critical volume which it can attain, and (4) the extent to which it can behave as a perfect osmometer.

The Effects of Electrolytes and Non-Electrolytes on Hemolysis. Red cells suspended in isotonic sugar solutions are always more resistant to saponin hemolysis than if they are suspended in isotonic saline. This inhibitory effect varies with the type of electrolyte, and the concentration of the lysin (Ponder, 1937). This reaction may change as the amount of electrolyte is increased, sometimes raising, other times lowering the titer.

There are almost as many degrees of resistance to hemolysis as there are hemolysins, even if the lysins are closely related. The first written record of saponin and hypotonic saline hemolysis showed an inverse relationship (Ponder, 1937). Sheep red blood cells are the most resistant to saponin hemolysis and guinea pig cells show the least resistance, however, guinea pig cells show the most resistance to hypotonic saline, while sheep cells show the least resistance.

Mechanism of Hemolysis. The complex hemolytic system differs from the simple system in that the cells are lysed only after they have been sensitized. When a hemolysin is used to sensitize the cells to the action of complement,

there is an interchangeable or reciprocal action of complement and hemolysin. A decrease in the concentration of either may be compensated for by an increase in the other (Eagle, 1929b). Several complex hemolytic systems have been studied; colloidal silicic acid (Ponder, 1933), tri-phenyl-methane dye (Browning and Mackie, 1914), and sodium taurocholate or glycocholate (Ponder, 1935), have been used as the sensitizing agents to the hemolytic action of complement.

During the lysis of the cell, the surface membrane is either partly or wholly destroyed. In the case of osmotic hemolysis this may be due to a mechanical stretching (Blum, 1930a and b). Frick (1934) postulated that during the lysis of the erythrocyte the cell membrane gives way at one or several localized points on the cell membrane.

Heidelberger (1956) indicated that hemolysin attached onto only about 0.25 to 1.0 percent of the red cell surface, and consequently postulated that the hemolysin is attached to only certain areas or "key spots" of the red cell with complement attached or fixed to the same positions.

Heidelberger (1956) postulated that the mechanism of hemolysis occurs in five steps. The antibody (A) combines with the red cell (E) to form an EA complex. The second stage takes place only in the presence of the calcium ion; the antigen-antibody complex combines with the first and fourth components of complement to form EAC'1,4. In the third step of hemolysis, the cells which have combined with hemolysin and the first and fourth components of complement, react with the second component in the presence of magnesium to form EAC'1,4,2. The fourth and fifth steps require the presence of the third component for the production of hemolysis. In the fourth step of hemolysis, the EAC'1,4,2 complex reacts with the third component of complement to form

the activated cell. The activated cell in the fifth stage is lysed with the resultant formation of the ghost cell and hemoglobin.

Saline

Margaria (1930) reported that the osmotic pressure of the blood of adult men to be equal to 0.945 percent saline, while that of women had a lower osmotic pressure equal to 0.927 percent saline. Other workers in the field have also verified that 0.90 percent saline is nearer to the average osmotic pressure of mammalian bloods than 0.85 percent saline. Osborn (1937) reported that there is little difference on the speed of hemolysis resulting from the use of 0.85 percent NaCl rather than 0.90 percent NaCl.

It has been shown that the magnesium ion is essential for the hemolytic action of complement (Mayer, et al., 1946). A hemolytic system under normal conditions does not contain sufficient magnesium ions for optimal activity, therefore, optimal activity can be obtained by the addition of extra magnesium ion. Certain substances, such as citrate and pyrophosphate, will bind the magnesium ion; therefore, to overcome this effect an extra amount of magnesium ion is added to the hemolytic system.

The Effects of Salt Concentration. Heidleberger (1956) showed the titer of complement varied markedly with the salt concentration of the hemolytic system. This study revealed the following titers as compared to the increasing molal salt concentrations: 143:0.115; 126:0.151; 118:155; 96:0.162 83:0.168; 75:0.173. This study indicated that a slight deviation in the amount of salt used in the preparation of isotonic saline would cause a marked reduction in the titer.

The Effects of Volume on Complement Activity. In studying the titer of a system as compared with the total volume of the hemolytic system

(Heidelberger, et al., 1941), the following titers were obtained: 272:2.0 ml; 211:3.0 ml; 148:5.0 ml.

Electrolytes in the Hemolytic System. The effects of certain salts in a hemolytic system were studied in detail (Purdy and Walbum, 1922), and it was shown that certain salts such as $MgSO_4$, $AgNO_3$, LiCl and $NiCl_2$ in specified concentrations favored hemolysis, while others such as $ZnSO_4$, $BiCl_2$, $FeCl_3$, and $CuSO_4$ inhibited hemolysis.

Hypertonic Hemolysis. At equal molor concentrations of various neutral salts, the degree of hypertonic hemolysis varies mainly with the type of anion present. Chlorides are the least effective, followed by bromides, iodides, and sulphocyanates (Jodlbauer, 1935).

In a study by Ridgon (1937), it was shown that rabbit erythrocytes were lysed by staphylococcus toxin in ten minutes when suspended in 0.7 percent saline, whereas an equal number of cells suspended in 6 percent saline under the same conditions were not completely lysed in fifteen hours.

