Journal of Clinical Investigation
Vol. 44, No. 1, 1965

pH Optima in Immune Hemolysis: A Comparison between Guinea Pig and Human Complement *

STANLEY YACHNIN † AND JANET M. RUTHENBERG

(From the Department of Medicine, University of Chicago, and the Argonne Cancer Research Hospital,‡ Chicago, Ill.)

Certain *in vitro* hemolytic systems involving human erythrocytes and human serum function more effectively if hemolysis is allowed to proceed in serum that has been slightly acidified, the optimal pH being approximately 6.5. Among such "pH-dependent" hemolytic systems are the hemolysis of normal erythrocytes by sera containing high titer cold agglutinins (1); the hemolysis by normal human serum of human erythrocytes whose membranes have been altered by a variety of proteolytic enzymes, chemicals, and viruses (2); the hemolysis of normal human red cells induced by the addition of poly I[1] to normal serum (3); and the hemolysis of red cells from patients afflicted with PNH (4). The diagnosis of PNH usually rests upon what has come to be known as the "acid hemolysis" test (4, 5).

The C′ system clearly plays a role in all these systems, since removal of any one of the four components of C′ from serum renders it incapable of participating in these reactions (1*a*, 2, 3, 6). On the other hand, it has been suggested that some other mechanism may be involved in red cell lysis, especially in the case of PNH hemolysis (7, 8, 1*b*). In the hemolytic system dependent on high titer cold agglutinins, Dacie has emphasized that lowering the pH may favor absorption of hemolysin and so contribute to increased hemolysis (1*c*). This, however, does not explain the enhancement of hemolysis by acidification in the PNH or poly I-induced hemolytic systems, where there is no evidence for the participation of red cell antibodies (amboceptor) (1*d*, 3).

Should the fact that the hemolytic systems already mentioned have a pH optimum at 6.5 suggest that their mechanism is in some way different from classical C′-dependent immune hemolysis and that factors other than C′ are involved? Boyd states that the pH optimum for C′ action is 6.3 to 7.8 (9). The first edition of Kabat and Mayer's text quotes earlier work indicating that C′ functions more effectively in EA hemolysis at pH 7.4 than at pH 6.9 (10). The second edition, however, states that between pH 7.15 and 8.52, C′ effectiveness increases with decreasing pH (11). All these data, however, are from studies using guinea pig serum, and it is difficult to find pH optimum data dealing with classical EA immune hemolysis where human serum is the source of C′. Both Dacie (1*e*) and Hinz, Picken, and Lepow (12) agree that the optimal pH for hemolysis of erythrocytes in the C′-dependent Donath-Landsteiner antibody hemolytic system is between 7 and 8. Recent studies on the pH optimum for C′-dependent bacteriolysis by human serum have shown that the system functions best at pH 8.4 (13). However, since lysozyme appears to participate in such bacteriolysis, the authors question whether this pH optimum can fairly be applied to human C′ action.

Our study was designed to obtain more definite data on the effect of pH on human C′ action as measured by classical amboceptor-coated sheep

---

* Submitted for publication July 27, 1964; accepted September 30, 1964.

Supported in part by the Helen and Joseph Regenstein Foundation.

† John and Mary R. Markle Scholar in Academic Medicine.

‡ Operated by the University of Chicago for the U. S. Atomic Energy Commission.

[1] The following abbreviations are used: C′, complement; C′H$_{50}$, 50% hemolytic unit of complement; C′1, C′2, C′3, C′4, the first, second, third, or fourth component of complement; C′1a, activated first component of complement; R3, serum lacking the third component of complement; E, sheep red cells; A, antibody or amboceptor; EA, sensitized sheep red cells; EAC′ . . ., sensitized sheep red cells bearing complement components as designated by numerical subscripts; E*, red cells irreversibly damaged by the action of complement; hu, human; g.p., guinea pig; PNH, paroxysmal nocturnal hemoglobinuria; poly I, polyinosinic acid.

red cell immune hemolysis. Parallel studies were carried out with guinea pig C′, and the results indicate that, in contrast to guinea pig C′, human C′ is substantially more effective at pH 6.5 than at pH 7.5. In addition we found that the enhancement of human C′-dependent immune hemolysis at pH 6.5 results primarily from a stimulation of C′3.

