STUDIES ON IMMUNE HUMAN HEMOLYSIS

II. The Donath-Landsteiner Reaction as a Model System for Studying the Mechanism of Action of Complement and the Role of C'1 and C'1 Esterase*

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INTRODUCTION

The role of serum complement in immune hemolytic reactions has been recognized for over sixty years (1). The manner of action and sequence of action of the several components of complement have been studied in a variety of systems involving human or guinea pig complement, sheep erythrocytes, and rabbit anti-sheep erythrocyte antiserum (2, 3). However, few studies have been done on hemolytic reactions involving human antibody, erythrocytes, and complement, or on immune hemolytic systems occurring in human diseases.

Studies on the role of human complement in human disease states and in the hemolysis of human cells have been hampered by lack of a convenient model system. The Donath-Landsteiner (D-L) reaction is suitable for this purpose, as previously indicated (4), because the antibody is an active hemolysin but a poor agglutinin and because the biphasic nature of the hemolytic reaction is readily susceptible to study of early and late phases of complement action separately.

Recent studies have indicated that C'1, the first component of complement, initiates the action of human complement in complement fixation by antigen-antibody aggregates (5); that C'1 is associated with esterase activity capable of hydrolyzing certain synthetic amino acid esters (6–8); and that there is in serum a naturally occurring inhibitor of the esterase (9). The current studies are an application to immune human hemolysis of the observations made on complement fixation. They indicate that the initial cold phase of the D-L

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reaction can be completed using serum, partially purified C'1, or C'1 esterase, the enzymatically active material derived from C'1. Further, the action of C'1 esterase can be blocked upon the addition of the inhibitor purified from normal serum.

MATERIAL AND METHODS

Material and methods were similar to those described in the preceding paper (4). In addition, the following are pertinent.

Antiserum.—An additional antiserum (complement-depleted antiserum III) was kindly supplied by Drs. W. S. Jordan, Jr. and J. H. Dingle from their patient II (10). The native serum had been stored at −20°C. since 1950. Purified antibody from case I was diluted in barbital buffer so that it was concentrated 2.5 times with respect to original serum; during preparation, complement-depleted antisera from cases I, II, and III were diluted 1/1.5 with respect to original serum and were thereafter diluted appropriately in barbital buffer as noted in individual protocols.

Fresh Normal Human Serum.—Human serum was obtained as described in the previous paper (4).

Fresh Guinea Pig Serum.—Fresh guinea pig serum was obtained by cardiac puncture, separated from clotted blood, and stored at −65°C. Several large pools of guinea pig serum which were obtained through the generosity of Dr. E. R. Arquilla had been adsorbed with sheep cell stroma and were free of hemolytic antibodies for human red cells.

Preparation of Complement Reagents.—R1, lacking C'1, the soluble portion of serum following dialysis against pH 5.4 acetate buffer, ionic strength 0.02, at 1°C. for 36 to 48 hours, was corrected to pH 7.4 ± 0.4 with 0.5 M NaOH, and adjusted to ionic strength 0.15 with 1.0 M NaCl in a final dilution of 1/1.5 with respect to original serum (11, 12). Cations were added to a concentration of 1.5×10^{-4} M Ca^{++} and 5×10^{-4} M Mg^{++}. The R1 was stored at −65°C.

R2, lacking C'2, the insoluble portion of serum following dialysis against the above acetate buffer, was washed twice with the acetate buffer at 2°C., corrected to pH 7.4 ± 0.4 with 0.5 M NaOH, adjusted to ionic strength 0.3 with 1.0 M NaCl, and stored frozen at −65°C. at a final volume of 0.75/1 with respect to original serum volume. At the time of use it was diluted with an equal volume of distilled water so that the final ionic strength was 0.15 and the final dilution was 1/1.5 (11, 12). Cations were added to a concentration of Ca^{++}, 1.5×10^{-4} M, and Mg^{++}, 5×10^{-4} M. The undiluted frozen material retained potency for at least 4 weeks.

R3, lacking C'3, was prepared from human serum by incubating selected normal sera with 2 mg./ml. of zymosan (Fleischmann No. 7B152) at 37°C. for 60 minutes, followed by centrifugation (3000 *G* for 30 minutes) at 1°C. to remove the particulate zymosan (13). In guinea pig serum, 2 mg. zymosan/ml. failed to inactivate C'3, and 10 to 20 mg. caused inactivation of other components of complement. Therefore, R3 was made from guinea pig serum by first preparing a properdin-zymosan (PZ) complex (14), by incubating 1 ml. of *human* serum with 2 mg. of zymosan for 60 minutes at 17°C., washing the complex twice at 0°C. with barbital buffer to remove occluded serum, and incubating the washed PZ complex with 1 ml. guinea pig serum at 37°C. for 1 hour.

R4, lacking C'4, was prepared from human and guinea pig serum by incubating 1 ml. of serum with 0.25 volume of 0.15 M NH_3 at 37°C. for 60 minutes and thereafter neutralizing with 0.25 volume of 0.15 M HCl (11).

Human serum heated at 51°C. for 30 minutes lacked C'2 but contained C'1, C'3, and C'4. *Human serum heated at 56°C. for 30 minutes* contained significant amounts only of C'4.

Criteria of Suitability of Reagents.—Titrations of the components of complement were

performed by the double dilution technique using a 50 per cent hemolytic end-point with amboceptor sensitized sheep cells (5, 12). Total complement was measured according to a 100 per cent hemolytic end-point, 1 unit being the least amount of serum capable of causing complete hemolysis of a standard number of sheep cells. "Complement-equivalent" units in serum reagents deficient in one or more components of complement were based on the total complement activity of the serum from which the reagents were prepared.

Human serum reagents were considered satisfactory if they caused no hemolysis of sensitized sheep cells when used at the 4 unit level, but did cause complete or nearly complete hemolysis when used in combination with other of the inactivated reagents at the 1 unit level, and if they did not have anticomplementary properties. Routinely only R1 + R2 and R3 + R4 recombinations were tested.

Human R1, R3, and R4 could be prepared which met the above criteria, but human R2 prepared in the usual manner often contained a small amount of C'2 (less then 5 to 10 units of an original 480 units) when measured by the dilution titration technique. As noted above, serum heated at 51°C. for 30 minutes lacked C'2, but contained the other components.

Satisfactory reagents from guinea pig serum could not be prepared in this fashion. Some preparations of guinea pig R1 were slightly hemolytic and did not recombine completely with guinea pig R2. R3 made with zymosan or human PZ was also slightly lytic; R4 was non-lytic and recombined satisfactorily with other reagents. Thus guinea pig reagents prepared in the prescribed fashion often failed to meet the requirements established for human reagents and results of this part of the study were therefore subject to these limitations in interpretation.

Partially purified C'1 was prepared from pooled human serum, as previously described (6), and stored at −20°C. at pH 5.5, ionic strength 0.30. Under these conditions there was no activation to the esterase form. Immediately prior to use, pH was adjusted to 7.4 ± 0.4 with 0.5 M NaOH and ionic strength to 0.15 by dilution with distilled water at 4°C.

C'1 esterase, the enzymatically active form of C'1, which has no detectable hemolytic C'1 activity but does possess esterase activity for certain synthetic amino acid esters, was prepared by incubation of partially purified C'1 at pH 7.4 and ionic strength 0.15 for 15 minutes at 37°C (6). Esterase activity of this preparation was determined as described previously (8, 15). One unit is that amount of esterase which liberates 0.5 micromole of titratable acid during incubation with *N*-acetyl-L-tyrosine ethyl ester for 15 minutes at 37°C. Approximately 1 unit of esterase activity is generated from 100 to 200 units of partially purified C'1 and corresponds to approximately 10 to 30 micrograms nitrogen.

