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Daniel Stats

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COLD HEMAGGLUTINATION AND COLD HEMOLYSIS. THE HEMOLYSIS PRODUCED BY SHAKING COLD AGGLUTINATED ERYTHROCYTES

By DANIEL STATS

(From The Laboratories and Medical Services of the Mount Sinai Hospital, New York City)

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INTRODUCTION

Cold hemagglutination is the reaction of clumping that occurs when serum and red blood cells are mixed at low (below 25° C.) temperatures. The agglutination is caused by an antigen-antibody reaction and is completely reversible by raising the temperature above 25° C. Cold hemolysis is an irreversible reaction in which hemoglobin is liberated from red blood cells at low temperatures. In the following paper, studies are presented which elucidate the relationship between these phenomena. A preliminary report of this work was published recently (1).

Little attention has been directed toward this problem in the work on cold hemagglutination (2). Nevertheless, cases of acute or chronic hemolytic anemia and paroxysmal cold hemoglobinuria associated with potent cold hemagglutination have been described. The relationship between the hemagglutination and the hemolysis in such cases was studied by Salén (3). This author was the only one to differentiate clearly the syphilitic cases of paroxysmal cold hemoglobinuria with positive Donath-Landsteiner reactions (4) from the non-luetic cases of paroxysmal cold hemoglobinuria with cold hemagglutination, in which the Donath-Landsteiner reaction is negative. After an intensive and detailed serological study of a non-luetic case of paroxysmal cold hemoglobinuria associated with cold hemagglutination, he stated: "the serum and plasma contained agglutinating and hemolyzing substances of high titer. The hemolyzing function was thermostable and 'not complex', *i.e.*, it reacted without complement." The relationship between cold hemagglutination and hemolysis was not delineated further nor was the significance of mechanical trauma considered. The experiments reported below show this re-

lationship as well as indicating the rôle of trauma (shaking) and other factors.

Various previous studies have neglected the importance of mechanical trauma in hemolysis. One investigator (5) described the morphological changes in mammalian erythrocytes during and after prolonged shaking, namely, the occurrence of spherical microcytes and numerous intermediate forms between these and normal discoidal erythrocytes. Another (6) observed that defibrinated blood of patients with hemolytic jaundice was always somewhat hemolyzed; he concluded that the mechanical fragility of blood of such patients was increased. Others (7) found that erythrocytes washed and resuspended in Ringer's solution were much more susceptible to hemolysis by shaking than the same cells in plasma. Human erythrocytes in Ringer's solution showed a trace of hemolysis when shaken for 15 minutes; similar cells in diluted plasma failed to reveal any hemolysis. Human erythrocytes had more resistance to mechanical injury (shaking) than sheep, dog, or rabbit erythrocytes. In a review of hemolysis (8), it was stated that a diminution in the osmotic resistance of erythrocytes was accompanied by an increase in their mechanical fragility. Preliminary studies (9) were reported in which blood was shaken 40,000 times at the rate of 560 times a minute. Normal human red blood cells in 0.85 per cent sodium chloride solution showed 0.7 per cent hemolysis while the greatest hemolysis observed in abnormal bloods was 4.0 per cent. Numerous experiments (10) have been described showing the hemolytic effect of shaking guinea pig erythrocytes previously agglutinated with various concentrations of silicic acid, concanavalin A, and immune serum. No data were presented to show that similar effects on human red cells were possible. At the same time, the

bloods were shaken for prolonged periods (3 hours) with glass beads and at high speed. Although none of these investigations bear directly on the question of hemolysis with potent cold hemagglutination, they show that the amount of shaking used was much greater than the shaking in the experiments reported below.

MATERIALS

The serums used in this study were obtained from patients with a variety of conditions. A brief summary of the cases follows:

The case of S. Y. has been published in detail in collaboration with the late Dr. J. G. M. Bullowa (11). This patient had a titer of cold hemagglutinins of 1/30,000 for over 2 years and is still under observation. He came to our attention because of the occurrence of symmetric gangrene of the tips of his extremities and hemoglobinuria after exposure to low environmental temperatures. No cause could be found to explain the presence of the cold hemagglutinin. A hemolytic anemia was not present.

V. A. was observed because of an acute hemolytic anemia without hemoglobinemia. The hemoglobin was 30 per cent.¹ This disease occurred about 2 weeks after the onset of a primary atypical pneumonia. Small doses of sulfadiazine had been administered 3 days before the onset of the anemia. She improved rapidly after blood transfusions. The cold hemagglutinin titer in this case varied from 1/10,000 at the height of the disease to 1/600 during convalescence.

R. A. was acutely ill with primary atypical pneumonia involving all lobes when first observed. This patient had not responded to large doses of sulfathiazol given before admission to the hospital. The cold hemagglutinin titer varied from 1/300 to 1/12,800, later falling to 1/640 in convalescence. She was acutely ill for about 2 weeks. At no time was there evidence of a hemolytic anemia; the lowest hemoglobin was 72 per cent. In this case, a striking polymorphonuclear leukocytosis of 25,000 was an unusual feature. Convalescence was prolonged but complete recovery ensued.