The Effects of Non-Electrolytes. The speed of hemolysis of dog red blood cells by sugar alcohols and their anhydride derivatives has been studied in detail (Kunkel, et al., 1939). It was shown that the three-carbon molecule derivatives caused rapid hemolysis, but five-and six-carbon molecules produced slow hemolysis.

The rate of osmotic hemolysis of ox red blood cells by certain non-electrolytes, such as glycerol and ethylene glycol, can be increased by the addition of certain electrolytes. Bivalent cations, $CaCl_2$, $BaCl_2$, $SrCl_2$, and $MgCl_2$ are more effective than univalent cations, LiCl, NaCl, and KCl, while bivalent and trivalent anions, Na_2SO_4, $MgSO_4$, and sodium citrate have a retarding effect (Jacobs, et al., 1937).

Hemolysis of human erythrocytes was studied in solutions of glucose of various concentrations in excess of hypotonic solution (Hendry, 1952). It was noted that there is a prolytic phase that lasts about 90 minutes which is followed by a hemolytic phase. During the prolytic phase the cells are readily permeable to potassium, at least 90 percent of the cell potassium can escape without the occurrence of lysis, therefore, the escape of cell potassium would not likely be an important factor in this type of hemolysis.

MATERIALS AND METHODS

Isotonic Saline Solution

Eight and one-half grams of dry sodium chloride (A.C.S.) and 0.1 gram of magnesium sulphate were added to a 1000 ml volumetric flask and brought to volume with distilled or de-ionized water. The saline was allowed to remain at room temperature. The hydrogen ion concentration was checked with bromo-thymol-blue indicator and adjusted to approximately pH 7.0 with 1N NaOH or 1N HCl if necessary.

Collection and Preservation of Sheep Blood

The sheep to be bled was immobilized in a standing position and the wool was removed from the puncture area. The area was sterilized with a five percent phenol solution. Bacterial contamination of sheep blood will cause unpredictable results and only aseptically collected and sterile blood was used through-out the experiment. The needle was inserted into the external jugular vein and 100 ml blood was collected into a bleeding flask containing 120 ml of 3.8 percent sodium citrate. In order to prevent clotting, the flask was rotated continuously during the collection of the blood and for five minutes afterwards.

The blood was allowed to cool at room temperature and transferred aseptically to sterile bottles. A portion of the blood was checked for sterility by placing an aliquot in nutrient broth. The collected blood was stored in a refrigerator until time of use.

Defibrinated blood used in the experiments was collected and preserved in essentially a similar way. Defibrination was accomplished by placing glass beads rather than citrate in the bleeding flask. The blood was stored in sterile bottles in the refrigerator until time of use.

Centrifugation of Cells

All centrifugation was performed at 5° C. Cells which were being washed in isotonic saline were centrifuged at 1700 r.p.m. for five minutes. Cells which were suspended in hypertonic solutions were centrifuged at 1200 r.p.m. for five minutes. Cells which were in isotonic saline suspension and were to be packed for calculation of a two percent cell suspension were centrifuged at 1700 r.p.m. for 10 minutes. The supernatant was removed by a suction pump.

Preparation of Sheep Red Cell Suspensions

Ten ml of citrated sheep blood were added to three to four volumes of isotonic saline. The suspension was centrifuged at 1700 r.p.m. for five minutes at a temperature of 5° C. The supernatant fluid was removed by suction through a 10 ml pipette. This process was repeated for a total of three washings. If on the third washing the uspernatant was not clear, the cells were too fragile and were discarded and a fresh supply was obtained. Cells over 30 days old were not used. After the third washing, an aliquot of cells was removed to be used as the control.

Preparation of a Two Percent Cell Suspension

All cell suspensions were washed into graduated 15 ml centrifuge tubes containing isotonic saline and centrifuged at 1700 r.p.m. for ten minutes in order to pack the cells firmly and evenly. The supernatant was removed, and the volume of packed cells was washed into 49 volumes of isotonic saline to give a two percent cell suspension.

Preparation and Preservation of Complement

Two types of complement were used during the experiments. Commercially prepared complement which was used had been dehydrated from the frozen state, in vacuo, by the lyophile method. This complement was reconstituted with neutral distilled water back to the original volume. The other type of complement was obtained by the direct bleeding of guinea pigs. Five guinea pigs were selected and five ml of blood were removed from the heart of each pig. The blood was allowed to clot at room temperature, ringed with an applicator stick, and refrigerated for one hour. The blood was centrifuged and the serum was removed from the clot. The serum lots were pooled to avoid hyperactive sera and recentrifuged. Aliquots sufficient for one day's use were placed in separate tubes to avoid complement destruction due to repeated thawing and freezing.

Guinea pig blood may possess complement activity which is less than or greater than prescribed standards. Low complement activity is generally caused by loss of activity through the storage of the guinea pig sera. However, occasionally improper feeding or housing of the guinea pigs will cause low titer sera.

Stock Hemolysin

A 1:100 stock hemolysin dilution was prepared by diluting 2.0 ml of glycerinized 50 percent hemolysin with 94.0 ml 0.85 percent saline and 4.0 ml of five percent phenol in saline solution.

Hemolysin Titration

The following procedure was used for each hemolysin titration:

1. The 1:100 stock hemolysin solution was diluted 1:1000 just prior to use.
2. The following amount of isotonic saline was added to each hemolysin titration:

Table 1. Hemolysin dilution.