Dilutions of either C'1 or C'1 esterase were made in barbital buffer and used in the cold phase of the reaction in place of whole human serum.

Esterase inhibitor (EI) was prepared from human serum by column chromatography as previously described (9, 16), and stored at −20°C. One unit of inhibitor is defined as that amount which will inhibit the esterolytic activity of 10 units of C'1 esterase under standard conditions (15) and corresponds to approximately 5 to 15 micrograms of nitrogen. Dilutions were made in barbital buffer. For studies in the cold phase, esterase inhibitor was either preincubated with C'1 esterase or serum for 15 minutes at 1–4°C., or was introduced into the cold phase of the hemolytic reaction with C'1 esterase, partially purified C'1, or serum just prior to addition of Donath-Landsteiner antibody and erythrocytes. The total volume of the reaction mixture was unchanged. To study the effect of inhibitor on cells after the cold phase, inhibitor was incubated with the erythrocytes at temperatures ranging from 1° to 42°C., usually in a total volume of 0.2 ml. of barbital buffer. Thereafter the cells were washed twice in barbital buffer at the same temperature and incubated in R1 at 37°C.

The *Donath-Landsteiner* reaction was performed as described in the previous paper with minor modifications (4). The initial reaction between cells, antibody, and serum or serum reagents in a volume of 0.30 ml. at 1°C. for 30 minutes is referred to as the *cold phase*. There-

after the cells were washed twice in barbital buffer at 2°C., and incubated in serum or serum reagents in a volume of 0.3 ml. at 37°C. for 30 minutes, referred to as the *warm phase.* In all experiments the indicator reaction was hemolysis at 37°C. On some occasions an additional reaction with esterase inhibitor was interposed between the cold and warm phases as described above. In most experiments, serum and serum reagents were diluted 1/1.5 with respect to original serum, and therefore 0.15 ml. was used instead of the 0.1 previously described. When a combination of reagents was used, 0.15 ml. of each was used in the reaction mixture.

The level of antibody and type of erythrocyte (PNH or normal) were selected so that hemolysis occurred only when fresh serum was present in both the cold phase and the warm phase of the reaction. In each instance the only source of any component of complement was the fresh serum or serum reagent, for none of the components of complement was present in measurable quantity in the purified antibody fraction or in the complement-depleted antisera. Based on the studies reported in the companion paper, it was possible to make observations on the following combinations of antibody and erythrocytes: complement-depleted antiserum I and normal red cells; complement-depleted antiserum II and normal cells; purified antibody I and PNH cells. Details of individual experiments are described separately below.

Similar to observations reported in the previous paper, these three combinations of erythrocytes and antibodies exhibited different degrees of sensitivity with respect to amounts of complement required for hemolysis. In the most sensitive reaction using PNH erythrocytes and purified antibody from case I there was at least a 5-fold excess of complement in both the cold and the warm phases. In the least sensitive reaction involving normal cells and complement-depleted antiserum I, the amount of complement was limiting, and dilution of serum, especially in the warm phase, resulted in decreased hemolysis. The reaction involving normal cells and the more potent complement-depleted antiserum II was intermediate in sensitivity.

These differences may account for some of the minor inconsistencies noted in the following experiments, particularly in experiments involving recombination of reagents deficient in one or more components of complement.

RESULTS

I. Requirement for the Components of Human Complement

The requirement for the individual components of complement in the total reaction was determined by using the various serum reagents lacking one component of complement in both the cold and warm phases; requirement for specific components in the cold phase was tested using various serum reagents in the cold phase and whole serum in the warm; and similarly, component requirements in the warm phase were tested using serum in the cold and various reagents in the warm.

Experimental.—In sequence the following were mixed and incubated at 1°C. for 30 minutes:

(*a*) 0.05 ml. of 25 per cent suspension of normal or PNH erythrocytes;
(*b*) 0.15 ml. of serum or serum reagent diluted 1/1.5;
(*c*) 0.05 ml. of purified antibody I, or 0.1 ml. undiluted complement-depleted antiserum I, or 0.1 ml. of complement-depleted antiserum II, diluted 1/4;
(*d*) barbital buffer to adjust the final volume to 0.30 ml.

Following the cold phase, the cells were washed twice in 3 ml. barbital buffer at 2°C.; 0.15 ml. of serum or serum reagent was added; the volume was corrected to 0.3 ml. with barbital buffer; and the mixture was incubated at 37°C. for 30 minutes. Following centrifugation,

hemolysis was scored visually from 0 to +++. Under these experimental conditions a score of +++ corresponded to approximately 50 per cent hemolysis.

A. Total Reaction.—There was a requirement for all four components of complement at some time during the hemolytic reaction (Table I). When incubation took place in a reagent in which any component of complement was lacking in both the cold and warm phases, no hemolysis occurred in any of the three reaction systems tested. An apparent discrepancy, hemolysis in

TABLE I

Requirement for the Components of Complement in both Phases of the Donath-Landsteiner Reaction

	Hemolysis in various reaction systems		
Erythrocytes Antibody	PNH* Purified antibody I	Normal Antiserum II	Normal Antiserum I
Serum reagent			
Whole serum	+++	+++	++
R1	0	0	0
R2	tr–++	0	0
51° serum	0	0	0
R3	0	0	0
R4	0	0	0
56° serum	0	0	0
R1 + R2	++	++	0
R3 + R4	++	+	0

The same reagents were used in both the cold and the warm phase of the reaction.

Hemolysis was scored visually from 0 to +++; + corresponded to approximately 5 to 10 per cent hemolysis; ++, 10 to 20 per cent hemolysis; and +++, 20 to 50 per cent hemolysis. Higher levels of hemolysis were not attainable under these experimental conditions.

* Paroxysmal nocturnal hemoglobinuria.

R2, was noted with the sensitive PNH erythrocytes and purified antibody I. This could be explained on the basis of the presence of small amounts of C'2 in the R2, sufficient to cause hemolysis of this, the most sensitive of the systems. When serum heated to 51°, lacking measurable C'2, was utilized, no hemolysis occurred.

Recombination of reagents resulted in restoration of hemolysis in two of the reaction systems, but failed to occur in the least sensitive reaction, that involving normal erythrocytes and the less potent complement-depleted antiserum I. The decrease in total complement activity in recombinations of serum reagents (17) was sufficient to account for the lack of hemolysis in this reaction system, which permitted little dilution of serum, particularly in the warm phase.

B. Cold Phase.—To test the requirement for specific components in the cold, various serum reagents were used in the cold phase and whole serum was used in the warm. Hemolysis failed to occur only when C'1 was absent in the cold (Table II). Thus, R1, or 56° serum, did not complete the cold phase successfully even though C'1 was present during the subsequent warm phase. Reagents lacking C'2, C'3, or C'4, but containing C'1 were fully active. Recombination of R1 with R2 resulted in a fully active system.

Therefore C'1 was the only component of complement required to complete the cold phase. If it was not present during the cold phase hemolysis failed to occur even when C'1 was present in the warm phase as whole serum.

TABLE II

Requirement for the Components of Complement in the Cold Phase of the Donath-Landsteiner Reaction

	Hemolyis in various reaction systems		
Erythrocyte Antibody	PNH Purified antibody I	Normal Antiseruum II	Normal Antiserum I
Serum reagent			
Whole serum	+++	+++	+++
R1	0	0	0
R2	+++	+++	+++
51° serum	+++	—	++
R3	+++	+++	+++
R4	+++	+++	+++
56° serum	tr	0	0
R1 + R2	+++	+++	+++
R3 + R4	+++	+++	+++

Various serum reagents were used in the cold phase. After the cells were washed, the reaction was completed in the warm phase in whole serum.