Methods

Standard buffer solutions. Stock (five times concentrated) barbital-buffered saline (BBS), pH 7.5, was prepared as described by Kabat and Mayer (11*a*). Before use it was diluted 1 : 5 with deionized distilled water, and Ca^{++} (1.5×10^{-4} M) and Mg^{++} (5×10^{-4} M) were added. Solutions of dilute BBS at various pH's were prepared by titrating the five times concentrated stock BBS to the desired pH with 1 N NaOH or HCl before dilution.

Buffer solution containing salts of EDTA. Na_3-HEDTA or Na_2MgEDTA[2] was dissolved in water, titrated to the desired pH, and adjusted to 0.15 M. Na_2MgEDTA-BBS contained 1.5×10^{-2} M Na_2Mg-EDTA plus the necessary amounts of Ca^{++} and Mg^{++}. Since this EDTA salt binds Ca^{++} but not Mg^{++}, serum diluted in Na_2MgEDTA-BBS will not support the hemolysis of EA, but will support EAC'_1 and $EAC'_{1,4}$ lysis. Na_3HEDTA-BBS contained 7.5×10^{-3} M Na_3-HEDTA; Ca^{++} and Mg^{++} were omitted. Since Na_3-HEDTA chelates both Mg^{++} and Ca^{++}, serum diluted in this reagent will not support EA, EAC'_1, or $EAC'_{1,4}$ lysis, but will hemolyze $EAC'_{1,4,2}$. Serum diluted in BBS was used to assay whole C′ activity; Na_2Mg-EDTA-BBS was used in conjunction with EAC'_1 to titer the effective combined titer of fluid phase C′4, C′2, and C′3 and, in conjunction with $EAC'_{1,4}$, to titer C′2 and C′3. The effective C′3 titer of serum was determined using $EAC'_{1,4,2}$ and serum diluted in Na_3HEDTA-BBS.

Serum and serum reagents. Guinea pig blood was collected by cardiac puncture and allowed to stand at room temperature for 4 hours. The serum was then separated by centrifugation, pooled, and frozen in portions at − 85° C in a mechanical freezer. Pooled human serum from blood collected by venipuncture was similarly stored. Two separate batches of human C′1*a* containing 48 and 37.6 U C′1 esterase, respectively, were prepared by previously described procedures (14, 15). Human R3 was prepared by absorption of serum with 2.5 mg zymosan per ml; guinea pig R3 was prepared using 13 mg zymosan per ml (11*b*). Both R3 preparations were excellent reagents for the preparation of their respective $EAC'_{1,4,2}$. Purified C′1*a* and R3 were also stored in portions at − 85° C.

Sheep red cells and amboceptor. Sterile sheep red blood cells collected in acid citrate dextrose solution and glycerinated amboceptor were obtained commercially[3] and kept at 4° C. Sheep red cells were usually discarded 3 weeks after drawing. A single batch of amboceptor was used throughout these studies.

Preparation of sensitized erythrocytes (EA). The coating of sheep red cells with hemolysin was always carried out at pH 7.5. The cells were washed three times in BBS and suspended in Na_3HEDTA-BBS to a cell concentration of 2×10^9 per ml. An equal volume of hemolysin appropriately diluted in Na_3HEDTA-BBS was added and the mixture allowed to stand at room temperature for 15 minutes. The EDTA was used during sensitization to avoid attachment to EA of rabbit C′1 sometimes present in commercial glycerinated amboceptors (16). The cells were then washed three times, once in Na_3HEDTA-BBS and twice in BBS, and resuspended in BBS to a concentration of either 1×10^9 or 5×10^8 per ml. EA to be tested directly in immune lysis were prepared using a 1 : 1,000 dilution of amboceptor. EA destined for use in formation of EAC′ . . . were sensitized using a 1 : 500 dilution of amboceptor.