Process	Tube Number									
	1	2	3	4	5	6	7	8	9	10
Ml. saline sol.	-	0.5	1.0	1.5	2.0	0.5	0.5	0.5	0.5	0.5
Ml. hemolysin (1-1000)	0.5	0.5	0.5	0.5	0.5	-	-	-	-	-

3. One-half ml of the 1:1000 hemolysin solution was placed into each of the first five tubes.
4. The following dilutions were then made:

Table 2. Hemolysin dilution.

Tube number	Process	Final hemolysin dilution
1	None	1:1000
2	Mix. Discard 0.5 ml.	1:2000
3	Mix. Transfer 0.5 ml. to tube 6, Discard 0.5 ml.	1:3000
4	Mix. Transfer 0.5 ml. to tube 7, Discard 1.0 ml.	1:4000
5	Mix. Transfer 0.5 ml. to tube 8, Discard 1.5 ml.	1:5000
6	Mix. Transfer 0.5 ml. to tube 9.	1:6000
7	Mix. Transfer 0.5 ml. to tube 10.	1:8000
8	Mix. Discard 0.5 ml.	1:10,000
9	Mix. Discard 0.5 ml.	1:12,000
10	Mix. Discard 0.5 ml.	1:16,000

5. 1.7 ml. of isotonic saline solution was added to each tube.

6. A 1:30 or 1:15 dilution of complement (depending on the activity of the complement) was made and 0.3 ml. of the diluted complement was added to each tube.

7. One-half ml. of the 2 percent sheep red cell suspension was added to each tube of the hemolysin titration.

8. All of the tubes were shaken to insure even distribution of the individual components, placed in a wire rack, and incubated in a 37° C. water bath for one hour.

9. After one hour of incubation, the tubes were removed from the water bath and the hemolysin titration read.

Reading of the Hemolysin Titration

The end point of the titration was a complete sparkling hemolysis and was given a numerical evaluation of 4. Other tubes in the titration were approximated with the following evaluations: 3 = 50 percent hemolysis, 2 = 25 percent hemolysis, 1 = 10 percent hemolysis, and 0 = 5 percent or less hemolysis.

Antibiotics in the Hemolytic System

The following crystalline antibiotics were diluted in sufficient isotonic saline to contain a specified number of units in 1.7 ml.: potassium penicillin, polymyxin B sulfate, and dihydrostreptomycin sulfate. This 1.7 ml amount was added to the hemolytic system and allowed to incubate for one hour. Defibrinated blood was used throughout these experiments. No antibiotic was added to the saline of the control titration.

Preparation of Hypertonic Solutions

All electrolytes and sugars were C.P. grade and were diluted in volumetric flasks with isotonic saline. The amount of electrolyte or sugar is expressed in milligrams per ml of isotonic saline.

Single Hypertonic Treatment of Cells

Citrated blood was used during this experiment. The blood was washed in isotonic saline and centrifuged at 1700 r.p.m. for five minutes. This process was repeated for a total of three washings. After three washings, the supernatant was clear in all cases. An aliquot was removed after the third washing to be used as a control, and placed in a graduated centrifuge tube containing isotonic saline. The remaining sediment of red blood cells was

divided into approximately one ml portions and washed into 49 volumes of hypertonic solution. These suspensions were allowed to incubate for one hour at 37° C. and then checked microscopically for crenation. After incubation the suspensions were centrifuged at 1200 r.p.m. for five minutes and the supernatant removed. The cells were resuspended in isotonic saline and allowed to remain in isotonic saline overnight at refrigerator temperature.

A slight variation in the above procedure was used in one experiment. After three washings in isotonic saline, one ml of packed cells was suspended in 50 ml of hypertonic solution. This suspension was dialyzed against 2000 ml of isotonic saline for 24 hours at refrigerator temperature. The saline was changed once at the end of 12 hours. The control cells were suspended in isotonic saline and dialyzed against isotonic saline.

In both experiments the control cells and cells which had been treated with hypertonic solutions were centrifuged at 1700 r.p.m. for five minutes, and the supernatant removed. If the pre-treated cells showed more than a slight degree of hypertonic hemolysis, they were washed once more in isotonic saline. The packed cells were washed into 15 ml graduated centrifuge tubes and centrifuged at 1700 r.p.m. for 10 minutes. A two percent cell suspension was calculated and added to the hemolysin titration.

Multiple Treatment of Cells

Citrated blood was used throughout this experiment. Cells were washed in isotonic saline three times before use. After the third washing, the supernatant was removed and a portion of the sediment was placed in isotonic saline to be used as a control. The remaining sediment was resuspended in the hypertonic solution and allowed to incubate for 15 minutes at room temperature. After

incubation the suspension was centrifuged at 1200 r.p.m. for five minutes and the supernatant was removed. The sediment was resuspended in isotonic saline, and centrifuged at 1700 r.p.m. for five minutes. The supernatant was removed and a portion of the sediment was removed and placed in a graduated centrifuge tube containing isotonic saline and labeled as one treatment. This process was repeated for a maximum of five treatments.