LIN. was studied at another hospital.² This patient ran the course of a severe primary atypical pneumonia. She had been ill for 3 weeks when, because of a hemoglobin of 65 per cent, blood transfusion was deemed advisable. A blood grouping test was reported Group AB but none of the available donors was compatible. At this time, the cold hemagglutinin titer was 1/6400. The blood group was redetermined as Group B and Groups O and B donors were compatible. A transfusion was not given, however, and the patient made an uneventful recovery. There was no evidence of a hemolytic anemia or hemoglobinuria.

¹ 100 per cent = 15.5 grams hemoglobin per 100 ml. of blood.

² This patient was observed through the courtesy of Dr. Peter Vogel at the Lincoln Hospital, Department of Hospitals, N. Y. C.

A. L. was an intern who had a severe prolonged primary atypical pneumonia. Sulfonamides were not administered in this instance. Early in the course of the disease, his cold hemagglutinin titer was 1/8; at the time of deferescence, the titer was 1/5120. This gradually diminished during convalescence. Neither hemolytic anemia nor hemoglobinuria was present.

METHODS

1. *The cold hemagglutination test*

The detailed technic of the cold hemagglutination test was the same as previously described (2). The serums examined were allowed to separate from their clots at 37° C. They were progressively diluted twofold in 0.4 ml. amounts from 1/2 to 1/60,000 with 0.85 per cent solution of sodium chloride. For most tests, homologous red blood cells, less than 1 day old, were washed 4 times or more with warm isotonic saline. The suspensions of cells were made to 1.5 per cent by volume and 0.1 ml. was added to the dilutions of serum.

Tests were routinely carried out in 10 × 75 mm. serological tubes. The reactants were well mixed by shaking; the tests were read after 18 hours at 4° C., the end-point being determined by microscopic examination. Most serums were studied on the day they were obtained. Several were stored at 4° C. for several days or longer before use.

To express varying degrees of hemagglutination the symbols 4+, 3+, 2+, +, ±, and - were used. These refer to progressively diminishing degrees of hemagglutination while the - sign indicates absence of agglutination. 4+ is equivalent to a firm button of agglutinated erythrocytes, 3+ to large clumps, 2+ to small clumps, + to clumps just visible with the naked eye and to clumps visible only microscopically, and ± to questionable clumping. The titer of a serum is the greatest dilution in which agglutination is observed. The titer is the actual dilution of the serum, not the final dilution after mixing with other reactants.

Ghosts of red cells were prepared by hemolyzing washed Group O erythrocytes with distilled water. Hypertonic sodium chloride solution was added until isotonicity was attained and the ghosts were centrifuged. The ghosts were rehemolyzed twice and separated by centrifugation. They were finally washed 4 times with isotonic sodium chloride solution until the supernate was clear. Ghosts prepared in this manner were pink in color when packed; this indicated that they were not entirely free of hemoglobin.

2. *The cold hemolysis test*

Whole oxalated blood or mixtures of serum and homologous or Group O erythrocytes were employed. Whole blood or erythrocytes were always less than 3 hours old when used. When, as occasionally happened, old stored serum was tested, red cells were obtained by venepuncture from healthy Group O donors. No attempt was made, in the experiments in which serum was used, to prevent

deterioration of complement, except in the early studies. This precaution was not taken for complement was found to play no rôle in the phenomenon of hemolysis (see below). Scrupulous care was always exercised to prevent hemolysis during the withdrawal, transportation, and centrifugation of blood. When erythrocytes were used with serum, they were washed 3 times with warm 0.85 per cent sodium chloride solution before use. Less than 20 minutes of centrifugation at a speed of 1200 r.p.m. was required. The packed erythrocytes were made up to the dilutions specified in the protocols by the addition of isotonic saline solution. The term 'RBC' applies to these suspensions, 50 per cent RBC having been made up by mixing equal volumes of packed erythrocytes and salt solution.

The great majority of the tests was performed in 10×75 mm. serological tubes. Some of the results were duplicated in 12×100 mm. tubes, 12×100 mm. paraffin-lined glass tubes, and lusteroid tubes of the same size.

After the ingredients had been added, the tubes were placed in a water bath at 37°C . for between 3 and 5 minutes. They were then shaken gently for purposes of mixing and transferred to a water bath set at the temperature of the experiment. This procedure was done to insure even distribution of the erythrocytes at the commencement of each test. However, in tests with whole blood, this was not necessary. The tubes in these instances were filled at room temperature (22°C .) and then immersed in water at the desired temperature.