C. Warm Phase.—When whole serum was used in the cold phase and various serum reagents in the warm phase, evidence for a requirement for C'2, C'3, and C'4 in the warm phase was obtained (Table III). In all these reaction systems some hemolysis occurred in R1, when serum had provided C'1 during the prior cold phase, although this was much more marked with the sensitive PNH erythrocytes than with normal cells.

When C'2 was lacking in the warm phase, no hemolysis occurred. Again the apparent discrepancy with the more sensitive PNH erythrocytes was probably due to small amounts of C'2 which were present in R2 preparations, for 51° serum, lacking only C'2, was inactive.

R3 was inactive in all reactions, indicating that C'3 is essential in the warm phase.

A requirement for C'4 in the warm phase was apparent in both reactions involving normal cells. With the sensitive PNH erythrocytes, hemolysis occurred in R4 when serum had been used in the prior cold phase, indicating that some C'4 had interacted during the cold phase and suggesting that C'4 might participate in either the cold or warm phase. This was supported by the observation that when R4, containing C'1 but lacking C'4, was used in the initial cold phase, hemolysis failed to occur in R4 in the warm, but did occur in whole serum. Observations reported below indicate that C'1 and C'4 may

TABLE III

Requirement for the Components of Complement in the Warm Phase of the Donath-Landsteiner Reaction

	Hemolysis in various reaction systems		
Erythrocyte Antibody	PNH Purified antibody I	Normal Antiserum II	Normal Antiserum I
Serum reagent			
Whole serum	+++	+++	+++
R1	+++	tr	+
R2	++	0	0
51° serum	0	0	—
R3	0	0	0
R4	++	0	0
56° serum	0	—	—
R1 + R2	+++	++	tr
R3 + R4	+++	++	+

The reaction in the cold phase was performed with whole serum. The various serum reagents were then used in place of whole serum in the warm phase.

interact at low temperatures, whereas C'2 and C'3 react only at higher temperatures.

Recombination of reagents showed restoration of hemolytic activity relative to the sensitivity of the reaction system to complement, as previously noted.

D. Recombination of Specifically Inactivated Serum Reagents.—A more detailed study of the recombination of the variously inactivated reagents in both phases of the reaction using PNH erythrocytes and purified antibody I demonstrated reactivation on recombination, indicating that the lack of hemolytic activity of the various reagents was due to a deficiency of the various components and not to the development of inhibitory properties in the reagents (Table IV). Restoration on recombination was not regularly achieved using the less sensitive normal cells, probably related to (*a*) the partial loss of activity of other components during the preparation of specifically inactivated

reagents (11), and (*b*) to the greater requirement for complement, especially in the warm phase, in those reactions utilizing normal cells.

E. Studies on Guinea Pig Complement.—In studies on the Donath-Landsteiner reaction using guinea pig complement, Jordan, Dingle, and Pillemer reported in 1951 that C'4 was the only component of complement required in the cold phase, and C'2 the only component required in the warm phase (10). Because the current results with human complement were inconsistent with this report, studies were attempted utilizing guinea pig serum as the source of complement and antisera from patients I

TABLE IV

Recombination of Serum-Reagents in the Donath-Landsteiner Reaction

Reagent	Hemolysis
Whole serum	+++
R1	0
R2	tr
R3	0
R4	0
50° serum	0
R1 + R2	++
R1 + R3	++
R1 + R4	++
R2 + R3	++
R2 + R4	tr
R3 + R4	+
50° serum + R1	++
50° " + R3	++
50° " + R4	++

Reagents or combinations were used in both the cold and warm phases of the reaction utilizing PNH cells and purified antibody I.

and II as well as the antiserum used by Jordan, Dingle, and Pillemer. These studies were sharply limited by two factors: first, guinea pig serum contained hemolytic antibodies active against human cells, particularly aginst PNH erythrocytes which have extreme susceptibility to hemolytic antibdies; and, second, there was difficulty in preparing guinea pig reagents in which there was complete inactivation of specific components and yet adequate activity when they were recombined. The first problem was met by utilizing guinea pig serum which had been adsorbed with sheep cell stroma, resulting in removal of the antibodies for human erythrocytes with only moderate loss of complement activity.

Within the limits imposed by the second problem, that of preparing adequate reagents, the requirements for specific components with guinea pig serum were consistent with those made on human serum.

Experimental.—Procedures were similar to those used in studies of human serum, modified slightly to reproduce the conditions described by Jordan *et al.* (10). The guinea pig sera were free of agglutinins for human cells and contained approximately one-half the original complement.

Antiserum levels and cells used were: 0.05 ml. complement-depleted antiserum I and PNH cells; 0.10 ml. complement-depleted antiserum I and normal cells; 0.10 ml. complement-depleted antiserum II, diluted 1/8, and normal cells; and 0.10 ml. complement-depleted antiserum III and normal cells.

The hemolytic reaction was performed by mixing in sequence 0.25 ml. of a 5 per cent suspension of erythrocytes, 0.1 ml. of serum or serum reagents, and antibody, and incubating

TABLE V

Requirement for the Components of Guinea Pig Complement in the Donath-Landsteiner Reaction

Serum reagent	Reagent in cold and warm	Reagent cold serum warm	Serum cold reagent warm
Guinea pig serum (without antiserum)	0	—	—
Guinea pig serum	+++	+++	+++
" " R1	0	0	++
" " R2	0	++	0
" " R3	0	+++	0
" " R4	0	+++	++
" " 56° Serum	0	0	0
" " R1 + R2	tr	++	++
" " R3 + R4	++	+++	++

The data depicted were from an experiment utilizing PNH erythrocytes and complement-depleted antiserum I. When a reagent was used in one phase of the reaction, whole serum was used in the alternate phase.

the mixture at 1°C. for 30 minutes. Thereafter the cells were washed twice in 3 ml. barbital buffer at 2°C. and incubated at 37°C. for 30 minutes with 0.1 ml. of serum or serum reagent in a total volume of 0.3 ml. in barbital buffer.

The specific differences from the method used by Jordan, Dingle, and Pillemer were that guinea pig serum was fresh frozen instead of lyophilized, and had been adsorbed with human erythrocytes; and that the antisera were treated with zymosan and ammonia prior to heating to insure total inactivation of all components of complement.

All the reaction systems tested showed requirements similar to those reported above for human serum (Table V). First, none of the reagents deficient in any component of complement resulted in hemolysis when it was used in both phases of the reaction, indicating that all four components were required. Second, R1 or serum heated to 56°C. for 30 minutes failed to complete successfully the initial cold phase, whereas R2, R3, and R4 were active; thus, reagents lacking C'1 were inactive, whereas reagents deficient in or lacking other components were active. Third, following exposure to serum in the cold, R1 was

hemolytic in the warm phase; R2 and R3 were not; and R4 was hemolytic on some occasions, indicating that C'4 reacted in either the cold or warm phase.

Summary.—These data on human and guinea pig serum complement are interpreted as indicating that C'1 is a requirement for the cold phase, is the only component so required, and therefore initiates the hemolytic process; that C'4 may interact during the cold or warm phase; and that C'2 and C'3 are required and must interact during the warm reaction. These observations emphasize the requirement for all components of complement and clearly eliminate C'4 as the sole requirement in the cold phase and C'2 as the sole requirement in the warm phase (10).