EXPERIMENTAL RESULTS

Antibiotics in the Hemolytic System

The results represent the effect of crystalline antibiotics added to a hemolytic system consisting of saline, complement, sheep red blood cells, and complement.

Potassium Penicillin. The results from the addition of potassium penicillin to the hemolytic system are shown in Table 3. The number of units of penicillin represents the amount of penicillin in each tube.

Table 3. Hemolysis exhibited with added penicillin in the hemolytic system.

Units of Penicillin	Tube number 1	2	3	4	5	6	7	8	9	10	Titer
0.25	4	4	4	4	4	4	3	3	2	2	1:6000
0.375	4	4	4	4	4	4	4	3	2	2	1:8000
0.5	4	4	4	4	4	4	3	3	2	2	1:6000
0.75	4	4	4	4	4	4	4	3	3	2	1:8000
1.0	4	4	4	4	4	4	4	3	2	3	1:8000
Control	4	4	4	4	4	4	4	3	2	2	1:8000
0.1	4	4	4	4	4	4	4	3	3	1	1:8000
1.0	4	4	4	4	4	4	4	3	1	1	1:8000
10.0	4	4	4	4	4	4	4	3	2	1	1:8000
100.0	4	4	4	4	4	4	4	3	2	1	1:8000
1000.0	4	4	4	4	4	4	3	3	1	1	1:6000
Control	4	4	4	4	4	4	4	3	3	1	1:8000

Dihydrostreptomycin Sulfate. The results from the addition of dihydrostreptomycin sulfate to the hemolytic system are shown in Table 4. The number of gammas of dihydrostreptomycin represents the amount of dihydrostreptomycin in each tube.

Table 4. Hemolysis with added dihydrostreptomycin sulfate in the hemolytic system.

Gammas of Dihydrostreptomycin	Tube number 1	2	3	4	5	6	7	8	9	10	Titer
0.1	4	4	4	4	4	4	4	3	3	2	1:8000
1.0	4	4	4	4	4	4	4	4	3	2	1:10,000
10.0	4	4	4	4	4	4	4	4	3	3	1:10,000
100.0	4	4	4	4	4	4	4	4	3	2	1:10,000
1000.0	4	4	4	4	4	4	4	3	2	2	1:8000
Control	4	4	4	4	4	4	4	3	3	2	1:8000

Polymyxin B Sulfate. The results from the addition of polymyxin B sulfate to the hemolytic system are shown in Table 5. The number of gammas represents the amount of polymyxin in each tube.

Table 5. Hemolysis with added polymyxin B sulfate in the hemolytic system.

Gammas of Polymyxin B Sulfate	Tube number 1	2	3	4	5	6	7	8	9	10	Titer
0.1	4	4	4	4	4	4	4	2	2	1	1:8000
1.0	4	4	4	4	4	4	4	3	2	1	1:8000
10.0	4	4	4	4	4	4	4	3	3	2	1:8000
100.0	4	4	4	4	4	4	4	1	2	1	1:8000
1000.0	4	4	4	4	4	4	4	3	2	2	1:8000
Control	4	4	4	4	4	4	4	3	2	2	1:8000

Sheep Blood Cells Crenated Once

The results represent the effect of hemolysin on sheep red blood cells treated with varying concentrations of hypertonic solutions. The cells after being suspended once in the hypertonic solutions were removed and placed in an isotonic saline solution and subsequently added to a hemolytic system consisting of saline, complement, and hemolysin.

Sodium Chloride. The results from the treatment of the cells with varying concentrations of sodium chloride solutions were shown in Table 6. The amount of sodium chloride shown represents the number of milligrams of sodium chloride per ml in the hypertonic solution in excess of 0.85 percent sodium chloride.

Table 6. Hemolysis results with red cells treated once with sodium chloride.

Milligrams NaCl/ml	Tube number 1	2	3	4	5	6	7	8	9	10	Titer
2	4	4	4	4	4	4	3	3	2	2	1:6000
4	4	4	4	4	4	4	3	3	2	2	1:6000
Control	4	4	4	4	4	4	3	3	3	2	1:6000
5	4	4	4	4	4	4	3	3	2	2	1:6000
10	4	4	4	4	4	4	3	2	1	1	1:6000
Control	4	4	4	4	4	4	3	3	3	1	1:6000
15	4	4	4	4	4	4	4	3	3	2	1:8000
25	4	4	4	4	4	4	4	3	3	2	1:8000
Control	4	4	4	4	4	4	4	3	3	2	1:8000

The results from the microscopic examination of the cells treated with varying concentrations of sodium chloride solutions are shown in Table 7.

Table 7. Microscopic examination of cells treated with NaCl.

Milligrams NaCl/ml	Microscopic examination (970 X)
2	No noticeable crenation.
4	No noticeable crenation.
5	No noticeable crenation.
10	No noticeable crenation.
15	No noticeable crenation.
25	Slight crenation.

Potassium Chloride. The results from the treatment of the cells with varying concentrations of potassium chloride are shown in Table 8. The amount of potassium chloride shown represents the number of milligrams of potassium chloride per ml in the hypertonic solution in excess of 0.85 percent sodium chloride.

Table 8. Hemolysis of red cells treated once with potassium chloride.