Preparation of sensitized red cell-complement component intermediates. $EAC'_{1,4,2}$ were prepared by reacting EA with the appropriate R3, and great care was subsequently taken to maintain them at 1° C to prevent temperature-dependent decay to $EAC'_{1,4}$ (17). $EAhuC'_{1,4,2}$ were prepared from human R3 as follows. A measured volume of R3 diluted 1 : 20 in BBS and previously warmed to 37° C (1 ml 1 : 20 R3/5×10^8 EA) was added to a centrifuged button of EA at 37°. The cells were resuspended, and after exactly 60 seconds the mixture was poured into 5 vol of BBS at 1° C. The cells were centrifuged, washed three times in cold BBS (1°), and resuspended in BBS (5×10^8 cells per ml). $EAg.p.C'_{1,4,2}$ were prepared from guinea pig R3 as follows. One ml of guinea pig R3 was added to 10 ml EA in BBS (1×10^9 EA per ml) at 1° C. After 30 minutes, the cells were centrifuged, washed three times in cold BBS, and finally resuspended in BBS at a cell concentration of 5×10^8 per ml. No more than 30 minutes was allowed to elapse between the preparation of $EAC'_{1,4,2}$ and their use.

$EAC'_{1,4}$ were prepared from $EAC'_{1,4,2}$ by allowing the latter to decay for 90 minutes at 37° C. The $EAC'_{1,4}$ were then washed and resuspended in BBS at a cell concentration of 5×10^8 per ml.

To prepare EAC'_1, .5 ml of an appropriate dilution (determined by pretitration) of purified huC′1*a* in BBS was added to a cell button of 5×10^8 EA. When $EAhuC'_1$ were prepared in bulk, proportionate volumes were employed. After incubation at 37° for periods of from 3 to 10 minutes, 10 vol of BBS prewarmed to 37° was added, and the cell suspension was centrifuged for 3 minutes. The $EAhuC'_1$ were resuspended in BBS (37° C) to a concentration of 5×10^8 per ml and tested immediately. Maintenance of the $EAhuC'_1$ at 37° C was necessary to prevent loss of huC'_1 activity, which occurs at lower temperatures (18).

---

[2] Geigy Industrial Chemicals, Ardsley, N. Y.

[3] Markham Laboratories, Chicago, Ill.

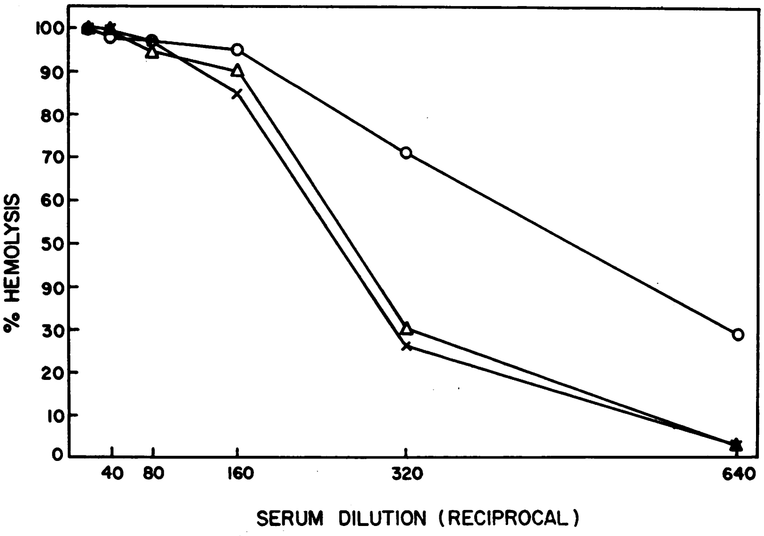

Fig. 1. The effect of pH EA lysis by human C′. ○——○, C′ titrated to pH 6.5, diluted in BBS, pH 6.5; ×——×, C′ titrated to pH 6.5, diluted in BBS, pH 7.5; △——△, C′ diluted in BBS, pH 7.5. See text, footnote 1, for abbreviations in all Figures. BBS = barbital-buffered saline.

