

STUDIES ON IMMUNE HUMAN HEMOLYSIS

I. THE KINETICS OF THE DONATH-LANDSTEINER REACTION AND THE REQUIREMENT FOR COMPLEMENT IN THE REACTION*

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Serum complement is of potential importance to clinical medicine and to human biology in immune reactions and hypersensitivity states. However, most studies on the characteristics and mechanism of action of complement have been performed on systems largely of non-human origin (1, 2). The present studies were undertaken to study a specific immune human hemolytic disorder and to use this as a model system for the study of human complement in a reaction system involving human erythrocytes and an antibody of human origin. The Donath-Landsteiner (D-L) reaction (3), responsible for the clinical syndrome of paroxysmal cold hemoglobinuria, is a reaction which requires for hemolysis that erythrocytes be incubated with antibody and fresh serum complement first at low temperatures, usually near 0°C., and then at higher temperatures, usually 37°C. It was chosen as the model for these studies because: (a) it involves human cells and antibody and avoids the use of reagents from multiple species (rabbit antibody, sheep red cells, guinea pig complement); (b) the D-L antibody is an active hemolysin but a weak agglutinin in contrast to the isoantibodies and cold agglutinins; and (c) the biphasic nature of the reaction permits the separation of the early and late phases of complement action.

The studies in the present paper were undertaken to define the kinetics of the Donath-Landsteiner reaction and served as the basis for the observations on the mechanism of action of complement in the succeeding paper. The data indicate that many conflicting earlier observations regarding the role of complement in the reaction (3-8) may be explained on the basis of variation in (a) potency and amount of antibody used, (b) sensitivity of cells, (c) amount

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and species source of serum complement, and (d) kinetic conditions such as temperature, pH, and ionic environment. The subsequent paper describes observations on the mechanism of action of complement, in particular the role of the first component of complement (C'1) and the esterase derived from it (C'1 esterase) in initiating immune human hemolysis.

MATERIALS AND METHODS

Donath-Landsteiner Antibody.—Serum was obtained from two patients with paroxysmal cold hemoglobinuria.

Case I was a 54 year old white male who was admitted to Cleveland Metropolitan General Hospital in December, 1958, because of paroxysms of hemoglobinuria following cold exposure and symptoms suggestive of paresis. Serologic tests for syphilis (Kline) in blood and spinal fluid were strongly positive, and the Donath-Landsteiner test was positive. Cold agglutinins were present in a titer of 1 to 4.

Case II was a 32 year old Negro woman who had paroxysmal cold hemoglobinuria, but who had no history or stigmata of luetic infection. Her blood serology was negative. The D-L reaction was positive. The patient had no Raynaud's-like phenomena, and cold agglutinins were weakly positive at a titer of 1 to 8. Characteristics of the antibody from this patient have been described briefly by Schmidt and Clifford (9).

Purification of Antibody.—(a) Alcohol fractionation: To obtain the D-L hemolysin free of the components of complement, gamma-2 globulin was prepared by ethanol fractionation of the serum of case I, according to the method of Deutsch and coworkers (10). Following the final precipitation the purified material was dissolved in barbital buffer containing 5×10^{-4} M Mg⁺⁺ and 1.5×10^{-4} M Ca⁺⁺, at pH 7.4 and ionic strength 0.15, unless otherwise noted. Two preparations contained 1.0 and 1.2 mg. protein N/ml. and represented approximately a fourfold purification with respect to serum. They contained no detectable amounts of any of the four components of serum complement. These preparations were designated "purified antibody I." The Kline test was negative on one and weakly positive on the other of the two preparations of the undiluted purified material, but was strongly positive on the untreated serum at a 1 to 10 dilution. The hemolytic antibody activity of serum and purified preparations was stable to heating at 56°C. for 30 minutes. At least half the original D-L antibody activity remained in the residual material from fraction II + III after the gamma-2 fraction was removed. This material had the same quantitative reaction in the Kline test as original serum.

(b) Column chromatography: Other preparations of partially purified antibody were obtained by chromatographic separation of serum from cases I and II on DEAE cellulose columns (Brown & Co., Berlin, New Hampshire, selectacel) according to the method described by Fahey *et al.* (11). Fractions were concentrated by lyophilization or ultrafiltration dialysis. Virtually all the activity was recovered in the fraction eluted with pH 8.0, 0.01 M phosphate buffer, which migrated on paper electrophoresis as gamma globulin. These preparations were designated "column-fractionated antibody I or II."