Milligrams KCl/ml	Tube number 1	2	3	4	5	6	7	8	9	10	Titer
10	4	4	4	4	4	4	3	3	1	1	1:6000
15	4	4	4	4	4	4	3	2	2	1	1:6000
20	4	4	4	4	4	4	2	2	2	1	1:6000
25	4	4	4	4	4	4	4	3	3	2	1:8000
30	4	4	4	4	4	4	3	3	2	2	1:6000
40	4	4	4	4	4	4	3	3	3	3	1:6000
50	4	4	4	4	4	4	4	3	3	3	1:8000
Control	4	4	4	4	4	4	3	3	2	2	1:6000

Potassium Chloride. The results from the treatment of cells with 50 milligrams potassium chloride per ml of isotonic saline are shown in Table 9. The hypertonic cell suspension was dialyzed against isotonic saline for 24 hours. After dialysis the cells were centrifuged and a two percent cell suspension calculated.

Table 9. Hemolysis of red cells subject to a single treatment with potassium chloride followed by dialysis.

Milligrams KCl/ml	Tube number 1	2	3	4	5	6	7	8	9	10	Titer
50	4	4	4	4	4	4	4	4	3	2	1:10,000
Control	4	4	4	4	4	4	4	3	3	2	1:8000

Microphotographs of cells suspended in 50 milligrams KCl per ml of isotonic saline and suspended in isotonic saline are shown in PLATES 1 and 2.

The results from the microscopic examination of the cells treated with varying concentrations of potassium chloride solutions are shown in Table 10.

Table 10. Microscopic examination of cells treated with KCl.

Milligrams KCl/ml	Microscopic examination (970 X)
10	No noticeable crenation.
15	No noticeable crenation.
20	Slight Crenation.
25	Crenated.
30	Crenated.
40	Crenated.
50	Crenated.

Magnesium Sulfate. The results from the treatment of the cells with varying concentrations of magnesium sulfate are shown in Table 11. The amount of magnesium sulfate represents the number of milligrams of magnesium sulphate per ml in the hypertonic solution in excess of 0.85 percent sodium chloride.

EXPLANATION OF PLATE I

Sheep erythrocytes suspended in isotonic saline. X970.

PLATE I

EXPLANATION OF PLATE II

Sheep erythrocytes suspended in 50 milligram potassium chloride per ml of isotonic saline. X970.

PLATE II

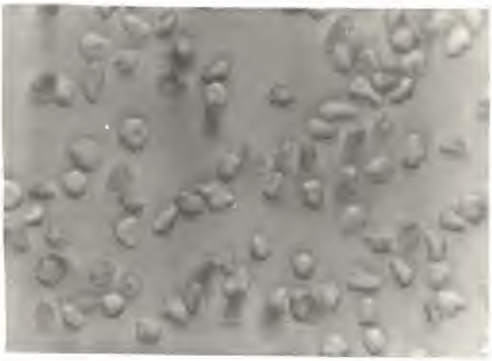

Table 11. Hemolysis of red cells treated once with magnesium sulfate.

Milligrams $MgSO_4$/ml	Tube number 1	2	3	4	5	6	7	8	9	10	Titer
10	4	4	4	4	4	4	4	3	2	1	1:8000
20	4	4	4	4	4	4	3	3	2	1	1:6000
30	4	4	4	4	4	4	2	2	2	1	1:6000
50	4	4	4	4	4	4	3	2	2	1	1:6000
Control	4	4	4	4	4	4	3	3	2	2	1:6000
60	4	4	4	4	4	4	3	2	2	2	1:6000
70	4	4	4	4	4	4	3	2	2	1	1:6000
80	4	4	4	4	4	4	3	3	3	2	1:6000
90	4	4	4	4	4	4	3	2	3	2	1:6000
100	4	4	4	4	4	4	3	3	3	2	1:6000
Control	4	4	4	4	4	4	3	3	2	2	1:6000

The results from the microscopic examination of the cells treated with varying concentrations of magnesium sulfate solutions are shown in Table 12.

Table 12. Microscopic examination of cells treated with $MgSO_4$.

Milligrams $MgSO_4$/ml	Microscopic Examination (970 X)
10	No noticeable crenation.
20	Slight crenation.
30	Slight crenation.
50	Crenated.
60	Crenated.
70	Crenated.
80	Crenated.
90	Crenated.
100	Crenated.

Sucrose. The results from the treatment of the cells with varying concentrations of sucrose are shown in Table 13. The amount of sucrose represents the number of milligrams of sucrose per ml in the hypertonic solution in excess of 0.85 percent sodium chloride.

Table 13. Hemolysis of red cells treated once with sucrose.

Milligrams sucrose/ml	Tube number 1	2	3	4	5	6	7	8	9	10	Titer
60	4	4	4	4	4	4	3	2	2	1	1:6000
70	4	4	4	4	4	4	4	3	3	2	1:8000
80	4	4	4	4	4	4	3	3	2	1	1:6000
90	4	4	4	4	4	4	3	3	2	2	1:6000
100	4	4	4	4	4	4	3	3	2	2	1:6000
Control	4	4	4	4	4	4	3	2	2	1	1:6000

The results from the microscopic examination of the cells treated with varying concentrations of sucrose solutions are shown in Table 14.

Table 14. Microscopic examination of cells treated with sucrose.