Reference to the protocols will reveal the frequent use of the terms "tapped" or "shaken". A tube so designated was removed to room temperature at the intervals stated in the individual experiments, held at the top between the thumb and index finger of the left hand and tapped gently and rapidly at the bottom with the index finger of the right hand. Depending upon the number of taps, this maneuver was completed in between 2 and 15 seconds. The tube was then replaced in the rack at the temperature of the experiment, or was centrifuged, depending upon the individual test.

In some tests, the results were read after centrifugation of the tubes. Centrifugation was usually done at room temperature (22°C .) at 700 r.p.m. for 3 minutes. In those tests in which the tubes contained potent cold hemagglutinating serum and erythrocytes, the amount of hemolysis was occasionally increased by even this short period of centrifugation. This was seen by observing a dark red rim of serum just above the junction of lightly packed erythrocytes and serum. In the great majority of the tests, however, this was not the case, for tubes tapped in the cold demonstrated diffuse redness of the supernates, whereas similar, but untapped, controls (see protocols) did not show any hemolysis after centrifugation. Despite this, the influence of centrifugation was eliminated in other control studies by allowing the tubes to stand and the erythrocytes to settle at the conclusion of an experiment. This procedure was applied frequently and did not affect the results materially.

An arbitrary scale for recording hemolysis was used. This was obtained by effecting complete hemolysis of 4 per cent to 6 per cent suspension of erythrocytes. The

terms "marked" or "4+" hemolysis in the protocols refer to such a dark ruby red color. Progressively diminishing degrees of hemolysis were denoted by the terms "moderate" or "slight" or 3+, 2+, +, tr. or ft. tr. The absence of hemolysis was indicated by a - sign. Complete hemolysis did not occur in any of the experiments.

EXPERIMENTAL OBSERVATIONS

1. *Demonstration of the cold hemolysis phenomenon*

The following experiments and results were observed in all cases in which the hemolytic phenomenon occurred. They serve to illustrate in a general way the nature of the phenomenon and will be an introduction to a detailed analysis.

A. Five tenths ml. of whole oxalated blood was kept at 4°C . for 30 minutes. During this time the tube was tapped 20 times every 5 minutes. Marked hemolysis was observed.

B. The same procedure as (A) but without tapping failed to show any hemolysis.

C. The same procedure as (A) but carried out at 37°C . failed to show any hemolysis.

2. *The influence of complement upon the cold hemolysis reaction*

A. Heat-inactivated serum ³

(1) Three tenths ml. of heat-inactivated serum was mixed with 0.2 ml. of 80 per cent RBC and then placed at 4°C . for 30 minutes. The tube was tapped 20 times every 5 minutes and then centrifuged. Marked hemolysis was observed.

(2) The degree of hemolysis was approximately the same as that observed in a similar test with fresh serum.

(3) As will be described later, the curve of diminishing hemolysis, when either the serum was progressively diluted with isotonic saline solution or the cellular concentration was progressively reduced, was the same with the fresh and the heat-inactivated serums.

(4) The same experiment as (1) carried out at 37°C . or at 4°C . but without tapping did not reveal any hemolysis.

(5) Both the heat-inactivated and fresh serums produced similar degrees of hemolysis in experiments in which minimal tapping was applied (see Section 5B).

³ Fresh serum was inactivated by heating at 56°C . for 1 hour.

B. Old serum ⁴

The experiments performed were identical with those described under "heat-inactivated serum" and the results were the same, *i.e.*, marked hemolysis occurred only when the cells were tapped in the cold.

C. Lyophilized serum ⁵

The experiments and results were the same as under "heat inactivated serum".

D. Resuspended or eluted antibody

Landsteiner (12) was the first to describe the fact that cold hemagglutinins can be absorbed from a serum and subsequently obtained free in salt solution. The reaction of cold hemagglutination is completely reversible by increasing the temperature from 4° C. to 37° C. Cold agglutinated erythrocytes freed from serum by centrifugation, decantation, and washing with 0.85 per cent sodium chloride solution in the cold, give up their absorbed agglutinins upon increasing the temperature. Using this technic, we (13) previously prepared cold hemagglutinins in salt solution in which there was reason to believe that practically all of the protein was antibody protein. We have utilized this technic in the following experiments.

In one series, equal parts of serum and erythrocytes were mixed gently and then placed at 4° C. for 2½ hours. Intense agglutination resulted.

⁴ Old serum was tested in only 1 case. This serum was collected aseptically 15 months before the experiments and stored at 6° C. in the dark.

⁵ Lyophilized serum was used in only 1 case. It was collected aseptically 15 months and lyophilized 14 months before the experiment. It was reconstituted to original volume with distilled water before use.