The effect of pH on immune hemolysis. The pH of thawed serum ranged from 7.5 to 7.7. We found that at the dilutions of serum employed, the pH was effectively controlled by the pH of the buffered saline used for dilution, so that in most experiments pH adjustments of C′ were an inherent part of the dilution process. In certain experiments, however, the pH adjustment of serum was initiated directly after thawing. This was done to ensure that altering the pH of native serum did not irreversibly affect its hemolytic C′ potential. Thus, in some experiments serum was adjusted to pH 6.5 with 0.3 N HCl, and the control serum (pH 7.5) was equally diluted with BBS (pH 7.5). The acidified serum was then serially diluted in pH 6.5 buffer (final pH 6.5), and pH 7.5 buffer (final pH 7.5), and the ability of these C′ dilutions to support immune hemolysis was compared with that of the unacidified serum diluted in pH 7.5 buffer and pH 6.5 buffer. Once it had been determined that preacidification of native serum did not permanently affect its hemolytic C′ capacity, and that any change in C′ activity was attributable entirely to the pH of the dilute C′ hemolytic milieu (*vide infra*), all pH adjustments of C′ reagents were made by dilution in the appropriate buffered saline.

Two basic questions were investigated. *1*) What effect does pH alteration have upon the capacity of C′ to support EA or EAC′ . . . hemolysis? The cells were prepared as outlined at a single pH (7.5), and samples were then subjected to hemolysis in equivalent serial dilutions of C′ at varying pH.

2) What effect does pH alteration have upon the formation of EAC′ . . .? To eliminate the effects of pH on the capacity of amboceptor to attach to the red cell, EA formation was always carried out at pH 7.5. In

TABLE I

*The effect of pH on the C′H₅₀ titer of human and guinea pig complement**

Dilution of C′ in BBS	Human C′ at pH						Dilution of C′ in BBS	Guinea pig C′ at pH				
	5.5	6.0	6.5	7.0	7.5	8.0		6.0	6.5	7.0	7.5	8.0
	% lysis							% lysis				
1:20	100	100	100	100	100	100	1:100	100	100	100	100	100
1:40	100	100	100	100	100	100	1:200	100	100	100	100	100
1:80	92	100	100	100	97	99	1:400	98	100	100	100	94
1:160	73	84	90	86	78	48	1:800	82.5	85.5	80	77.6	70
1:320	32.5	38	46	33	22	6.5	1:1,600	5.0	19	24	21.6	17.5
1:640	14	16	15	5.5	1.4	0	1:3,200	1.8	0.9	1.7	0	1.6
$C'H_{50}$ titer	237	272	304	274	225	157	$C'H_{50}$ titer	1,125	1,180	1,180	1,125	1,020
Ratio $\frac{C'H_{50}}{C'H_{50} \text{ at pH } 7.5}$	1.05	1.21	1.35	1.22	1.0	0.70		1.00	1.05	1.05	1.00	0.907

* See text, footnote 1, for abbreviations in all Tables. Also, BBS = barbital-buffered saline.

TABLE II

The effect of pH on the lysis of EAhuC′$_1$ and EAhuC′$_{1,4}$

Dilution of human C′ in Na$_2$Mg-EDTA-BBS	EAhuC′$_1$						Dilution of human C′ in Na$_2$Mg-EDTA-BBS	EAhuC′$_{1,4}$					
	pH 6.5			pH 7.5				pH 6.5			pH 7.5		
	OD 540 mμ	OD 540 mμ −blank	Lysis	OD 540 mμ	OD 540 mμ −blank	Lysis		OD 540 mμ	OD 540 mμ −blank	Lysis	OD 540 mμ	OD 540 mμ −blank	Lysis
			%			%				%			%
1:50	1.106	1.086	97	0.771	0.751	67	1:20	1.079	1.050	44	0.800	0.771	32
1:100	0.582	0.562	50	0.183	0.163	14.5	1:40	0.578	0.549	23	0.432	0.403	17
1:200	0.082	0.062	6	0.030	0.010	1	1:80	0.190	0.161	6.7	0.144	0.116	4.5
1:400	0.019	0	0	0.013	0	0	1:160	0.072	0.043	2	0.055	0.026	1
Blank	0.016			0.018				0.029					
EA blank	0.020			0.020				0.027					
100%	1.142	1.122		1.142	1.122			2.390	2.361				
C′H$_{50}$ at pH 6.5* / C′H$_{50}$ at pH 7.5	1.64							1.60					