Treatment of Serum Containing Antibody to Inactivate the Components of Complement (Complement-Depleted Antiserum).—Parts of these studies were performed with unfractionated antiserum. For this purpose serum from cases I and II was incubated at 37°C. for 60 minutes with 2 mg./ml. of zymosan (Fleischmann No. 7B152) (12) to inactivate C'3, the third component of complement. After centrifugation to remove zymosan, the serum was incubated for 60 minutes at 37°C. with 0.25 ml. of 0.15 M NH₃ per ml. of serum to inactivate C'4, the fourth component of complement; 0.25 ml. of 0.15 M HCl per ml. of serum was then added to neutralize the NH₃, and the serum was heated at 56°C. for 30 minutes to inactivate C'1 and C'2, the first and second components of complement (13). The final dilution was 1 to 1.5 with respect to the original serum volume. These sera contained none of the components of

complement, as measured by the dilution titration technique (13, 14), and therefore were considered adequate for use in studies on the role of complement in the D-L reaction. The treated sera from cases I and II were designated complement-depleted antiserum I or II and contained D-L antibody activity equal to the original serum when tested with added fresh normal human serum as a source of complement.

Cold agglutinins in the sera containing D-L antibody were determined by incubating 0.2 ml. volumes of serial twofold dilutions of antiserum in barbital buffer containing 1.5×10^{-4} M Ca^{++} and 5×10^{-4} M Mg^{++} with 0.2 ml. of a 2 per cent suspension of normal group O erythrocytes in the same barbital buffer at room temperature for 1 hour and then overnight in a refrigerator at 5°C. Upon removal from the refrigerator the degree of agglutination was determined macroscopically by disruption of the cell button by gentle inversion. The titer was the highest dilution of antiserum causing any agglutination.

Erythrocytes.—Erythrocytes were obtained from a normal person of blood group O and a patient with paroxysmal nocturnal hemoglobinuria (PNH), who was also of blood group O. Cells from PNH patients have been used regularly (15, 16) for the study of hemolysins since they are more susceptible to hemolytic antibodies than are normal cells. Although the PNH abnormality renders the erythrocytes subject to hemolysis by acidified undiluted normal human serum, normal serum does not cause lysis of PNH cells at pH 7.4 and a serum dilution of 1 to 3, as was used in the present study (*vide infra*). Blood was defibrinated and centrifuged at room temperature to avoid adsorption of incomplete cold antibody to the cells. The packed unwashed cells were stored in an equal volume of modified Alsevers (13) solution at 4°C. for periods less than 1 week. Prior to use they were washed three times with 0.15 M NaCl, once with pH 7.4 barbital buffer, containing 5×10^{-4} M Mg^{++} and 1.5×10^{-4} M Ca^{++} , and suspended to 25 per cent by volume in the same buffer, unless otherwise noted. For experiments involving alteration of pH, cells were suspended in 0.15 M NaCl.

Serum.—Individual *human sera*, as a source of complement, were obtained from defibrinated blood and were used immediately or stored at -65°C . in a mechanical deep freeze.

Other Reagents.—Barbital buffer, ionic strength 0.15, pH 7.4, containing 5×10^{-4} M Mg^{++} and 1.5×10^{-4} M Ca^{++} , was prepared as described previously (13, 14). Barbital buffer without added Mg^{++} or Ca^{++} was used in experiments on the requirement for those ions in the reaction. Unless otherwise designated, the term barbital buffer refers to the buffer with added ions. Isotonic NaCl (0.15 M) was used in place of barbital buffer in experiments when pH was varied.

Michaelis buffer, ionic strength 0.15 at various pH values, was used in experiments in which pH was altered (17). One M CaCl_2 and MgCl_2 were diluted in 0.15 M NaCl for experiments involving various concentrations of Ca^{++} and Mg^{++} . The ionic strength of reaction mixtures was altered by adding appropriate amounts of NaCl and distilled water prior to the addition of erythrocytes.

Ethylenediamine tetra acetic acid (EDTA) and its various salts were obtained as Sequestrene® from Geigy Chemicals Co., New York, as Na_3EDTA , Na_2MgEDTA , and Na_2CaEDTA . They were made up at 0.15 M concentrations in 0.15 M NaCl, and further dilutions were made in 0.15 M NaCl.

Resin-treated serum was prepared by exposing serum to an equal volume of amberlite IRC-50 (Rohm and Haas), Na cycle, for 10 minutes at 4°C., followed by aspiration and centrifugation of the serum to remove particulate resin, and was used immediately.

An International refrigerated centrifuge (PR-2) at 2°C. was used for centrifugation after the cold phase; centrifugation after the warm phase was in International table-model centrifuges at room temperature.