II. Partially Purified C'1 and C'1 Esterase as a Source of Complement in the Cold Phase, and Inhibition of Hemolysis by Serum Inhibitor of C'1 Esterase

A. Partially Purified C'1 and C'1 Esterase.—A partially purified fraction of C'1 has been isolated from serum free of the other components of complement (6). At pH 7.4, ionic strength 0.15, and 37°C. this preparation rapidly loses hemolytic C'1 activity and acquires enzymatic properties capable of hydrolyzing certain synthetic amino acid esters and interacting with C'4 and C'2 in serum (6, 8). It is heat labile and its esterolytic and complement-interacting properties are blocked by an inhibitor present in fresh serum. If C'1 is the only component required in the cold phase of the Donath-Landsteiner reaction, as the previous observations indicate, then it should be possible to substitute purified C'1 for whole serum in the cold phase of the reaction. Further, if the esterase properties are responsible for the functional role of C'1, then the preparations with active esterase properties and no demonstrable C'1 activity should also complete the cold phase successfully. Experiments were designed to test these hypotheses.

Experimental.—The reaction mixtures for the cold phase were similar to those previously described, except that partially purified C'1 or C'1 esterase was substituted for whole human serum in some tubes in amounts indicated in Table VI. Mixtures contained 0.05 ml. 25 per cent PNH cells, 0.05 ml. purified antibody I, and 0.1 ml. of serum, C'1 or C'1 esterase as indicated, in a total volume of 0.3 ml. The warm phase was performed as previously described using 0.10 ml. of undiluted serum or 0.15 ml. of serum reagents.

Antibody, partially purified C'1, or C'1 esterase had no effect in the cold phase individually, but either the C'1 or the C'1 esterase in conjunction with antibody completed the cold phase successfully so that hemolysis occurred subsequently in the warm phase in either serum or R1 (Table VI). Various amounts of esterase were required to complete the cold phase depending upon the potency of the antibody and sensitivity of the cells. As little as 0.05 unit of C'1 esterase, corresponding to approximately 5 to 10 units of C'1 and 0.5 to 1.5 micrograms nitrogen, was required in the reaction mixture when PNH cells

and the potent antiserum II were used; as much as 4 units were required with normal cells and the less potent purified antibody I. Increasing amounts of esterase caused progressively greater amounts of hemolysis (Fig. 1).

When C'1 esterase was the source of complement in the cold phase, C'2, C'3, and C'4 were required for hemolysis in the warm, but C'1 was not. When the preparations of C'1 esterase were inactivated by heating at 56°C. for 30 minutes, they were no longer effective in completing the cold phase (Table VI).

TABLE VI

Partially Purified C'1 and C'1 Esterase in the Cold Phase of the Donath-Landsteiner Reaction

Antibody	Source of complement		Hemolysis
Purified antibody I	Cold phase	Warm phase	
0.05 ml.	—	Serum	0
—	Partially purified C'1 (equivalent to 0.9 unit C'1 esterase)	"	0
—	0.9 unit C'1 esterase	"	0
0.05 ml.	serum	"	+++
0.05 "	Partially purified C'1 (equivalent to 0.9 unit C'1 esterase)	"	+++
0.05 "	0.9 unit C'1 esterase	"	+++
0.05 "	0.9 " " "	R1	++
0.05 "	0.9 " " "	R2	+
0.05 "	0.9 " " "	51° serum	0
0.05 "	0.9 " " "	R3	0
0.05 "	0.9 " " "	R4	0
0.05 "	0.9 unit C'1 esterase heated to 56°C.	Serum	0

Serum = fresh human serum.

The kinetic characteristics of the cold phase of the reaction were the same whether serum or C'1 esterase was used as the source of complement (Table VII). In both instances, the reaction proceeded best at 1°C. and was nearly complete in 30 minutes. It had an apparent pH optimum between pH 7 and 8, and was inhibited by increases in ionic strength. Similarly, the cold phase of the reaction was blocked by addition of 3×10^{-3} M Na_3EDTA or $Na_2MgEDTA$ to the system, but it was not blocked by $Na_2CaEDTA$, indicating that Ca^{++} was required. The rates of hemolysis in the warm phase were similar whether whole serum or C'1 esterase was used in the cold (Fig. 2).

B. Other sources of Esterase Activity.—Eluates of antigen-antibody aggregates previously "activated" by exposure to human serum have also been observed to have properties similar to C'1 esterase (7). They are capable of hydrolyzing the same synthetic amino acid esters and interacting with C'4, are heat labile, and blocked by the

serum inhibitor of C'1 esterase. Preparations of this "active eluate" were also effective in completing the cold phase of the Donath-Landsteiner reaction in place of serum or C'1 esterase. Although detailed studies were not performed, in the D-L reaction the preparations of "active eluate" had less than one-half the activity of C'1 esterase

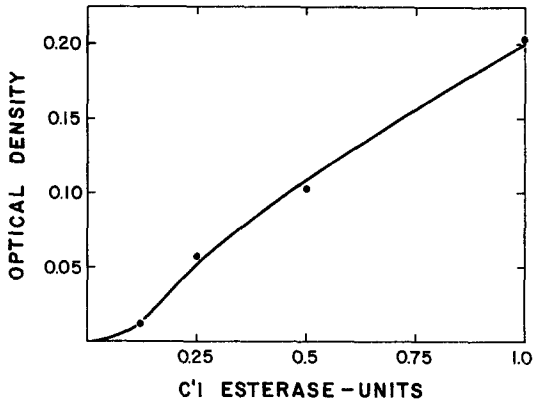

FIG. 1. Quantitative effect of C'1 esterase in the cold phase of the Donath-Landsteiner reaction. The reaction mixture contained PNH erythrocytes, 0.05 ml. purified antibody I, and C'1 esterase, in a volume of 0.3 ml. Warm phase contained 0.3 ml. R1.

TABLE VII

Comparison of Kinetic Characteristics of Cold Phase using C'1 Esterase and Whole Serum

Conditions	Source of complement in cold	
	C'1-esterase	Serum
Temperature	1°C.	1°C.
Time	30 min.	30 min.
pH	7–8	6.5–8
Ionic strength	Inhibited $\geqq$ 0.30	Inhibited $\geqq$ 0.40
Cation requirement	Ca^{++}	Ca^{++}

For the studies using C'1 esterase, PNH erythrocytes, 0.05 ml. of purified antibody I, and 0.5 unit C'1 esterase were used in the cold phase, and 0.3 ml. of R1 in the warm phase. For studies with serum in the cold, PNH erythrocytes, 0.05 ml. of purified antibody I, and 0.1 ml. serum were used in the cold phase, and 0.1 ml. undiluted serum in a total volume of 0.3 ml. in the warm phase.

preparations relative to their esterolytic activity as determined by the standard assay (15).

The residual material from ethanol Fraction II + III of antiserum I, after separation of the gamma-2 globulin fraction which was used as purified antibody I, was not only observed to contain some residual antibody activity, as reported by Deutsch and coworkers (19), but also contained 25 units per ml. of esterase activity at original

serum concentration. It was therefore of interest to determine whether this fraction of serum containing both antibody and C'1 esterase was capable of completing the cold phase of the D-L reaction in the absence of other sources of complement or antibody.

At the level of 0.05 ml. the fraction was active alone in completing the cold phase of the D-L reaction. It was inactivated by heating at 56°C. for 30 minutes, a procedure which inactivates esterase properties but not antibody. The activity of the heated fraction could accordingly be restored by addition of fresh normal serum. The activity of the unheated fraction was also blocked by an inhibitor of C'1 esterase derived from fresh human serum (see below).

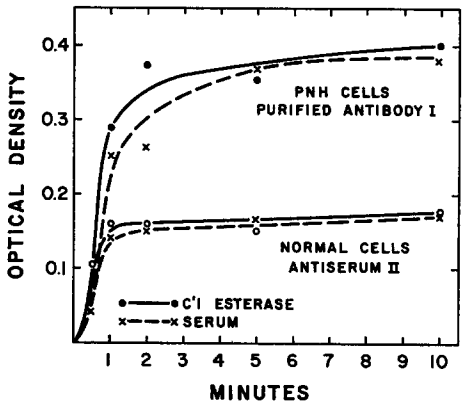

FIG. 2. Relative rates of hemolysis in the warm phase using C'1 esterase or serum in the cold phase. Reaction mixtures included normal erythrocytes with 0.1 ml. of complement-depleted antiserum II, or PNH cells and 0.05 ml. purified antibody I. Source of complement in cold phase was 0.1 ml. of serum or 1.0 unit of C'1 esterase. In the warm phase 0.1 ml. of serum was used in a total volume of 0.3 ml.