* Estimated by graphic methods. This ratio represents, in the case of EAhuC′$_1$, the relative potency at the two pH's of fluid phases C′4, C′2, and C′3; in the case of EAhuC′$_{1,4}$, the relative hemolytic potency of fluid phases C′2 and C′3.

these experiments (involving EAhuC′$_1$, and EAhuC′$_{1,4,2}$) EAC′ . . . formation was done at varying pH's, and lysis was carried out with serial dilutions of C′ at a single pH (7.5). Since excess C′1 was present in substantial amounts during the single wash, EAhuC′$_1$ prepared at a given pH were subjected to washing in buffer of an equivalent pH. However, since excess R3 was removed in the initial centrifugation following EAC′$_{1,4,2}$ formation, the three washes in these experiments were carried out at pH 7.5. The effect of pH on the formation of EAC′$_{1,4}$ was not investigated, since these cells were prepared from EAC′$_{1,4,2}$ and we assumed that their behavior would parallel that of the parent cell.

All hemolytic tests were carried out by adding 4 ml of the appropriate C′ dilution to a thoroughly drained button of 5×10^8 EA, EAC′$_1$, or EAC′$_{1,4,2}$ and incubating the cell suspension at 32° C for 60 minutes with occasional mixing. In the experiments with EAhuC′$_{1,4}$, a 1×10^9 cell button was employed. Cell blanks in buffer alone at all pH's tested were always run. One hundred per cent hemolysis was determined by the addition of 4 ml 0.1% Na_2CO_3 solution to a button of 5×10^8 cells. When C′ diluted in Na$_2$MgEDTA-BBS or Na$_3$HEDTA-BBS was used, an EA blank was always run at the lowest C′ dilution. Hemolysis was measured by determining the optical density of the supernatant fluid at 540 mμ. The OD (100% hemolysis) of 5×10^8 cells in a 4-ml vol is approximately 1.3. C′H$_{50}$ titers of whole C′ were determined as outlined by Kabat and Mayer (11*c*). The proportions of volume, amount of C′, and cell concentration were different from those usually employed, and therefore the C′H$_{50}$ titers are higher than these authors report. Since we were interested primarily in relative titers at various pH's, this was not important. In instances where the effects of pH on the effective cumulative titer of fluid phase C′ subcomponents were being assessed with EAC′ . . ., C′H$_{50}$ titers were approximated by graphic methods employing either logarithmic probability graph paper (titer versus per cent hemolysis) or a log-log plot of titer versus $y/1 - y$ where $y =$ fraction of cells lysed. Although both methods of plotting the data resulted in

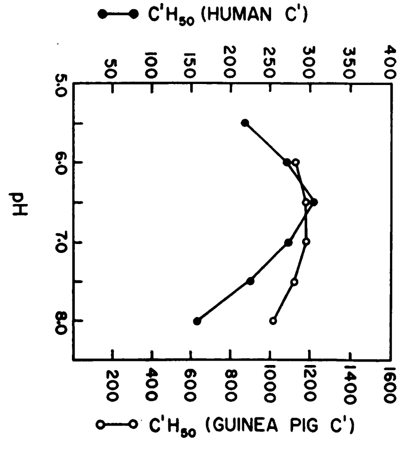

FIG. 2. THE EFFECT OF pH ON THE COMPLEMENT TITERS OF HUMAN AND GUINEA PIG SERUM.