Cold incubation was in a refrigerated cold bath thermostatically controlled to $1^{\circ}\text{C} \pm 1^{\circ}$, or at various temperatures in Dewar flasks. Warm incubation was in a thermostatically controlled water bath at $37^{\circ}\text{C} \pm 0.5^{\circ}$ or at various temperatures in Dewar flasks.

The *Donath-Landsteiner hemolytic reaction* was performed as follows:

Cold phase: To 10 x 100 mm. test tubes in a tray of melting ice at 5°C., in sequence were added 0.05 ml. of a 25 per cent suspension of erythrocytes, 0.1 ml. of fresh human serum, 0.05 ml. or 0.10 ml. of an appropriate dilution of complement-depleted antiserum or purified antibody, and barbital buffer to correct the final volume to 0.30 or 0.35 ml. After mixing, the tubes were incubated at 1°C. for 30 minutes with frequent mixing by agitation; 3 ml. of barbital buffer at 1°C. were added; the cells were centrifuged at 1500 *G* for 5 minutes at 2°C. and washed a second time in similar fashion with 3 ml. of cold buffer. No hemolysis occurred during the cold phase.

Warm phase: After the second wash, the tubes containing the cell buttons were transferred to an ice bath, 0.1 ml. of human serum was added, and the volume was adjusted to 0.3 ml. with barbital buffer; the tubes were mixed by agitation and incubated at 37°C. for 30 minutes, during which time hemolysis occurred.

Quantitation of hemolysis: (a) qualitative hemolysis was scored visually on a 0 to +++ scale, covering a range from 0 to approximately 50 per cent hemolysis. The nature of the D-L reaction is such that complete hemolysis was not attainable under the conditions of these tests; (b) quantitative determination of hemolysis was performed by diluting 0.1 ml. of supernatant with 0.9 ml. distilled water and reading the optical density at 540 millimicrons along a light path of 12 mm. in a Bausch and Lomb Spectronic 20 colorimeter. One hundred per cent hemolysis was equivalent to an optical density of approximately 0.70.

When incubations were done at various temperatures, the cells were washed at those temperatures. Incubation for various times in the cold was followed immediately by centrifugation at 2°C.

Appropriate control tubes lacking serum or antibody were included in each experiment. Under the conditions of dilution and pH in these experiments there was no hemolysis of the normal erythrocytes or erythrocytes from patients with paroxysmal nocturnal hemoglobinuria in the presence of normal human serum, but in the absence of the Donath-Landsteiner antibody. Thus, as noted above, at pH 7.4 with serum complement diluted one to three, no acid hemolysis of PNH cells occurred. Hemolysis occurred only when D-L antibody was added.

Effect of Concentration of Complement and Erythrocytes.—The conditions of both the cold and warm phase were selected on the basis of a series of preliminary experiments in which the effect of variations in the amount of complement, number of erythrocytes, and volume of reaction was studied.

A fivefold increase or eightfold decrease in the amount of fresh serum in the cold phase did not alter the degree of hemolysis, indicating that an excess of complement was present in the usual experimental conditions. Increase in the volume of the reaction mixture resulted in diminished hemolysis. Utilization of larger numbers of cells resulted in greater total hemolysis, but a decreased percentage of cells was hemolyzed.

Conditions involving an excess of complement were chosen to be comparable to previously reported studies. The final selection of conditions was influenced by a limited supply of antibody and by the amount of hemolysis which could be detected colorimetrically. The system was usually limiting with respect to antibody and cells, but there was excess complement, as noted. Under these conditions there was almost complete utilization of antibody, but no measurable fixation of complement during either the cold or warm phase.

RESULTS

I. Influence of Antibody Potency, Erythrocyte Sensitivity, and the Presence of Complement on the Donath-Landsteiner Reaction

A. Antibody Potency and Erythrocyte Sensitivity.—Serial twofold dilutions of the several preparations of D-L antibody were tested for hemolytic activity

against normal and PNH erythrocytes when fresh serum was present as a source of complement in both the cold and warm phase of the reaction (Table I). Against normal erythrocytes, complement-depleted antiserum I, from the luetic patient, caused appreciable hemolysis only in the undiluted state (expressed as a titer of 1); purified antibody I or column-fractionated antibody I were only active when concentrated twofold with respect to the original serum. Complement-depleted antiserum II, however, caused hemolysis of normal cells at a dilution of 1 to 32.