C. Inhibition of the Donath-Landsteiner Reaction by Partially Purified Serum Inhibitor of C'1 Esterase.—The foregoing data strongly suggested that C'1 esterase was responsible for the first stage of the hemolytic action of human complement in the Donath-Landsteiner reaction. Additional supporting evidence was obtained from studies on inhibition of the hemolytic reaction. Recently Pensky and Levy (9) have obtained in partially purified form an inhibitor of C'1 esterase previously demonstrated to be present in normal human serum. The inhibitory property is completely inactivated at 60°C. for 30 minutes and is contained in an alpha-2 globulin fraction of serum which has been purified 100- to 200-fold. It blocks stoichiometrically the esterolytic and complement-interacting properties of C'1 esterase (15). One unit of esterase inhibitor (EI) is designated as that amount capable of inactivating 10 units of C'1 esterase under standard conditions. This material is also capable of blocking the Donath-Landsteiner reaction.

1. Cold phase:

Experimental.—Just prior to performance of the cold phase, esterase inhibitor was mixed with equivalent amounts of C'1 esterase, partially purified C'1, or human serum at 4°C. in an ice bath; 0.05 ml. PNH erythrocytes, 0.05 ml. purified antibody I, and barbital buffer were then added to a total volume of 0.30 ml. and the mixtures incubated for 30 minutes at 1°C. The cells were washed twice in 3 ml. of barbital buffer at 2°C. and incubated at 37°C. with 0.3 ml. R1.

In the absence of inhibitor, equivalent amounts of C'1 esterase, partially purified C'1, or whole serum caused equivalent hemolysis (Table VIII). Addition of twice the stoichiometric concentration of esterase inhibitor to C'1 esterase just prior to the cold phase prevented hemolysis, but the same amount of inhibitor had no effect on the reaction when serum or partially purified C'1 was the source of complement in the cold phase.

TABLE VIII

Effect of Esterase Inhibitor on the Cold Phase of Donath-Landsteiner Reaction

Reagent in cold phase	Hemolysis	
	No inhibitor	With inhibitor (0.18 unit)
0.9 unit C'1 esterase	+++	tr
Partially purified C'1 (equivalent to 0.9 unit C'1 esterase)	+++	+++
Serum (containing 90 to 120 units C'1)	+++	+++

To avoid the possibility that the lack of effect of inhibitor on whole serum in the cold phase was due to excess of serum a constant amount of inhibitor was used with serum serially diluted to a point where it no longer had hemolytic activity. At no dilution of serum did inhibitor affect the cold phase activity of the serum. The quantitative effect of inhibitor on C'1 esterase was similar to that demonstrated in the esterase assay, approximately 0.10 unit of inhibitor blocking 1.0 unit of esterase (Fig. 3).

Prior incubation of cells with inhibitor had no effect if the cells were washed before introduction into the cold phase.

The data were interpreted to indicate that the inhibitor blocked the participation of the free enzyme in the cold phase, but was without effect when activation of C'1 to the enzymatic form evolved from serum or partially purified C'1 at the erythrocyte surface. This is consistent with observations described above indicating that the cold phase is completed successfully when whole serum, R3, or R4, all of which contain inhibitor, is used in the cold phase. The data offer further evidence that enzymatic activity is involved in the hemolytic effect of C'1.

2. Effect of inhibitor on cells after completion of cold phase:

The esterase inhibitor also blocked the hemolytic reaction when it was incubated with cells after they had been "activated" by exposure to antibody and a source of C'1 in the cold phase.

Experimental.—After completion of the usual cold phase (utilizing PNH erythrocytes, 0.05 ml. of purified antibody I, and 1.0 unit C'1 esterase, or equivalent amounts of serum or partially purified C'1), the cell complexes, referred to as "activated" cells, were washed twice in 3 ml. barbital buffer at 2°C. These were incubated with various amounts of esterase inhibitor, usually 0.1 unit, under various conditions of time, temperature, pH, and ionic strength, in a total volume of 0.2 ml. The cells were again washed twice in 3 ml. barbital buffer at room temperature, and finally incubated in 0.3 ml. R1 at 37°C. for 30 minutes. In each experiment, cells labeled "control" in the figures were incubated in barbital buffer in place of inhibitor under similar conditions. Adjustments of temperature, pH, and ionic strength were performed as previously described.

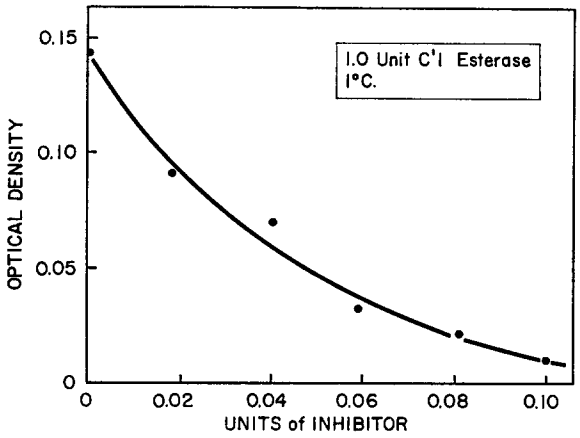

FIG. 3. Quantitative effect of esterase inhibitor on C'1 esterase in the cold phase of the Donath-Landsteiner reaction.

The effect of esterase inhibitor on "activated" cells under these conditions was time, temperature and concentration dependent. When cells which had been exposed in the cold phase to 1.0 unit of esterase were subsequently exposed to 0.10 unit of inhibitor, there was no effect at 1°C. in 30 minutes and only slight effect after 20 hours. At higher temperatures the inhibitor rendered cells progressively less susceptible to hemolysis, complete inactivation taking place in 60 minutes at 32°C. (Figs. 4, 5). A larger amount of inhibitor resulted in more rapid inhibitory effect (Fig. 6). Under similar conditions cells incubated with buffer in place of inhibitor underwent little or no decrease in sensitivity to subsequent hemolysis in R1. No conclusions can be drawn regarding stoichiometric relationships, because there are no data on the quantity of C'1 esterase which actually reacted with erythrocytes during the cold phase.

This effect of inhibitor on "activated" cells was the same whether C'1 esterase, partially purified C'1, or serum was used in the cold phase, in contrast to the observations in the cold phase, in which only C'1 esterase was affected.

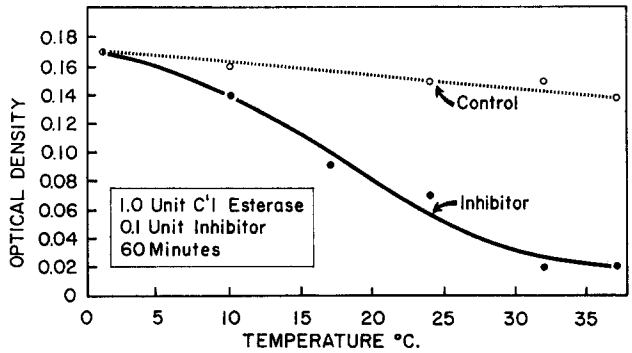

FIG. 4. Interaction of "activated" cells with esterase inhibitor in the Donath-Landsteiner reaction: effect of temperature.