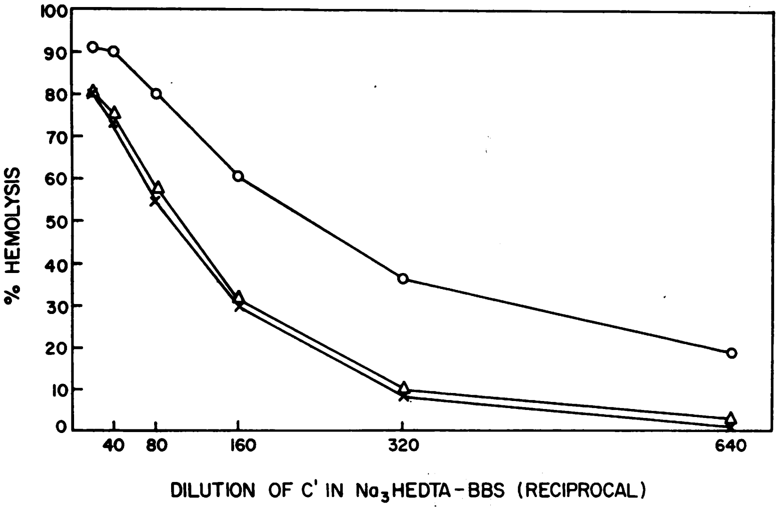

FIG. 3. THE EFFECT OF pH ON THE ABILITY OF HUMAN C' DILUTED IN NA_3HEDTA-BBS TO HEMOLYZE EAhuC$'_{1,4,2}$. O——O, C' titrated to pH 6.5, diluted in Na_3HEDTA-BBS, pH 6.5; ×——×, C' titrated to pH 6.5, diluted in N_3HEDTA-BBS, pH 7.5; △——△, C' diluted in Na_3HEDTA-BBS, pH 7.5.

departure from linearity at the extremes of hemolysis (0 to 20% and 80 to 100% hemolysis), agreement between these two arbitrary methods was good if the results were based on the values nearest to 50% hemolysis.

All experiments were performed on at least two separate occasions, and the results obtained were always comparable.

A Zeiss PMQ II spectrophotometer and silica cuvettes with a 1-cm light path were used for all optical density measurements. pH determinations were made with a Beckman Zeromatic pH meter.

Results

The effect of pH on EA and EAC' . . . lysis. A comparison between the ability of human C' to support EA lysis at pH 7.5 and pH 6.5 is shown in Figure 1. The C$'H_{50}$ titer of human C' is substantially increased at pH 6.5. This enhancement is not seen if the human serum serving as a C' source is titrated to pH 6.5 with HCl and its pH subsequently raised by dilution in pH 7.5 BBS.

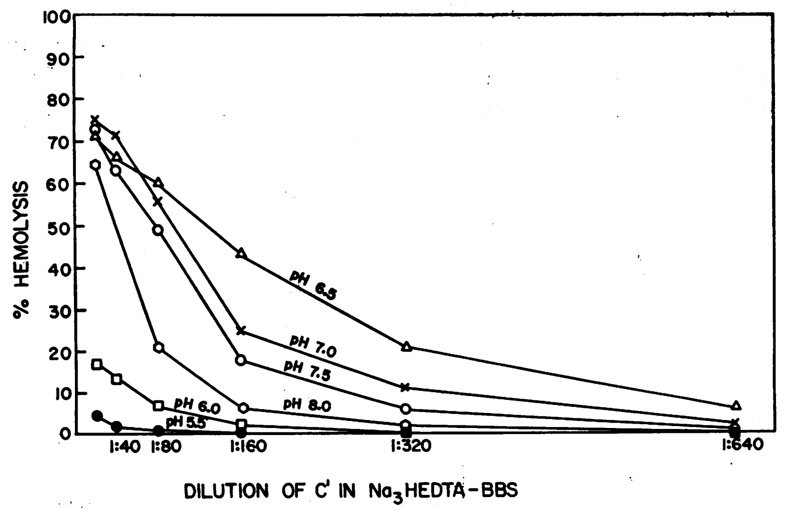

FIG. 4. THE EFFECT OF pH ON THE ABILITY OF HUMAN C' DILUTED IN NA_3HEDTA-BBS TO HEMOLYZE EAhuC$'_{1,4,2}$. pH adjustments were accomplished by dilution in the appropriate buffer.