TABLE I
Relative Activities of Several Preparations of Donath-Landsteiner Antibody—Complement Present in Cold and Warm Phase

Antibody preparation	Titer of hemolytic activity	
	Normal cells	PNH cells
Case I		
Complement-depleted antiserum I.....	1	6
Purified antibody I.....	$\frac{1}{2}$	2
Column-fractionated antibody I.....	$\frac{1}{2}$	4
Case II		
Complement-depleted antiserum II.....	32	128
Column-fractionated antibody II.....	—	64

The titer of hemolytic activity is expressed as the reciprocal of the dilution of 0.1 ml. of antibody or antiserum resulting in optical density of 0.12 under conditions described in methods. Twofold dilutions were made serially, and end points between two tubes were expressed as the interpolated half dilution. When more than 0.1 ml. of antiserum was required to cause lysis, the volume of antiserum was increased to 0.2 ml. or a concentrated preparation of partially purified antibody was used.

In contrast, the titer of hemolytic activity of all the antibody preparations was four to eight times higher when PNH erythrocytes were used. This greater sensitivity of PNH erythrocytes to hemolytic antibodies is well known (16), and made possible a variety of studies with purified antibody I that would not have been possible with normal cells. These observations emphasize that hemolysis in the D-L reaction is a function of both activity of the antibody and sensitivity of the erythrocytes.

B. The Requirement for Complement During the Cold and Warm Phases of the Donath-Landsteiner Reaction.—A major point of controversy about the D-L reaction has been concerned with the requirement for complement during the initial cold phase of the reaction. It is agreed that complement must participate at some point in the D-L reaction. In experiments to be described, complement was omitted during the cold phase when cells and antibody were incubated together, but was present during the subsequent warm phase. The cells were always washed between the cold and warm phase to be certain that

none of the reactants was carried over from the cold to the warm phase. Using normal erythrocytes, none of the antibody preparations caused more than a trace of warm phase hemolysis when fresh serum had been omitted in the prior cold phase. However, as shown in Fig. 1 using PNH cells, appreciable warm phase hemolysis occurred even when complement had been omitted in the cold phase, when complement depleted antiserum II was used at a dilution as great as $\frac{1}{16}$. The addition of serum complement in the cold phase not only increased appreciably the amount of hemolysis, but also permitted lysis at much higher

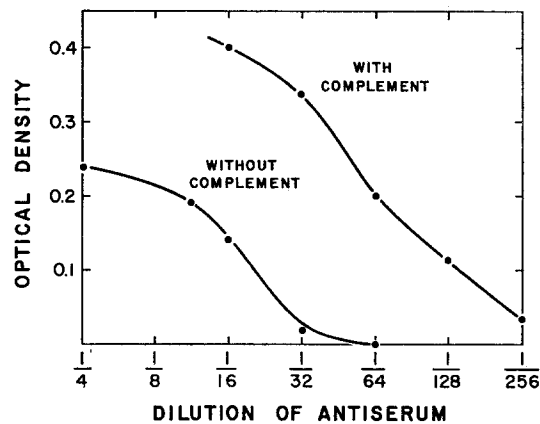


FIG. 1. The effect of complement in the cold phase of the Donath-Landsteiner reaction on PNH erythrocytes. Various dilutions of complement-depleted antiserum II and PNH erythrocytes were incubated with or without serum complement at 1°C . for 30 minutes. Hemolysis was measured following subsequent incubation with serum complement at 37°C . for 30 minutes. An arithmetic plot of antibody dilutions on the abscissa was chosen for ease of graphic representation.

dilutions of antibody. Similar results were obtained with PNH cells using the several antibody preparations from case I. Thus, the use of large amounts of antibody permitted completion of the cold phase in the absence of complement; the requirement for complement in the cold phase, therefore, is a quantitative rather than a qualitative one, depending on potency of antibody and sensitivity of erythrocytes.

When the several antibodies were incubated with cells in the presence of excess fresh serum complement in the cold phase, washed, and tested for the requirement for complement in the warm phase, similar results were observed. At none of the concentrations of antibody used did normal cells hemolyze in the warm phase in the absence of complement. However, when PNH cells, fresh serum, and undiluted complement-depleted antiserum II were incubated together in the cold, some hemolysis occurred in the warm phase even in the

absence of added complement. This was comparable in amount to the hemolysis caused by a $1/128$ dilution of the antiserum when complement was present in both the cold and warm phases. Similarly, when an eightfold concentrated preparation of column-fractionated antibody I was incubated with fresh serum and PNH cells in the cold, some hemolysis occurred even in the absence of complement in the warm phase. Thus, greater amounts of antibody were required to cause hemolysis when complement was absent in the warm phase than when it was absent in the cold phase. In each instance, the degree of hemolysis was increased markedly when complement was added in the warm phase.