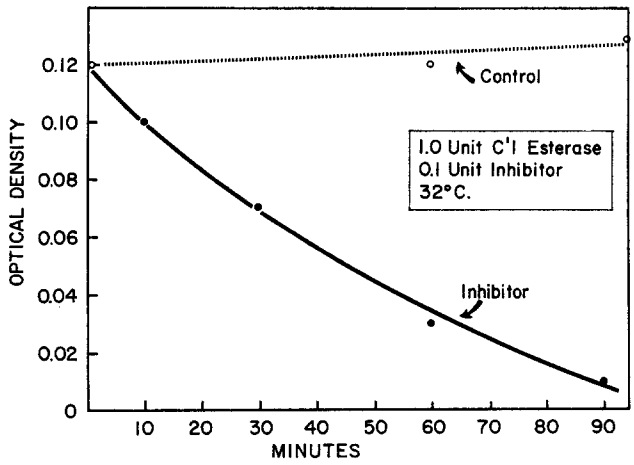

FIG. 5. Interaction of "activated" cells with esterase inhibitor in the Donath-Landsteiner reaction: effect of time of incubation.

D. Elution of Esterase Activity from Erythrocytes.—Becker has demonstrated that C'1 esterase activity can be eluted from sensitized sheep cells after they have been exposed to guinea pig serum at 0°C. (20). Similar observations have been made in the Donath-Landsteiner system by increasing the amount of antibody, and using an excess of whole human serum during the initial cold phase.

Experimental.—Replicate samples of 0.05 ml. of a 25 per cent suspension of PNH erythrocytes, 0.10 ml. of human serum, and 0.20 ml. of purified antibody I were incubated at 1°C. for 30 minutes and the cells washed twice with barbital buffer. To each tube 0.2 ml. of 5×10^{-3} M Na_3EDTA in barbital buffer without divalent cations was added and the cells incubated at 1°C. for 15 minutes. After centrifugation the supernatants from all tubes were pooled, and $CaCl_2$ was added to a final concentration of 5×10^{-3} M. Similar reaction mixtures lacking either antibody or fresh serum were tested in identical fashion.

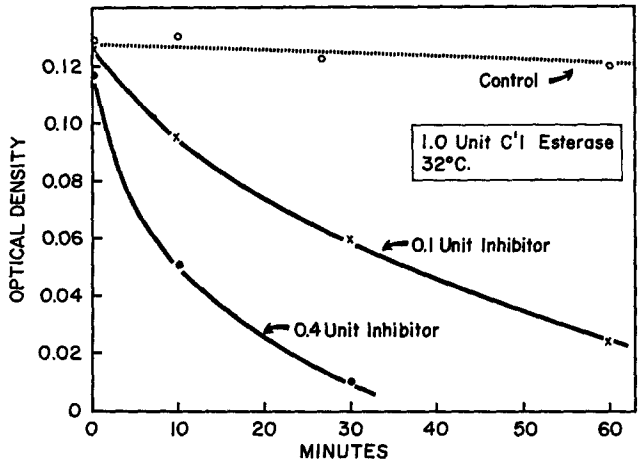

FIG. 6. Interaction of "activated" cells with esterase inhibitor in the Donath-Landsteiner reaction: effect of concentration of inhibitor.

The eluate was tested for C'1 esterase activity in the standard assay (15). It was tested for activity in the cold phase of the D-L reaction in the usual manner, utilizing PNH erythrocytes and purified antibody I. It was also tested for antibody activity in the D-L reaction by using it in the cold phase in conjunction with PNH erythrocytes and fresh serum. The ability to inactivate C'4 was determined by incubating 0.05 ml. of eluate with 0.05 ml. of heated serum (56°C., 30 minutes) or R1 for 30 minutes at 1°C., and then measuring the C'4 activity of the resulting mixture with R4.

The eluate from "activated" cells contained (Table IX): (*a*) esterase activity capable of hydrolyzing acetyl-L-tyrosine ethyl ester in the usual assay for esterase; (*b*) activity which could be substituted for C'1 esterase in the cold phase of the Donath-Landsteiner reaction, but no antibody activity; and (*c*) activity capable of inactivating C'4 in heated serum or R1. Thus, the eluate from "activated" erythrocytes had similar properties to C'1 esterase. The eluate from cells incubated in the initial cold phase with serum alone or antibody alone did not possess these activities.

It is to be emphasized that the amount of activity recovered was small relative to the amount of potential C'1 esterase activity present in the volume of serum initially used. Particularly in the esterase assay, the amounts of activity

detected were near the lower limits of sensitivity of the assay procedure. Nonetheless, activity by all three parameters was detectable in the eluate from "activated" cells and was not detectable in the controls.

Summary.—Partially purified C'1 or the activated enzyme, C'1 esterase, successfully completed the cold phase of the hemolytic reaction in the absence of the other components of complement. Serum inhibitor of C'1 esterase blocked the effect of C'1 esterase in the cold phase of the reaction, and diminished hemolysis when exposed to "activated" cells after the completion of the cold phase. Esterase activity was eluted from erythrocytes following the cold phase when whole serum was the source of complement in the cold.

TABLE IX

Properties Eluted from Erythrocytes after the Initial Cold Phase

Reactants in cold phase	Activity in eluate		
	Esterase	C'4-inactivating property	C'1-activity for PCH hemolysis
	units/ml.		
Antibody-erythrocyte-normal serum	3.6	Present	Present
Antibody-erythrocyte- —	Not measurable	Absent	Absent
— -erythrocyte-normal serum	" "	"	"

III. The Sequential Interaction of the Components of Complement and Requirement for Divalent Cations in Separate Steps

Experiments were designed to determine the order in which the several components of complement interacted in the Donath-Landsteiner reaction.

Experimental.—Two to four successive reactions were performed as follows. The cells were washed twice with 3 ml. of barbital buffer at 2°C. between each step.

Reaction I. 0.05 ml. of a 25 per cent suspension of PNH cells, 0.08 ml. of purified antibody I, and 1.5 units of C'1 esterase, in a total volume of 0.3 ml. were incubated at 1°C. for 30 minutes in the usual cold phase. After washing, the cells were incubated in the intermediate reactions, or were incubated directly in reaction IV in R1 at 37°C.

Reaction II. Cells from reaction I were incubated with 0.1 ml. of heated serum (56°C., 30 minutes) and 0.1 ml. barbital buffer at 1°C. for 30 minutes. C'4 was the only measurable component of complement in the heated serum.

Reaction III. After washing, cells from reaction II were incubated at 25°C. for 30 minutes with 0.1 ml. R3 or equivalent amounts of other serum reagents, as noted in the tables, and 0.1 ml. barbital buffer.

Reaction IV. After washing, cells from one of the previous reactions were incubated in 0.1 ml. of serum, or the equivalent of R1 or other serum reagent at 37°C. for 30 minutes. R4, which had been heated to 51°C. for 30 minutes, contained C'3, but lacked C'4 and C'2.

When the various salts of EDTA were introduced, barbital buffer without added divalent cations was used as diluent. EDTA was made up in 0.15 M NaCl and used in a final concentration of 3×10^{-3} M. The volumes of reaction mixtures were unchanged.

Utilizing C'1 esterase in the initial step of the reaction, it was possible to interact the cells sequentially with the four components of complement to demonstrate that the interaction occurred in the order C'1, C'4, C'2, C'3 (Table X).

As previously noted, partially purified C'1 or C'1 esterase lacked the other components of complement. It interacted optimally at 1°C. in the presence of cells and antibody (reaction I), rendering those cells susceptible to lysis in R1 or serum (reaction IV). C'1 was the only component required during this initial stage at 1°C. The initial reaction required Ca^{++}, as indicated by the fact that it was blocked by 3×10^{-3} M Na_3EDTA and $Na_2MgEDTA$, but not by $Na_2CaEDTA$ (Table XI).