The mixture was then centrifuged at 4° C. (the centrifuge cups were packed with ice) at 1200 r.p.m. for 15 minutes. The supernatant serum was removed (serum absorbed by RBC) and the cold agglutinated erythrocytes were washed 4 times with ice-cold normal saline solution. Throughout these procedures, the temperature of the cells was maintained below 6° C. One half volume of 0.85 per cent sodium chloride solution was added to the washed agglutinated erythrocytes and the tube was placed at 37° C. for 1 hour. Gentle agitation of the mixture was carried out. Following 15 minutes of centrifugation at 25° C., the supernate was removed (resuspended antibody from RBC).

In another series, the same manipulations were carried out with serum and ghosts of erythrocytes. In this instance, centrifugations were more prolonged because of the low density of the ghosts. The eluted antibody in this experiment was labelled "resuspended antibody from ghosts" and the absorbed serum was designated "serum absorbed by ghosts".

Each of the above 4 specimens was tested for cold hemagglutinins and hemolysis. The results are given in Table I.

3. The influence of the concentration of erythrocytes upon the cold hemolysis reaction

Two tenths ml. amounts of suspensions of washed Group O erythrocytes, varying in concentration from 80 per cent to 10 per cent by volume, were mixed with 0.3 ml. amounts of undiluted serum whose titer of cold hemagglutinins was 1/30,000. The tubes were placed at

TABLE I

The relationship between cold hemagglutination and cold hemolysis in absorbed serum and resuspended or eluted antibody

| Specimen | Cold hemagglutinin titer | Undiluted serum 4° C. Tapping* Hemolysis | Undiluted serum 4° C. No tapping Hemolysis | Undiluted serum 37° C. Tapping Hemolysis | ½ dilution of serum 4° C. Tapping Hemolysis | ½ dilution of serum 4° C. Tapping Hemolysis |
|----------------------------------|--------------------------|--|--|--|---|---|
| Serum absorbed by RBC | 1/25,600 | 3+ | — | — | 3+ | 2+ |
| Resuspended antibody from RBC | 1/25,600 | 3+ | + | + | 3+ | 2+ |
| Serum absorbed by ghosts | 1/25,600 | 4+ | — | — | 3+ | 2+ |
| Resuspended antibody from ghosts | 1/25,600 | 2+ | ft. tr. | ft. tr. | + | tr. |

* Tapping refers to 20 taps of each tube every 5 minutes for the duration of the test. All hemolysis tests ran for 30 minutes.

No tapping is self explanatory.

The slight degrees of hemolysis observed in the resuspended antibody tests at 4° C. without tapping and at 37° C. with tapping do not indicate actual hemolysis produced by the test. These specimens were tinged with hemoglobin during their preparation.

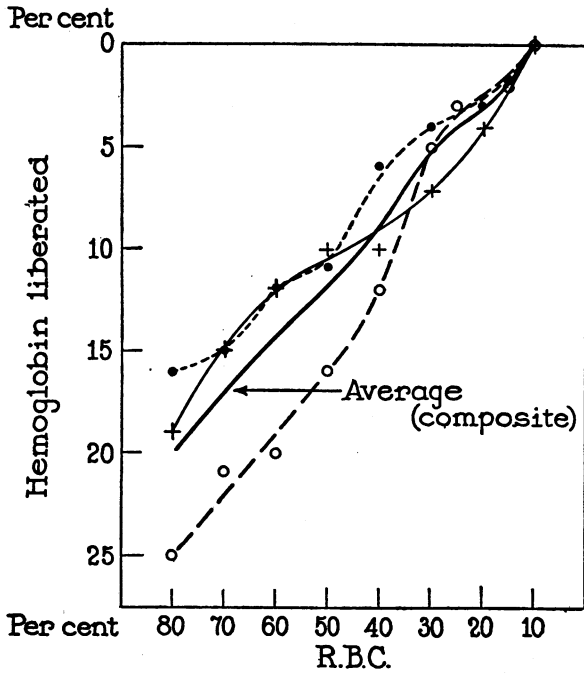


FIG. 1. RELATIONSHIP BETWEEN THE NUMBER OF ERYTHROCYTES AND THE HEMOGLOBIN LIBERATED IN THE COLD HEMOLYSIS PHENOMENON

4° C. for 20 minutes, during which time they were tapped 20 times every 5 minutes. This experiment was performed on 3 different occasions. The results of each are given in Figure 1.

The average of the determinations is also given. The ordinates (percentage of hemoglobin liberated) were obtained by determining the absolute quantity of hemoglobin in the supernates, using a Coleman spectrophotometer and monochromatic (576 mu) light. This was compared with the total amount of hemoglobin calculated to be present in the erythrocytes before hemolysis.