In none of the experiments reported here was it possible to cause hemolysis when complement was lacking from both phases. Even using high concentrations of antibody and the sensitive PNH cells, it was necessary that fresh serum containing complement be present in either the cold or the warm phase of the reaction for lysis to occur. Data demonstrating the requirement for all of the components of hemolytic complement in the D-L reaction will be presented in the following paper.

C. Effect of Heated Serum in the Cold Phase.—When purified D-L antibody was used in the cold phase of the reaction with fresh serum as the source of complement, the addition of heated normal serum to the reaction mixture in the cold inconstantly resulted in a moderate decrease in hemolysis in the subsequent warm phase, even though the cells were washed and incubated in fresh serum in the warm. Thus, heated normal serum had a variable inhibitory effect on the cold phase of the reaction, an effect which may have been due to the anticomplementary properties of some heated sera. This observation may account for some previous reports on the apparent thermolability of the D-L antibody (6, 18, 19), since heated antiserum has been frequently used as the source of antibody.

II. The Kinetics of the Hemolytic Reaction

Observations were made on the effects of time, temperature, pH, ionic strength, and Ca^{++} and Mg^{++} on the cold and warm phases of the D-L reaction. In each instance these characteristics were studied using several combinations of antibody and normal or PNH cells, including (a) PNH cells and purified antibody I at a dilution of 1 to 2, a level requiring fresh serum in both the cold and warm phases; (b) normal cells and complement-depleted antiserum II at a dilution of 1 to 8, a level requiring fresh serum in both phases; and (c) PNH cells and complement-depleted antiserum II at a dilution of 1 to 8, a level that did not require fresh serum in the cold phase. In all the experiments, excess complement was supplied in both phases of the reaction as described in methods. The term "activation" in the cold phase is used to indicate preparation of the erythrocytes for hemolysis during incubation with serum in the subsequent warm phase.

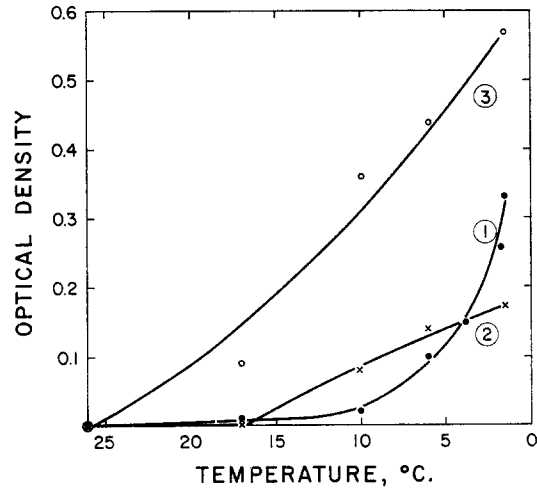


FIG. 2. Effect of temperature on the cold phase of the Donath-Landsteiner reaction, using several antibody preparations and cell types in the presence of excess complement.

Key: (1) PNH cells and purified antibody I; (2) Normal cells and complement-depleted antiserum II; (3) PNH cells and complement-depleted antiserum II.

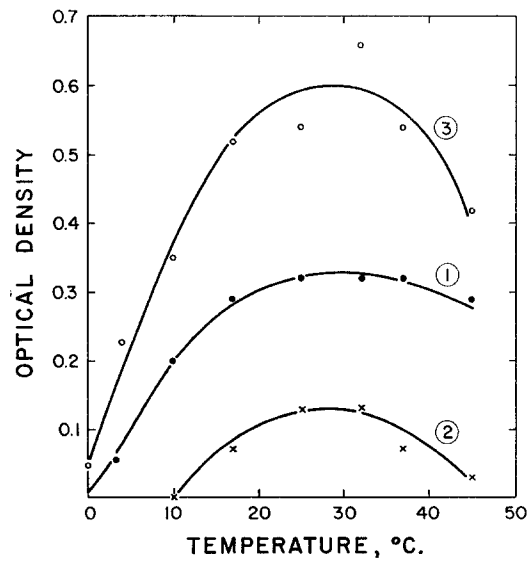


FIG. 3. Effect of temperature on the warm phase of the Donath-Landsteiner reaction.

Key: (1) PNH cell and purified antibody I; (2) Normal cells and complement-depleted antiserum II; (3) PNH cells and complement-depleted antiserum II.