TABLE X

Sequential Interaction of Components of Complement in the Donath-Landsteiner Reaction

Reaction				Hemolysis
I	II	III	IV	
1°C.; 30 min	*1°C.; 30 min.*		*37°C.; 30 min.*	
C'1 esterase	—	—	Serum	+++
Buffer	—	—	Serum	0
C'1 esterase	—	—	R1	+++
" "	—	—	R4	0
" "	56° serum	—	Buffer	0
" "	56° "	—	R4	+++
56° serum	C'1esterase	—	R4	0
C'1-esterase	R4	—	R4	0
" "	56° serum	—	R3	0
" "	56° "	—	R4-51°	0
" "	Buffer	—	R4	0
" "	R4	—	51° serum	0
" "	56° serum	R3 (1°C.)	51° "	0
" "	56° "	R3 (25°C.)	51° "	++
" "	56° "	R3 (25° C.)	R4-51°	++
" "	56° "	51° (25°C.)	R3	0
" "	56° "	R3 (25°C.)	R3	0

A dashed line indicates that the reaction step was omitted.

R4–51° serum refers to R4 which was subsequently heated to 51° for 30 minutes. 51° serum and 56° serum were heated at the appropriate temperature for 30 minutes.

The temperature at which reaction III took place is indicated in parentheses.

Following exposure to C'1 or C'1 esterase the cells were incubated at 1°C. for 30 minutes in serum heated to 56°C. (reaction II, Table X). This serum contained considerable C'4 activity, no detectable C'3 activity, and no C'1 or C'2. Thereafter, the cells could be hemolyzed in R4 in reaction IV, indicating that C'4 had interacted during exposure to the 56°C. serum. The 56°C. serum could not be reacted successfully before C'1 esterase. When C'4 was lacking during reaction II, hemolysis failed to occur subsequently if C'4 were excluded from the subsequent steps. After reaction

with C'4, both C'2 and C'3 were required for hemolysis, since subsequent exposure to R3 or R4 heated to 51°C. failed to cause lysis. C'2 could not be interacted before C'4, as evidenced by the fact that hemolysis failed to occur if the cells were exposed to reagent lacking C'4 but containing C'2 (R4) and then to one lacking C'2 but con-

TABLE XI

The Effect of Various Salts of EDTA on the Several Stages of the Action of Human Complement

Reaction				Hemolysis
I	II	III	IV	
1°C.; 30 min.	*1°C.; 30 min.*	*25°C.; 30 min.*	*37°C.; 30 min.*	
C'1 esterase	—	—	R1	+++
C'1 esterase + Na_3EDTA	—	—	R1	0
C'1 esterase + $Na_2MgEDTA$	—	—	R1	0
C'1 esterase + $Na_2CaEDTA$	—	—	R1	+++
C'1 esterase	56° serum	R3	R4-51°	+++
" "	56° serum + Na_3EDTA	R3	R4-51°	+
" "	56° serum + $Na_2MgEDTA$	R3	R4-51°	+++
" "	56° serum + $Na_2CaEDTA$	R3	R4-51°	+++
" "	56° serum	R3 + Na_3EDTA	R4-51°	0
" "	56° "	R2 + $Na_2MgEDTA$	R4-51°	+++
" "	56° "	R2 + $Na_2CaEDTA$	R4-51°	+++
" "	56° "	R3	R4-51° + Na_3EDTA	+++
" "	56° "	R3	R4-51° + $Na_2MgEDTA$	+++
" "	56° "	R3	R4-51° + $Na_2CaEDTA$	+++

The various salts of EDTA were used at a concentration of 3×10^{-3} M.

taining C'3 and C'4 (51°C. serum). The presence of Na_3EDTA in the incubation with C'4 decreased hemolysis in the subsequent warm phase, but $Na_2MgEDTA$ and $Na_2CaEDTA$ were without effect in this step (Table XI), suggesting that the reaction was dependent on Mg^{++}. An absolute requirement for Mg^{++} could not be concluded with certainty, because it was not possible to exclude C'4 from subsequent steps of the reaction. (For unexplained reasons, R3 which had been treated with ammonia to inactivate C'4 could not be used as a source of C'2 in reaction III.)

Following interaction with C'1 and C'4, C'2 was interacted by exposure of cells (reaction III) to R3, a reagent containing C'2 but lacking C'3 (Table X). This reaction failed to occur at 1°C., but was completed at 25°C., consistent with Leon's observation (18) that human serum reacts with sheep cells to form EAC'_A cells at relatively high temperature. Intermediate temperatures were not tested. Na_3EDTA blocked this step, but $Na_2MgEDTA$ and $Na_2CaEDTA$ did not (Table XI), indicating a requirement for Mg^{++} during the interaction of C'2. The successful completion of this step was indicated by the fact that the cells treated in this manner could be subsequently hemolyzed by R4-51 C. serum which contains C'3 but lacks C'2 and C'4. If C'3 were present but C'2 lacking (51°C. serum) in this third step, hemolysis failed to occur in the subsequent reaction, further emphasizing the participation of C'2 in reaction III prior to reaction with C'3.

Finally, after completion of the first three reactions involving C'1, C'4 and C'2, the cells were hemolyzed in the presence of C'3 by incubating them with serum or a reagent containing C'3 but lacking C'4 and C'2 (R4 heated to 51°C.) (Table X). A reagent lacking C'3 (R3) failed to cause hemolysis. The final reaction was routinely performed at 37°C., and hemolysis occurred even in the presence of Na_3EDTA (Table XI), indicating that there was no divalent cation requirement in this step.

Summary.—Hemolysis occurred only when the cells were interacted consecutively with C'1 or C'1 esterase at 1°C., C'4 at 1°C. or higher temperature, C'2 at a higher temperature, and finally C'3 at higher temperature. Ca^{++} was required for the initial interaction with C'1 esterase, and Mg^{++} was required for the reaction with C'2. There was no divalent cation requirement for the interaction with C'3.

DISCUSSION

Evidence has been presented that the action of human complement in a naturally occurring human hemolytic reaction is initiated by C'1 and that the activity of C'1 may be related to its esterase properties. The data are these:

A. The inactivation of C'1 in serum prevents the completion of the initial cold phase of the Donath-Landsteiner reaction, and therefore prevents hemolysis.

B. The active enzyme, C'1 esterase, derived from several sources, can be substituted for serum as a source of complement in the cold phase.

C. The effect of C'1 esterase can be blocked by an inhibitor of the esterase properties.

D. C'1 esterase activity can be recovered from erythrocytes after they have been exposed to antibody and serum complement in the initial cold phase of the reaction.

These observations are consistent with the hypothesis that the action of human complement is initiated by activation of C'1 from its naturally occurring proenzyme to the active enzyme, C'1 esterase (5). They are strikingly similar to current observations on the role of complement in immune cytotoxicity,

studies in which the deleterious effects of complement on human amnion cells in the presence of specific antibody were initiated by C'1 esterase (17, 21). They are also similar to the data previously reported on the sequence of events in complement fixation by antigen-antibody aggregates (5).