4. The influence of the titer of cold hemagglutinins upon the cold hemolysis reaction

A series of bloods from normal individuals and from patients with high titers of cold hemagglutinins was tested for cold hemagglutinins and the titers ascertained. In the cold hemolysis test, 0.3 ml. of progressive twofold dilutions of the serums was mixed with 0.2 ml. amounts of suspensions of 80 per cent washed homologous erythrocytes. These were kept at 4° C. for 30 minutes and were tapped 20 times every 5 minutes. The relationship between the titers of cold hemagglutinins and cold hemolysis is given in Figure 2. In this illustration, each set of paired rectangles shows the cold hemagglutinin and cold hemolysis titer of one serum. The left rectangle of each (with H on top) is the result of the hemolysis titration and should be referred to the left axis. The right rectangle of each (with

Relationship Between Titers of Cold Agglutinins and Cold Hemolysis

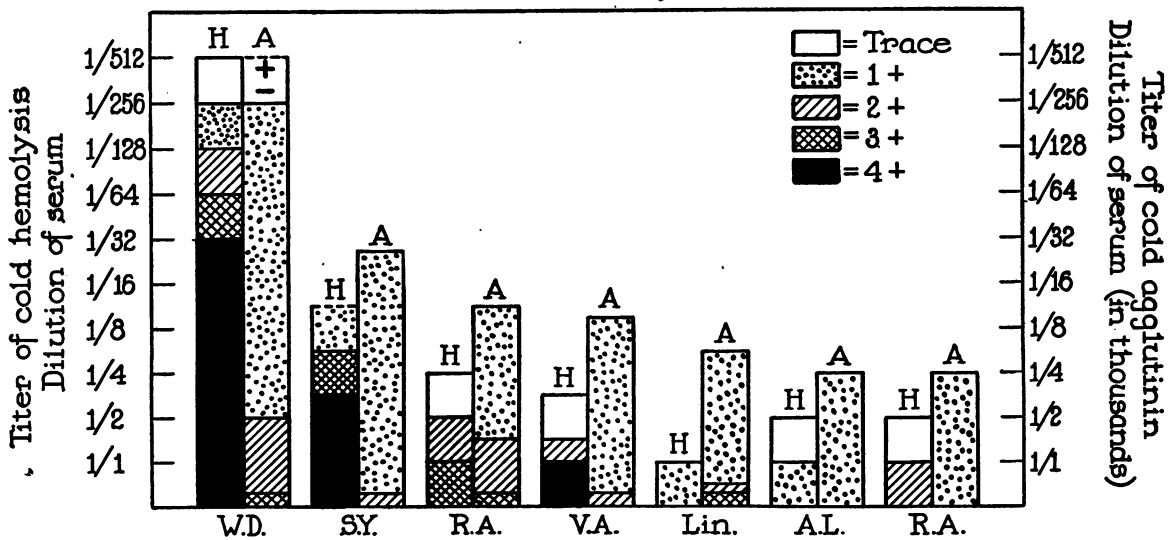


FIG. 2

A on top) is the finding of the hemagglutinin titration and should be referred to the right axis. The initials at the bottom of each refer to the individual patient from whom the serum was obtained. Serum "W.D." was provided by Dr. William Dameshek of Boston from a case of hemolytic anemia.

In 3 of the cases (V. A., LIN., and R. A.) in Figure 2, tests were also performed with serums drawn at later dates when the titers of cold hemagglutinins had fallen to 1/1500 or less. At this time, the tests for hemolysis were negative.

5. The influence of temperature upon the reaction

A. Hemagglutination and hemolysis tests at various temperatures

Hemagglutination and hemolysis tests were performed at several different temperatures with blood from a patient with a high titer of cold hemagglutinins. In the hemolysis tests, tubes containing 0.5 ml. of whole oxalated blood were immersed in water at the indicated temperatures. The test ran for 30 minutes, during which time the tubes were tapped 20 times at the intervals that are noted. The results of these tests are summarized in Tables II and III.

TABLE II

The degree of hemolysis at various temperatures

| Temperature °C. | Tapped every 10 min. | Tapped every 15 min. | Tapped once at the end of 30 min. | Not tapped |
|-----------------|----------------------|----------------------|-----------------------------------|------------|
| 4 | 3+ | 2+ | 2+ | — |
| 7 | 3+ | 2+ | + | — |
| 13 | 3+ | + | + | — |
| 18 | 2+ | + | tr. | — |
| 22 | ft. tr. | ft. tr. | — | — |
| 30 | — | — | — | — |
| 37 | — | — | — | — |

TABLE III

Hemagglutination at various temperatures

| Dilution of serum | 1/10 | 1/20 | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 | 1/1280 | 1/2560 | 1/5120 | 1/10,240 | 1/20,480 |
|-------------------|------|------|------|------|-------|-------|-------|--------|--------|--------|----------|----------|
| Temperature, °C. | | | | | | | | | | | | |
| 37 | — | | | | | | | | | | | |
| 30 | ± | — | | | | | | | | | | |
| 22 | + | + | + | + | ± | ± | — | | | | | |
| 16.5 | 3+ | 3+ | 3+ | 2+ | 2+ | + | + | + | ± | — | | |
| 7 | 3+ | 3+ | 3+ | 2+ | 2+ | 2+ | 2+ | + | + | + | ± | — |
| 4 | 2+ | 3+ | 2+ | 3+ | 3+ | 2+ | 2+ | + | + | + | + | — |