Milligrams sucrose/ml	Microscopic Examination (970 X)
60	Crenated.
70	Crenated.
80	Crenated.
90	Crenated.
100	Crenated.

Lysozyme. The results from the treatment of the cells with crystalline lysozyme are shown in Table 15. The amount of lysozyme represents the the number of milligrams of lysozyme per ml of isotonic saline.

Table 15. Hemolysis of red cells treated once with lysozyme.

Milligrams Lysozyme/ml	Tube number 1	2	3	4	5	6	7	8	9	10	Titer
4	4	4	4	4	2	2	2	1	1	1	1:4000
2	4	4	4	4	4	3	2	3	2	1	1:5000
1	4	4	4	4	3	2	2	2	1	0	1:4000
Control	4	4	4	4	2	2	2	1	0	0	1:4000

Multiple Treatment of Sheep Blood Cells

The results represent the effect of hemolysin on sheep red blood cells treated more than once with the same concentration of hypertonic solution. The cells after being suspended in the hypertonic solution were removed and placed in isotonic saline and designated as one treatment. This process was repeated for a maximum of five treatments.

Sodium Chloride. The results from the multiple treatment of cells with sodium chloride are shown in Table 16. The hypertonic solution contained 20 milligrams sodium chloride per ml of isotonic saline.

Table 16. Hemolysis of red cells following multiple treatment with sodium chloride.

Number of Treatments	Tube number 1	2	3	4	5	6	7	8	9	10	Titer
1	4	4	4	4	4	3	3	2	2	1	1:5000
2	4	4	4	4	4	3	2	2	1	1	1:5000
3	4	4	4	4	4	3	3	2	2	1	1:5000
Control	4	4	4	4	4	4	3	2	2	1	1:6000

Magnesium Chloride. The results from the multiple treatment of cells with magnesium chloride are shown in Table 17. The hypertonic solution contained 20 milligrams magnesium chloride per ml of isotonic saline.

Table 17. Hemolysis of red cells following multiple treatment with magnesium chloride.

Number of Treatments	Tube number 1	2	3	4	5	6	7	8	9	10	Titer
1	4	4	4	4	4	3	3	2	2	1	1:5000
2	4	4	4	4	4	3	2	4	1	1	1:5000
3	4	4	4	4	4	3	2	2	1	1	1:5000
4	4	4	4	4	4	3	2	2	1	1	1:5000
5	4	4	4	4	4	4	3	3	2	2	1:6000
Control	4	4	4	4	4	4	2	2	2	1	1:6000

Potassium Chloride. The results from the multiple treatment of cells with potassium chloride are shown in Table 18. The hypertonic solution contained 20 milligrams potassium chloride per ml of isotonic saline.

Table 18. Hemolysis of red cells following multiple treatment of cells with potassium chloride.

Number of Treatments	Tube number 1	2	3	4	5	6	7	8	9	10	Titer
1	4	4	4	4	3	3	2	2	2	2	1:4000
2	4	4	4	4	3	2	2	2	1	0	1:4000
3	4	4	4	4	3	2	2	2	1	0	1:4000
4	4	4	4	4	3	2	2	2	0	0	1:4000
5	4	4	4	4	4	3	3	2	2	1	1:5000
Control	4	4	4	4	3	2	2	1	1	0	1:4000

Potassium Nitrate. The results from the multiple treatment of cells with potassium nitrate are shown in Table 19. The hypertonic solution contained 20 milligrams of potassium nitrate per ml of isotonic saline.

Table 19. Hemolysis of red cells following multiple treatment of cells with potassium nitrate.

Number of Treatments	Tube number 1	2	3	4	5	6	7	8	9	10	Titer
1	4	4	4	4	4	4	3	3	2	2	1:6000
2	4	4	4	4	4	4	3	3	2	2	1:6000
3	4	4	4	4	4	4	3	3	2	2	1:6000
Control	4	4	4	4	4	4	3	3	3	2	1:6000

Potassium Oxalate. The results from the multiple treatment of cells with potassium oxalate are shown in Table 20. The hypertonic solution contained 20 milligrams potassium oxalate per ml of isotonic saline.

Table 20. Hemolysis of red cells following multiple treatment of cells with potassium oxalate.

Number of Treatments	Tube number 1	2	3	4	5	6	7	8	9	10	Titer
1	4	4	4	4	4	4	3	3	3	2	1:6000
2	4	4	4	4	4	4	3	3	2	2	1:6000
3	4	4	4	4	4	4	3	3	2	2	1:6000
Control	4	4	4	4	4	4	3	3	3	2	1:6000

Sodium Sulfate. The results from the multiple treatment of cells with sodium sulfate are shown in Table 21. The hypertonic solution contained 20 milligrams sodium sulfate per ml of isotonic saline.

Table 21. Hemolysis of red cells following multiple treatment of cells with sodium sulfate.

Number of Treatments	Tube number 1	2	3	4	5	6	7	8	9	10	Titer
1	4	4	4	4	4	4	4	4	3	3	1:10,000
2	4	4	4	4	4	4	4	3	3	3	1:8000
3	4	4	4	4	4	4	4	3	3	3	1:8000
Control	4	4	4	4	4	4	4	3	3	2	1:8000

Potassium Iodide. The results from the multiple treatment of cells with potassium iodide are shown in Table 22. The hypertonic solution contained 20 milligrams potassium iodide per ml of isotonic saline.