A. Effect of Temperature of Incubation.—

Cold phase: During incubation for 30 minutes at various temperatures, there was a progressive increase in the activation of the cells with decreasing temperature. Maximum activation occurred when the cold phase was carried out at 1°C. (Fig. 2). Only with the most sensitive system involving PNH cells and the more potent antiserum did appreciable activation occur above 10°C., again emphasizing the importance of both antibody potency and cell sensitivity in determining conditions under which the reaction will proceed.

Warm phase: Following a cold phase at 1°C. there was considerable hemolysis during subsequent incubation at temperatures as low as 10°C. (Fig. 3). There was a progressive increase in the amount of hemolysis as the temperature was increased to 32°C. At temperatures above 32°C. there was a progressive decrease in hemolysis. The same relative degree of sensitivity existed as was evident in the earlier studies; the antiserum from case II acting on PNH cells resulted in greater hemolysis at lower temperatures than did the others. Although the optimum appeared to be 32°C., most subsequent reactions were carried out at 37°C., which was nearly optimal.

B. Time of Incubation.—

Cold phase: During the cold phase at 1°C. there was a rapid increase in degree of activation during the first 10 to 15 minutes of incubation with a relatively slow increase thereafter (Fig. 4). At variable times up to 60 minutes there was no observed diminution in the activity of the cell-antibody complex, as had been observed by Yorke and MacFie (18). Longer periods of incubation were not attempted. In all subsequent experiments a period of 30 minutes at 1°C. was used as a standard period of cold incubation.

Warm phase: Following incubation at 1°C. for 30 minutes, hemolysis occurred rapidly during incubation in serum at 37°C. (Fig. 5). After the cold phase, when washed cell buttons were transferred to the 37°C. bath and serum warmed to 37°C. was added, there was considerable hemolysis in a sample obtained after 30 seconds, and hemolysis was nearly complete at the end of 1 minute. Although there were absolute differences of degree in the amount of hemolysis which occurred, the rates of hemolysis were approximately the same in the several systems under these conditions. A 30-minute period of incubation at 37°C. was used in all subsequent experiments.

*C. pH.—*Alteration of pH at constant ionic strength of 0.15 was effected with Michaelis buffers added to serum in which the pH had been adjusted with 0.15 M HCl or NaOH. The two phases were studied separately with incubation in the alternate phase at pH 7.4 in barbital buffer. Reactions utilizing PNH cells and purified antibody I, and normal cells with complement-depleted antiserum II were studied. In both instances the hemolytic reaction was inhibited at pH values below 5.2 and above 9.7 in either the cold or warm phase. For normal cells and complement-depleted antiserum II the optimum range

in both the cold and warm phases was between pH 7.5 and 8.5. For PNH cells and purified antibody I, a broader optimum was observed in both the cold and warm phases with maximum activity between pH 6.0 and 8.0 in the cold phase and between pH 6.5 and 8.5 in the warm phase. PNH cells, which characteristically undergo hemolysis in undiluted serum at pH 7.0, were not lysed in the presence of normal human serum and in the absence of D-L antibody under the conditions of these experiments, even at pH 7.0. These data on pH optima are in agreement with those reported by Dacie (16).

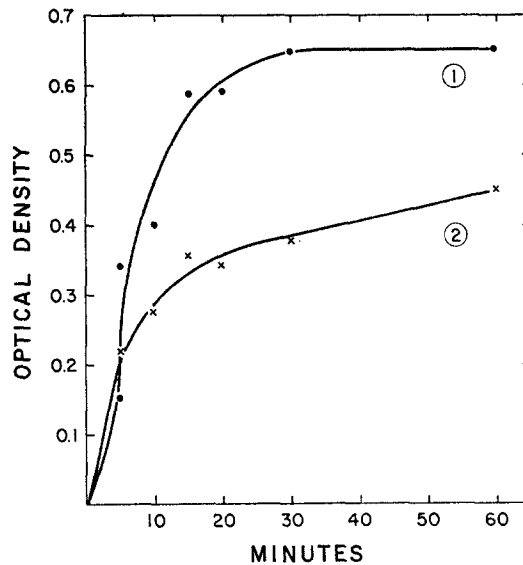


FIG. 4. Effect of time of incubation during the cold phase of the Donath-Landsteiner reaction.

Key: (1) PNH cells and purified antibody I; (2) Normal cells and complement-depleted antiserum II.

D. Ionic Strength.—An increase in ionic strength had a marked inhibitory effect on the D-L reaction, particularly on the two systems requiring fresh serum in the cold phase. In the cold phase there was progressive decrease in activity as the ionic strength was increased, decreasing to about one-half activity at ionic strength 0.30. The effect was more marked in the warm phase, where there was progressive diminution in hemolytic activity at any increase above ionic strength 0.15 and marked diminution above 0.25. The most active system, that utilizing PNH cells and complement-depleted antiserum II, was less sensitive to changes in ionic strength, there being no decrease in activity in the cold phase at ionic strength 0.30, and decrease in the warm phase to slightly less than one-half the original activity at ionic strength 0.40. It is this reaction which does not require fresh serum in the cold phase.