A variety of observations in the present study lend support to the concept that the esterase properties of C'1 have functional significance in the initiation of the hemolytic action of complement. Both the esterase preparation made by serum fractionation and by elution from an antigen-antibody aggregate lacked directly demonstrable C'1 activity, as measured by the reconstitution of hemolytic activity with R1, but were capable of completing the cold phase of the Donath-Landsteiner reaction, and therefore had functional activity. A third source of C'1 esterase was observed in a crude serum fraction containing antibody, and this fraction by itself completed the cold phase. Further, an eluate made from erythrocytes after they were exposed to antibody and serum in the cold phase of the Donath-Landsteiner reaction contained esterase activity and was capable of substituting for serum in a subsequent Donath-Landsteiner reaction. Thus, the correlation between esterase activity and activity in the cold phase of the Donath-Landsteiner reaction was excellent. It was noted earlier, however, that the esterase preparation eluted from an antigen-antibody aggregate (pneumococcal S III—rabbit anti-S III) was less effective in the Donath-Landsteiner reaction per unit of esterase activity than the preparation prepared by serum fractionation. Although direct proof is lacking, it is possible that the sites responsible for combination with the erythrocyte-antibody complex may have been altered in the preparation which had been eluted from another antigen-antibody complex. Becker (20) has offered evidence that the sites responsible for esterase activity and combination may be different.

Inhibition of the cold phase of the D-L reaction by a partially purified preparation of a serum inhibitor of C'1 esterase provided an important line of evidence for the functional participation of C'1 esterase in immune human hemolysis. It is emphasized, however, that such inhibition could be achieved only with appropriately separated reactions and under selected experimental conditions. The serum inhibitor of C'1 esterase effectively blocked the cold phase of the D-L reaction only when C'1 esterase was used as the source of complement. The inhibitor had no effect on completion of the cold phase when partially purified C'1 or whole serum were used. It is noteworthy that whole serum contains large amounts of inhibitor (15).

Stated in another way, the inhibitor did not affect the cold phase when activation of C'1 to C'1 esterase occurred at the surface of the erythrocyte-antibody complex. This was in agreement with previous observations (5) that "activated aggregates" containing enzymatic activity could be prepared by interacting antigen-antibody complexes at 0°C. for 30 minutes with C'1 in whole serum which contains inhibitor. The explanation for the failure of inhibitor to block

the activity of C'1 esterase at 0°C. after the enzyme had reacted with the erythrocyte-antibody complex is unknown. It may be speculated that stereochemical effects may be involved. However, after successful completion of the cold phase, the inhibitor blocked the activity of activated erythrocytes as a function of temperature, time, and concentration of inhibitor. In view of effective inhibition of activated erythrocytes by inhibitor at 32–37°C. (Fig. 4), the question arises of why serum or R1, both of which contain inhibitor, can cause hemolysis during the warm phase. The explanation may lie in the relative rates of the inhibitory and hemolytic reactions.

Parallel studies with a variety of antigen-antibody aggregates activated in a similar fashion with C'1 or C'1 esterase indicate that the rate of interaction of such aggregates with C'4 is greater than the rate of inhibition by inhibitor. The observations on the effect of inhibitor in the D-L reaction were therefore similar to other studies on the role of inhibitor in complement fixation (22) and immune cytotoxicity (21).

The present data do not suggest the manner of action of C'1 in the early stages of hemolysis. It is possible that the esterase attacks a substrate in the cell, although no substrate has been demonstrated. It is more probable that C'1 esterase serves only as a point of contact or attachment for other components of complement. Becker has recently suggested that the latter interpretation is correct, for he has offered evidence that guinea pig C'1 activity can be removed almost completely from sensitized sheep cells after C'4 and C'2 have interacted, without interfering with the subsequent hemolytic reaction (23).

The use of partially purified preparations of C'1 and C'1 esterase has also permitted a more direct study of the sequence of action of the components of human complement in an all-human hemolytic reaction. Although Pillemer, Seifter, and Ecker offered evidence eighteen years ago that the action of guinea pig complement was initiated by C'1 and C'4 (2) and Mayer and his colleagues (3) have published a series of kinetic studies on hemolysis by guinea pig complement of sheep cells sensitized with rabbit antibody, few detailed studies have been done in an entirely human system. The studies of Mayer and his collaborators have shown that the sequence of action of the components of guinea pig complement is as follows: C'1 and C'4 react first in the presence of Ca^{++}, C'2 then reacts in a step requiring Mg^{++}, and C'3 (consisting of at least two components) reacts in the absence of divalent cations, following which the cells hemolyze spontaneously.

The current observations indicate that in the Donath-Landsteiner hemolytic reaction in which cells, antibody, and complement are of human origin, and which is responsible for a specific human disease, the sequence of events is similar. Thus, using C'1 esterase it was shown that C'1 reacts first in a step requiring Ca^{++} ions and is followed by C'4. Both can react at low temperatures.

Similar to the observations on guinea pig complement, C'2 then interacts in a step requiring Mg^{++}. However, C'2 fails to interact at 0°C. as guinea pig serum does, but requires higher temperatures. This is similar to the observation made by Leon on the lysis of sheep cells by human serum (18). Finally, after reaction with C'1, C'4, and C'2, human C'3 completes the Donath-Landsteiner reaction in the absence of divalent cations and results in hemolysis.

Therefore, these observations on human complement made with sequential exposure of cells to variously inactivated reagents confirm the kinetic studies on guinea pig complement regarding both the sequence of action and ion requirement, and indicate further similarities between guinea pig and human complement whether acting in a mixed system or one of entirely human origin. They clarify the sequence of action in the early steps of complement action, pointing out that C'1 clearly acts before C'4, in full agreement with Becker (23).

The current data regarding the requirement for the four components of complement for the D-L reaction are similar to those for other immune hemolytic reactions and reactions requiring complement. They differ from those reported by Jordan, Dingle, and Pillemer (10) using guinea pig serum as a source of complement, which indicated that C'4 was required for the cold phase, and C'2 for the warm phase, with no requirement for C'1 or C'3. Attempts to repeat their experiment, utilizing guinea pig serum as a source of complement and several antisera including the antibody preparation which they had used (which had been stored frozen for 10 years) and an identical experimental design, were unsuccessful. In each instance the observations were similar to those made in the present studies with human serum and indicated a requirement for C'1 in the cold phase and for the other three components in the warm. There was agreement that serum heated to 56°C., lacking C'1 but containing C'4, failed to complete the cold phase. Heated serum would have been expected to have been active in the cold phase if C'4 were the sole cold phase requirement as they proposed (10). The discrepancies between the two studies remain unexplained despite numerous discussions with Drs. Jordan and Dingle, and their assistance in designing experiments and in supplying antibody.

It is concluded that in the Donath-Landsteiner reaction, an immune hemolytic reaction responsible for a specific human disease, paroxysmal cold hemoglobinuria, hemolysis is mediated by specific antibody, all of the components of complement and Ca^{++} and Mg^{++}, and that an early event in the mechanism of hemolysis is activation of C'1 to C'1 esterase. This reaction provides a useful model for studying the mechanism of action of complement and of the enzymatic nature of immune reactions in human disease.

SUMMARY

1. The inactivation of C'1 blocks the completion of the cold phase of the Donath-Landsteiner reaction; inactivation of the other components of complement does not interfere with the cold phase of the reaction.

2. C'2, C'3, and C'4 are required for the completion of the hemolytic reaction. C'4 reacts in either the cold or warm phase, but C'2 and C'3 must react in the warm phase.

3. Partially purified C'1 or C'1 esterase can be substituted for whole serum in the cold phase, although neither reagent contains any of the other components of complement

4. Partially purified serum inhibitor of C'1 esterase blocks the effect of C'1 esterase in the cold phase, and reverses the effect of complement, C'1 or C'1 esterase when incubated with "activated" cells after the cold phase.

5. C'1 esterase activity can be eluted from "activated" erythrocytes with Na_3EDTA.

6. The components of human complement in this human hemolytic reaction act in the order C'1, C'4, C'2, C'3. Ca^{++} is required for the reaction with C'1, and Mg^{++} is required for the reaction with C'2.

7. Accordingly, a functional role of C'1 esterase in a human disease state is demonstrated, and a human model is established for the study of the mechanism of action of complement.

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