Table 22. Hemolysis of red cells following multiple treatment of cells with potassium iodide.

Number of Treatments	Tube number 1	2	3	4	5	6	7	8	9	10	Titer
1	4	4	4	4	4	4	4	4	3	3	1:10,000
2	4	4	4	4	4	4	4	4	3	3	1:10,000
3	4	4	4	4	4	4	4	4	3	2	1:10,000
Control	4	4	4	4	4	4	4	3	2	2	1:8000

Dextrose. The results from the multiple treatment of cells with dextrose are shown in Table 23. The hypertonic solution contained 20 milligrams dextrose per ml of isotonic saline.

Table 23. Hemolysis of red cells following multiple treatment of cells with dextrose.

Number of Treatments	Tube number 1	2	3	4	5	6	7	8	9	10	Titer
1	4	4	4	4	4	4	4	4	3	3	1:10,000
2	4	4	4	4	4	4	4	4	3	3	1:10,000
3	4	4	4	4	4	4	4	3	3	3	1:8000
Control	4	4	4	4	4	4	4	3	3	3	1:8000

Sucrose. The results from the multiple treatment of cells with sucrose are shown in Table 24. The hypertonic solution contained 20 milligrams sucrose per ml of isotonic saline.

Table 24. Hemolysis of red cells following multiple treatment of cells with sucrose.

Number of Treatments	Tube number 1	2	3	4	5	6	7	8	9	10	Titer
1	4	4	4	4	4	4	3	3	2	2	1:6000
2	4	4	4	4	4	4	4	3	2	2	1:8000
3	4	4	4	4	4	4	4	3	2	1	1:8000
Control	4	4	4	4	4	4	4	3	2	2	1:8000

Lactose. The results from the multiple treatment of cells with lactose are shown in Table 25. The hypertonic solution contained 20 milligrams lactose per ml of isotonic saline.

Table 25. Hemolysis of red cells following multiple treatment of cells with lactose.

Number of Treatments	Tube number 1	2	3	4	5	6	7	8	9	10	Titer
1	4	4	4	4	4	4	4	3	3	3	1:8000
2	4	4	4	4	4	4	4	3	2	2	1:8000
3	4	4	4	4	4	4	4	4	3	3	1:10,000
Control	4	4	4	4	4	4	4	3	4	3	1:8000

Maltose. The results from the multiple treatment of cells with maltose are shown in Table 26. The hypertonic solution contained 20 milligrams maltose per ml of isotonic saline.

Table 26. Hemolysis of red cells following multiple treatment of cells with maltose.

Number of Treatments	Tube number 1	2	3	4	5	6	7	8	9	10	Titer
1	4	4	4	4	4	4	4	3	2	2	1:8000
2	4	4	4	4	4	4	4	3	3	2	1:8000
3	4	4	4	4	4	4	4	3	3	2	1:8000
Control	4	4	4	4	4	4	4	3	3	3	1:8000

DISCUSSION

The sheep blood cell hemolytic system is a widely used indicator system in the complement-fixation test. Unlike other antigen-antibody reactions, the hemolytic reaction is an interaction of three components rather than two, being composed of : the antigen (sheep erythrocytes), antibody (hemolysin), and guinea pig complement. There are numerous variables which may be encountered in the study of this system. Such factors as concentration, temperature, and stability of the individual components contribute to the final titer of the hemolysin titration.

The first series of experiments was designed to show what effect an antibiotic would have on the hemolytic system. This involved the addition of a particular antibiotic to the hemolytic system. All factors such as the age of the cells, hydrogen ion concentration of the system, time of incubation,

complement activity, hemolysin activity, and final volume were the same as the control. Within experimental error, the results indicated that there was no significant change in the titer of the hemolytic system upon the addition of the specified concentrations of potassium penicillin, dihydrostreptomycin sulfate, or polymyxin B suflate.

The second series of experiments was designed to show what effect hemolysin and complement would have upon blood cells which had been crenated once with varying concentrations of hypertonic solutions. Solutions of both electrolytes and non-electrolytes were used as the crenating agents. The cells were resuspended in isotonic saline after being crenated by the hypertonic solution. The amount of crenation of the cell, as determined by microscopic examination, was proportional to the concentration of the hypertonic solution. In all cases microscopic examination of cells resuspended in isotonic saline indicated no physical abnormalities was compared with those of the control. Within experimental error, there was very little difference in the activity of complement and hemolysin on sheep cells crenated once with the tested hypertonic solutions as compared with the cells of the control itiration. Cells suspended in potassium chloride at a concentration of 50 milligrams per ml showed an increase in titer of one tube.

The third series of experiments was designed to show what effect complement and hemolysin would have upon blood cells which were treated more than once with a hypertonic solution. The erythrocytes were suspended in a hypertonic solution, the supernatant removed, and the cells resuspended in isotonic saline. This process was repeated for a maximum of five times. If the multiple treatment of cells with the various indicated hypertonic solutions produces only a physical effect in which the cell membrane is disrupted due to a mechanical stress and strain with a resultant increase in cell fragility, multiple

treatment of cells should show an increase in the hemolysin titer with an increase in the number of treatments. An increase in the number of treatments gave an increase in the amount of hypertonic hemolysis of the cells. In three cases (magnesium chloride, potassium chloride, and lactose) there was an increase in the titer of one tube with an increase in the number of treatments. It is felt that this increase could be attributed to the physical disruption of the cell membrane with repeated treatments.