E. Divalent Cations.—Studies on the requirements for divalent cations in the two phases of the reaction utilized PNH erythrocytes with purified antibody I at a level equivalent to 0.1 ml. of original serum. This antibody level required the presence of fresh serum in both the cold and warm phases of the reaction.

In the presence of undiluted serum and an excess of complement, resin treatment of the reagents in both phases with the cation exchange resin amberlite IRC-50 resulted in about a 50 per cent decrease in over-all hemolytic activity as reported previously (20).

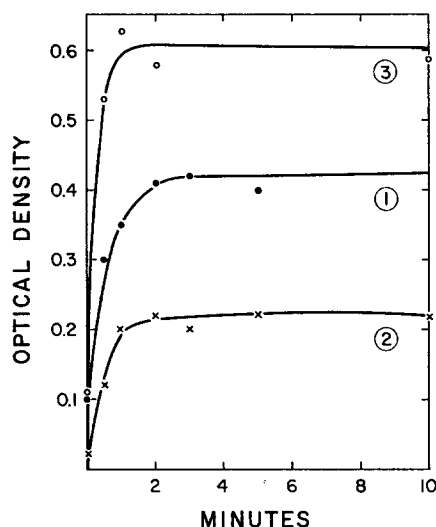


FIG. 5. Effect of time of incubation during the warm phase of the Donath-Landsteiner reaction.

Key: (1) PNH cells and purified antibody I; (2) Normal cells and complement-depleted antiserum II; (3) PNH cells and complement-depleted antiserum II.

Resin treatment in the cold phase alone had little effect, but resin treatment in the warm phase reduced hemolytic activity approximately one-half. This was restored completely by addition of 10^{-4} M Mg^{++} , but was not affected by low levels of Ca^{++} , and was further inhibited at a concentration of 10^{-8} M Ca^{++} .

The effect of EDTA was more pronounced. With undiluted fresh serum, 3×10^{-3} M Na_3EDTA or $Na_2BaEDTA$ blocked completely the initial cold phase of the reaction. $Na_2MgEDTA$ inhibited partially, and $Na_2CaEDTA$ was without effect. On diluting fresh serum 1 to 4, Na_3EDTA , $Na_2BaEDTA$, and $Na_2MgEDTA$ blocked the cold phase completely, and $Na_2CaEDTA$ was without effect, indicating a requirement for Ca^{++} in the initial cold phase of the reaction.

In the warm phase of the reaction with the usual concentration of fresh serum, 10^{-8} M Na_3EDTA and $Na_2BaEDTA$ blocked completely the hemolytic

reaction, but comparable levels of Na_2MgEDTA and Na_2CaEDTA were without effect. In each instance addition of equimolar Mg^{++} restored hemolytic activity. These data indicate that Mg^{++} but not Ca^{++} was required in the warm phase. Data are presented in the subsequent paper which indicate that C'1 acts in the cold phase, C'4 may interact in either the cold or warm phase, and C'2 and C'3 in the warm. The observations reported here on the requirement for Ca^{++} in the cold phase and Mg^{++} in the warm phase are consistent with published observations on the role of cations in the sequential action of the components of complement (2).

DISCUSSION

There have been many conflicting reports concerning the properties of the D-L antibody and the characteristics of the hemolytic reaction. A number of early investigators (7, 8) in their original observations demonstrated that hemolysis occurred even though fresh serum was not present in the initial cold phase. Hoover and Stone, Moss, and Cooke (4-6) demonstrated that fresh serum as a source of complement was required in the cold phase with the antibodies they were studying. There have also been differences concerning the critical temperature and rate of the cold phase of the reaction.

The present study confirms the surmise made originally by Mackenzie (21) and repeated by Dacie (16) that many of the discrepancies previously observed may be related to characteristics of the several constituents of the hemolytic system and to the kinetics of the reaction.