B. The relationship between the duration of incubation at 4° C. and hemolysis

The effect of varying the time of incubation at 4° C. on the resulting hemolysis was determined. The results in Table IV were obtained using 0.5

TABLE IV

The relationship between the duration of incubation at 4° C. and cold hemolysis with varying amounts of tapping (whole oxalated blood)

| Duration of incubation at 4° C., minutes | 1 Tap result | 4 Tap result | 8 Tap result |
|--|--------------|--------------|--------------|
| $\frac{1}{2}$ | — | tr. | tr. |
| $\frac{1}{4}$ | + | 3+ | 2+ |
| $\frac{1}{2}$ | 2+ | 4+ | 4+ |
| $\frac{3}{4}$ | + | 4+ | 4+ |
| 1 | + | 4+ | 4+ |
| 1½ | 3+ | 4+ | 4+ |
| 2 | 3+ | 3+ | 4+ |
| 3 | 3+ | 4+ | 4+ |
| 4 | 4+ | 3+ | 4+ |
| 10 | 4+ | 4+ | 4+ |
| 40 | 4+ | 4+ | 4+ |
| | | 2 tap result | 3 tap result |
| 90 | + | 2+ | 4+ |

Note: The column heading 'Tap Result' denotes the degree of hemolysis after the respective time intervals when the indicated number of taps were applied to the specimen.

ml. amounts of whole oxalated blood, whereas 0.3 ml. of fresh or old serum plus 0.2 ml. of an 80 per cent suspension of erythrocytes was employed in each tube to obtain the results in Table V. The tapping in each instance was performed at the end of the indicated time interval at 4° C.

C. Further studies of the hemolytic reaction at 37° C.

The following experiments were performed to determine the influence on hemolysis of a temperature of 37° C. Five tenths ml. of whole oxa-

TABLE V

The relationship between the duration of incubation at 4° C. and cold hemolysis with varying amounts of tapping (serum and erythrocytes)

| Duration of incubation at 4° C., minutes | 1 Tap result | 2 Tap result | 3 Tap result |
|--|--------------|--------------|--------------|
| 1½ | — | tr. | tr. |
| 2 | — | + | 2+ |
| 3 | tr. | + | + |
| 4 | tr. | + | 2+ |
| 10 | 4+ | 4+ | 4+ |
| 15 | 2+ | 3+ | 4+ |
| 20 | + | 3+ | 4+ |
| 30 | + | 2+ | 2+ |

Note: The columns 'Tap Result' have the same significance as in the preceding table.

lated blood of one of the patients was used in each test unless specified otherwise.

(1) A sample of blood was kept at 4° C. for 20 minutes; at the end of this time, the tube was tapped once. Two + hemolysis was observed.

(2) Another sample was subjected to the same procedure as in (1) but, following the tapping, the blood was kept at 37° C. for 1 hour. Two + hemolysis was observed.

(3) A similar 0.5 ml. sample was kept at 4° C. for 20 minutes, but the tube was not tapped. The tube was then transferred to a water bath at 37° C. After 1 minute in this bath, the tube was tapped 20 times. This tapping was repeated every 5 minutes during the succeeding 30 minutes, the temperature being maintained at 37° C. There was no hemolysis.

(4) Three samples were stored at 4° C., 20° C., and 37° C., respectively, for 24 hours, care being taken to prevent agitation of the tubes. There was no hemolysis at 4° C. and 20° C. whereas slight hemolysis was present at 37° C. These samples, however, were not sterile.

(5) Various samples were kept at 4° C. for from 10 to 90 minutes. The tubes were then transferred to 37° C. without any tapping having been applied. After 6 hours at this temperature, no hemolysis was observed.

(6) A sample of whole oxalated blood was kept at 37° C. for 1 hour. It was then transferred to 4° C. where it was kept without tapping for 24 hours. There was no hemolysis. This experiment was also performed with 0.3 ml. of progressive dilutions of serum and 0.2 ml. of an

80 per cent suspension of erythrocytes with the same result.

(7) In a series, 0.3 ml. of fresh serum was mixed with 0.2 ml. of suspensions of erythrocytes, varying in concentration from 80 per cent to 1 per cent by volume. The tubes were then transferred to 4° C. where they were kept without tapping for 30 minutes. They were then placed at 37° C. for 2 hours. There was no hemolysis. A similar experiment, in which 0.2 ml. of fresh serum and 0.1 ml. of a 1/5 dilution of pooled fresh guinea pig serum (complement) were mixed with 0.2 ml. of suspensions of erythrocytes, was likewise negative for hemolysis.