Cells treated three times with potassium iodide gave a rise in titer of one tube in all three cases. This increase in cell fragility might be attributed to either a physical alteration or a chemical change in the cell membrane. Since the fragility did not increase with repeated treatment, it is felt that this increase in titer is, therefore, due to a chemical reaction of the potassium iodide and the cell.

Cells treated three times with glucose showed a rise in titer of one tube with one and two treatments, but on the third treatment the titer was equal to that of the control titration. Somewhat similar results were obtained with the treatment of cells with sodium sulfate.

Multiple treatment of cells with sodium chloride, potassium nitrate, potassium oxalate, sucrose, and maltose seemed to indicate no significant differences in the titer of the hemolysin titration.

SUMMARY

Various concentrations of potassium penicillin, dihydrostreptomycin sulfate, and polymyxin B sulfate were added to a hemolysin titration consisting of hemolysin, complement, and sheep erythrocytes. Within experimental error, the results indicated that the addition of antibiotics

to this hemolytic system does not appreciably affect the final hemolysin titer.

Sheep red blood cells were treated with varying concentrations of hypertonic solutions. After suspension in the hypertonic solution, the cells were suspended in isotonic saline and added to the hemolytic system. With the exception of treatment of sheep erythrocytes with a 50 milligram per ml solution of potassium chloride, experimental results indicated that the pre-treatment of cells with the indicated electrolytes and non-electrolytes did not appreciably effect the final titer of the hemolysin titration.

Sheep red blood cells were suspended in various two percent solutions of electrolytes and non-electrolytes. After suspension in the hypertonic solution, the cells were resuspended in isotonic saline. This process was repeated to a maximum of five times. With two exceptions, the fragility of the cell (as indicated by the hemolysin titration) either increased with the number of treatments, or there was no significant change. Treatment of sheep erythrocytes three times with potassium iodide caused a slight increase in cell fragility, but was independent of the number of treatments.

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THE EFFECT OF PRE-TREATMENT OF CELLS AND THE PRESENCE OF ANTIBIOTICS ON THE SHEEP CELL HEMOLYTIC SYSTEM

by

LAWRENCE ALLEN CHITWOOD

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The sheep red blood cell hemolytic system consists of three reactive components: hemolysin (antibody), sheep erythrocytes (antigen), and guinea pig complement. This indicator system is used in the complement-fixation tests. Numerous varibles will effect the final titer of this hemolytic system. Salt concentration, total volume, hydrogen ion concentration, presence of certain organic and inorganic compounds, activity of complement, fragility of the erythrocyte, total number of erythrocytes, temperature and time of incubation, and the relative amount of antibody are perhaps the most important factors.

With so many factors, it is difficult to state what the optimum condition is with respect to any one of the other factors, since it may differ with a variation of the other factors. The purpose of this study was to determine what effect antibiotics and pre-treated cells had on the hemolytic system while holding other variables constant. The titration of hemolysin was carried out by varying the amount of hemolysin from 1:1000 to 1:16,000 in the presence of constant amounts of sheep erythrocytes and complement.

The following antibiotics were diluted in isotonic saline: potassium penicillin, polymyxin B sulfate, and dihydrostreptomycin sulfate. No antibiotic was added to the saline of the control titration. Within experimental error, the results indicated that there is no significant change in the titer of the hemolytic system upon the addition of potassium penicillin, dihydrostreptomycin sulfate, or polymyxin B sulfate.

A second series of experiments was designed to show the effect of hemolysin and complement on blood cells which had been pre-treated with varying concentrations of certain electrolytes and non-electrolytes in excess of isotonicity. The sheep erythrocytes were suspended in various hypertonic solutions and

incubated in a 37° C. water bath for one hour. The suspension was centrifuged and the hypertonic supernatant removed. The cells were resuspended in isotonic saline and allowed to remain in isotonic saline overnight at refrigerator temperature. The suspension was centrifuged and the supernatant removed. The sediment of cells was placed in a graduated centrifuge tube containing isotonic saline and after centrifugation a two percent cell suspension was calculated and added to the hemolysin titration. A slight variation was used in one experiment. The hypertonic solution was removed by dialysis against isotonic saline. The following electrolytes and non-electrolytes were used: sodium chloride, potassium chloride, magnesium sulfate, sucrose, and lysozyme. With the exception of pre-treatment of cells with a 50 milligram KCl per ml of solution, experimental results indicated that the tested compounds did not appreciably affect the final titer of the hemolysin titration.

In the third series of experiments the sheep erythrocytes was suspended in various two percent solutions of organic and inorganic compounds which were dissolved in isotonic saline. After suspension in the hypertonic solution, the cells were resuspended in isotonic saline. This process was repeated for a maximum of five times. An aliquot of cells was removed after each treatment and used in the hemolysin titration. With two exceptions the fragility of the cell, as indicated by the hemolysin titration, either increased with the number of treatments or there was no significant change. Treatment of cells three times with potassium iodide caused a slight increase in cell fragility but was independent of the number of treatments.