For example, the potency of antibodies varies, as indicated by the two sera studied here which differed strikingly in hemolytic titer against the same cells. The sensitivity of erythrocytes to hemolytic antibody varies greatly as emphasized by the comparison between normal and PNH erythrocytes in the present studies, and by others in noting differences in the hemolysis of isologous and heterologous cells by D-L antibodies (22). The current observations clearly emphasize that the requirement for complement in both phases of the Donath-Landsteiner reaction is quantitative and not qualitative, depending upon the level of antibody and sensitivity of erythrocytes. Under any circumstances the presence of complement during the cold phase resulted in markedly increased hemolysis. Although this effect of complement is especially marked in the Donath-Landsteiner reaction, it is not unlike earlier observations by Maurer and Talmage, who offered evidence indicating increased antigen-antibody interaction in the presence of non-immune serum (23). The antibody level and cell sensitivity also influenced the critical temperature and rates of reaction.

The selection of kinetic conditions in the two phases of the reaction also influences profoundly the course of the reaction, and there is little evidence in many reports that the kinetic characteristics were taken into consideration.

For example, striking differences in the cold phase were noted in the present study between temperatures of 1° and 5°C., and as recorded here, the relative amounts of antibody and complement are also critical factors. Further, in many studies the source of antibody was heated serum which may have an inhibitory effect on the initial stage of the reaction, as noted here when heated normal serum was added to the reaction mixture.

It is apparent from a review of reports of the various D-L hemolysins which have been studied that there are other serologic properties than those considered here which differ from patient to patient. In some cases of luetic cold hemoglobinuria, there have been moderately high titers of cold agglutinins (3, 24, 25), although none have been as high as reported with cold hemoglobinuria due to cold agglutinins. In most patients the direct antiglobulin test is positive only immediately after chilling, but in a few the direct antiglobulin test has been consistently positive even in the absence of chilling (26). There is no reason to doubt that these patients had cold hemolysins of the D-L type. Some antibodies have been reported to be heat labile (6, 18, 19), whereas most others have been heat stable, variations which may have been due to inhibitory properties acquired by serum during heating or possibly to small but critical losses of antibody during heating of low potency sera. These varied serologic characteristics are similar to the apparent variable nature of the serum protein abnormalities which occur in syphilis and to the great variation and lack of consistency of serologic tests for syphilis.

It will be noted that in neither of the patients reported here was there a high titer of cold agglutinins, consistent with the conclusion that both antibodies were D-L biphasic cold hemolysins. Agglutination following the cold phase was slight and the cells were easily resuspended, in contrast to the marked agglutinating properties of hemolytic amounts of isoantibodies or to cold agglutinins. It is further noted that hemolysin activity was present in a fraction which contained little or none of the Kline antibody, corroborating observations made many times previously (8, 27) that the D-L antibody is distinct from the antibody responsible for the serologic tests for syphilis.

Other observations reported here may also contribute to an understanding of the discrepant observations reported previously regarding the maximum temperature at which the cold phase of the reaction will proceed. Although temperatures under 10°C. were required using normal cells, in confirmation of the observations of Mackenzie (20) and Dacie, erythrocyte sensitization occurred at higher temperatures when a more sensitive system was used, *i.e.*, PNH erythrocytes and the high titer non-luetic antiserum II. This is similar to the observation of Grafe in 1911 (28).

The relatively low temperature at which hemolysis occurs after completion of the cold phase also may explain the previously observed monophasic hemolysis which has been observed to occur at 17°C., a temperature low enough to

result in reaction of antibody with cell and high enough to result in lysis by complement (9, 29).

One objective of the present study was to establish conditions suitable for the study of the mechanism of action of human complement on human erythrocytes, particularly related to a naturally occurring human disease. As pointed out, it was possible to adjust the concentration of the various reactants so that fresh serum was required in both the initial cold and final warm phases of the reaction. It may be emphasized that despite all the variables which may influence this biphasic system, it is similar to other immune hemolytic systems in requiring antibody, all four components of complement, and Ca^{++} and Mg^{++} for activity. The accompanying report offers evidence that C'1 participates in the cold phase of the reaction and is thus responsible for the initiation of hemolytic complement activity as has been proposed previously (1, 30).

SUMMARY

The Donath-Landsteiner reaction was studied using low and high titer antisera and purified antibody, normal and PNH erythrocytes, and human serum complement.

The requirement for complement in both the cold and warm phases of the reaction depended upon the level of antibody used and the sensitivity of the cells to hemolytic antibodies. Complement was not necessary in the cold phase using PNH cells and a potent source of antibody, but complement was required to be present at some stage if hemolysis were to occur.

Optimal conditions for the cold phase were at 1°C. for 30 minutes at pH 7.4. Ca^{++} ions were required. Hemolysis in the warm phase occurred within one minute, was optimal at 32°C., and required Mg^{++} .

The relation of these observations to previous reports is discussed with respect to discrepant observations on the nature of the Donath-Landsteiner reaction.

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