6. The influence of tapping upon the cold hemolysis reaction

These experiments were performed to elucidate further the importance of tapping upon the reaction. Many of the previous tests, especially those in Section 5B, provide information on the same point.

A. Five tenths ml. samples of whole oxalated blood from 12 healthy individuals were kept at 37° C. and 4° C. for 45 minutes. The tubes were tapped 20 times every 3 minutes during this period. The blood from 1 subject was slightly hemolyzed at both temperatures. In none of the others was hemolysis observed.

B. An experiment similar to that reported in Table IV was carried out except that the tubes were tapped 20 times at the end of each time-interval. The degree of hemolysis in each tube was identical with that reported under "8 tap result."

SUMMARY OF RESULTS

The foregoing experiments elucidate an unusual type of hemolysis, previously reported briefly. Cold hemolysis depends jointly upon a high titer (over 1/3000) of cold hemagglutinins, a high concentration of erythrocytes (over 4 per cent in the final mixture), and tapping of the cold agglutinated erythrocytes.

The importance of the high titer of cold hemagglutinins was demonstrated in several ways. Numerous serums with agglutinin titers of 1/2500 or less did not produce hemolysis, when the other essential factors were operating. On the other hand, all serums whose agglutinin

titers were over 1/3000 (Figure 2) did reveal hemolysis when appropriately tested. Considering the grossness of the methods, there was a remarkable correlation between the titers of cold hemagglutination and cold hemolysis. Above a cold hemagglutinin titer of 1/3000, there was a rough direct linear proportion between the hemagglutination and hemolysis titers. As the titer of cold hemagglutination fell with a rise in temperature, there was a concomitant decrease in the titer of cold hemolysis. At a temperature of 22° C., the titer of agglutinins had fallen to 1/320 and no significant hemolysis could be produced, whereas at lower temperatures, intense hemagglutination and hemolysis were observed (Tables II and III).

The experimental results in Figure 1 show that as the number of red blood cells in a mixture of potent cold hemagglutinating serum and erythrocytes was diminished, the degree of hemolysis was decreased. No significant hemolysis occurred when the final concentration of erythrocytes was less than 4 per cent. (0.3 ml. serum + 0.2 ml. 10 per cent RBC.) Photoelectric determinations of the hemoglobin of the supernates from that experiment, clearly brought out the relationship; there was an absolute as well as a relative diminution in hemolysis, coincident with a decrease in the number of red blood cells. This relationship was shown to be linear. Thus, when 80 per cent RBC (32 per cent in the final mixture) was employed, 20 per cent of the hemoglobin of the erythrocytes was liberated. However, with 20 per cent RBC (8 per cent in the final mixture), only 3 per cent of the hemoglobin was liberated.

The necessity of tapping the cold agglutinated blood was shown in practically all of the experiments. One tap of blood kept at 4° C. for 4 minutes was followed by 4 + hemolysis (Table IV), whereas 20 taps of the same blood every 5 minutes for 30 minutes at 37° C. did not result in any hemolysis. Many controls, using blood from normal individuals or from patients with low titers of cold hemagglutinins, yielded negative results.

Increasing the number of tappings of the blood increased the degree of hemolysis up to a certain point. Beyond this, no further hemolysis resulted. This was shown in Table IV in which

hemolysis was produced sooner and to a greater degree after short incubation in the cold when the blood was tapped 4 times, as opposed to the hemolysis obtained when it was tapped only once. On the other hand, hemolysis was not appreciably increased when the number of taps was increased from 4 to 20.

When blood with potent cold hemagglutinins remained at 4° C. for long periods (90 minutes), there was a diminution in the degree of hemolysis after very slight tapping (Table IV).

Hemolysis occurred only when agglutinated cells were tapped. If a specimen were kept at 4° C. for 20 minutes without tapping, then transferred to a water bath at 37° C., and the tapping applied repeatedly after 1 minute at this temperature, hemolysis did not result.

The fact that intense hemolysis was readily produced with heat-inactivated serum, old serum, old lyophilized serum, and resuspended or eluted saline solutions of cold hemagglutinins, indicated that complement was not an essential agent in this hemolytic system. Other confirmatory evidence of this was previously found in the observations that complement was not fixed or absorbed during cold hemagglutination (2), that the hemolysis occurred at 4° C. and did not occur at 37° C., and that intense hemolysis could be produced in the short period of 1 minute.

COMMENT

The following may be deduced from the observations detailed in this paper.

It is apparent that during the stage of aggregation of erythrocytes into clumps and masses, slight agitation causes the liberation of hemoglobin. Unpublished studies and others previously reported (2) have shown that no alteration of the hypotonic fragility of erythrocytes was caused by cold agglutination. The present studies demonstrated that when the agglutinated erythrocytes were relatively well packed by sedimentation, hemoglobin was not readily liberated by slight tapping. This was at least in part explained by the fact that they moved as one mass when tapped only once. If the tapping was continued and the mass broken, hemoglobin was liberated.

Through the courtesy of Dr. G. K. Hirst,⁶ a highly concentrated suspension of the Lee strain of influenza B virus was made available and was tested against human Group O erythrocytes. Hemagglutination was observed after 18 hours at 4° C. with a dilution of virus of 1/16,000. Despite this, hemolysis was not produced when the undiluted virus was shaken with erythrocytes in the cold. As pointed out by Dr. Hirst (14), the hemagglutination produced by influenza virus is fine and does not assume the characteristics of a "button". Massive agglutination, as observed with potent cold hemagglutinating serum, does not occur. These facts suggest that it is not so much the titer of a serum that is important in regard to hemolysis, but rather the type and intensity of cellular aggregation.

There is no clear understanding of the rôle that mechanical trauma plays in determining the duration of life of the erythrocyte. At the same time, the significance of this factor in clinical hemolytic syndromes has not been studied adequately. The experiments reported here indicate that, under certain clearly defined conditions, mechanical trauma (*e.g.*, tapping of blood) of even very slight degree causes the liberation of large quantities of hemoglobin. The mechanism by which this occurs was not studied, rather was the emphasis placed on the conditions for optimal hemolysis. The direct demonstration of the importance of mechanical trauma in producing hemolysis reopens the question of the participation of this factor in other types of hemolysis.

Even though some of the intricacies of the hemolytic mechanism accompanying cold hemagglutination were elucidated, many clinical facts remained unexplained. As mentioned under "Materials", only one of the cases studied had a hemolytic anemia—and this patient had previously received a small dose of sulfadiazine. In this case, a striking parallelism between the severity of the hemolytic process, the titer of the cold hemagglutinins, and the intensity of the *in vitro* hemolytic phenomenon was observed. As the patient improved and the abnormal hemolysis subsided, the cold hemagglutinin titer

fell and the cold hemolysis phenomenon was no longer positive. One of the other cases had paroxysmal cold hemoglobinuria due to potent cold hemagglutination; hemolytic anemia was not present. In the other cases, despite the presence of high titers of cold hemagglutinins and a positive *in vitro* test for cold hemolysis, neither hemoglobinuria nor anemia occurred. It is obvious, therefore, that, if this hemolytic phenomenon is indicative of a mechanism operative *in vivo*, other facts, as yet undisclosed, must be of importance. Another case,⁷ of severe chronic hemolytic anemia associated with pregnancy, had a cold hemagglutinin titer of 1/1600 for several years before and after splenectomy. The anemia was cured by splenectomy. Cold hemolysis of this blood was not demonstrable. Even though cold hemagglutinins in high titer may be observed in rare patients with hemolytic anemia and a hemolytic mechanism may be shown, it is not proven that the agglutinin is pathogenetically related to the hemolytic syndrome.

Cold hemolysis with cold hemagglutination must be sharply differentiated from the cold—warm hemolysis of Donath and Landsteiner (4). The use of the term "cold hemolysis test" for the latter phenomenon is not accurate and, indeed, is more applicable to the test described in this communication. In the Donath and Landsteiner test for syphilitic paroxysmal cold hemoglobinuria, hemolysis is independent of hemagglutination; tapping (or mechanical trauma) is of no importance; hemolysis occurs only after warming the chilled blood; hemolysis is of the so-called complex nature requiring both amboceptor and complement. Cold hemolysis with cold hemagglutination is dependent upon potent cold hemagglutination, a large number of erythrocytes, and tapping. Cold hemolysis is not complex since complement is not required; hemolysis occurs in the cold; mechanical trauma is a necessary prerequisite to cold hemolysis.

CONCLUSIONS

1. Blood containing potent (titer over 1/3000) cold hemagglutinins was readily hemolyzed if shaken in the cold.

⁶ International Health Division of the Rockefeller Foundation, New York.

⁷ Previously studied by Dr. Louis R. Wasserman.

2. Detailed studies indicated the facts that normal blood or blood with low titers of cold hemagglutinins was not hemolyzed by shaking; as the number of erythrocytes was reduced, hemolysis diminished absolutely and relatively; the reaction of hemolysis was unaffected by complement and occurred with heat inactivated serum.

3. As erythrocytes undergo rearrangement and form aggregates due to the action of the cold hemagglutinin, they are hemolyzable by slight shaking. When the agglutinated cells have sedimented, more shaking is required to effect hemolysis.

4. Hemoglobinemia and hemolytic anemia are not invariably present in patients whose serums exhibit a high titer of cold hemagglutinins, even though such serums are capable of causing cold hemolysis *in vitro*.

5. Paroxysmal cold hemoglobinuria may occur in patients with potent cold hemagglutinins. In these patients, the cold hemolysis test with tapping is positive. Paroxysmal cold hemoglobinuria in syphilitics is associated with a positive Donath-Landsteiner test and is different serologically from the cases with hemagglutination.

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