TABLE III

The effect of pH on the hemolysis of $EAg.p.C'_{1,4,2}$

Dilution of guinea pig C′ in Na_3HEDTA-BBS	pH 6.5			pH 7.5		
	OD 540 mu	OD 540 mμ−blank	Lysis	OD 540 mμ	OD 540 mμ−blank	Lysis
			%			%
1:100	1.182	1.151	91	1.177	1.153	91
1:200	1.057	1.026	81	1.017	0.993	78
1:400	0.760	0.729	58	0.761	0.737	58
1:800	0.321	0.291	23	0.354	0.330	26
1:1,600	0.078	0.047	4	0.112	0.088	7
1:3,200	0.041	0.010	1	0.030	0.004	0
Blank	0.031			0.024		
100%	1.296	1.265		1.296	1.272	

Thus acidification of whole serum to pH 6.5 has no permanent or irreversible effects upon whole C′ activity. More extensive data on the effects of pH on human C′ activity in EA lysis, together with similar observations made on guinea pig C′, are shown in Table I and Figure 2. Our results concerning the effect of pH on the C′ titer of guinea pig serum are in substantial agreement with those of Kabat and Mayer (11). There is a slight but definite increase in guinea pig C′ potency with decreasing pH, a broad optimum being reached between pH 6.5 and 7.0. The effect of decreasing pH on human C′ potency is, in contrast, much greater, with a narrower optimum, at about pH 6.5.

Having demonstrated substantial enhancement of hemolytic human C′ potency at pH 6.5, we studied the effect of pH on the lysis of various EAC′ . . . in the hope of determining which of the intermediate stages in immune hemolysis were most affected by such pH alterations. The data in Table II illustrate the effect of pH on the hemolysis of $EAhuC'_1$ and $EAhuC'_{1,4}$ in human C′ diluted in $Na_2MgEDTA$-BBS. Since both of these C′ component-EA intermediates hemolyze more effectively at pH 6.5, and since both are equally enhanced to approximately the same degree as noted earlier with EA lysis, we presumed that the stimulatory effect of pH reduction depends primarily upon enhancement of either C′2 or C′3 or both. From the results shown in Figures 3 and 4 we concluded that pH alteration affects primarily the last stage in immune hemolysis, namely, the reaction $EAhuC'_{1,4,2} + huC'3 \rightarrow E^*$. It is apparent again, in a fashion analogous to EA lysis, that acidification of whole serum to pH 6.5, followed by dilution at pH 7.5, does not permanently alter the effective C′3 titer of serum. By comparison with EA lysis, $EAhuC'_{1,4,2}$ hemolysis diminishes rather sharply as the pH is lowered from the optimum at 6.5. In contrast to human C′, the reaction $EAg.p.C'_{1,4,2} + g.p.C'3 \rightarrow E^*$ is

TABLE IV

Comparison of the hemolytic potency of human C′ and C′ subcomponents at pH 6.5 and pH 7.5

Cell type	Experiment no.	$C'H_{50}$		$\frac{C'H_{50} \text{ at pH } 6.5}{C'H_{50} \text{ at pH } 7.5}$	$\frac{C'H_{50} \text{ at pH } 6.5}{C'H_{50} \text{ at pH } 7.5}$ (average)
		pH 6.5	pH 7.5		
I. EA	1	391	294	1.33	
	2	445	265	1.68	1.40
	3	304	223	1.35	
	4	435	355	1.22	
II. $EAhuC'_1$	1	100*	61*	1.64*	
III. $EAhuC'_{1,4}$	1	16.8*	10.5*	1.60*	
IV. $EAhuC'_{1,4,2}$	1	219*	93*	2.35*	
	2	120*	77*	1.56*	1.80*
	3	178*	119*	1.49*	

* Estimated by graphic methods. The ratio represents, in the case of $EAhuC'_1$, the relative potency at the two pH's of fluid phases C′4, C′2, and C′3; in the case of $EAhuC'_{1,4}$, the relative hemolytic potency of fluid phases C′2 and C′3; in the case of $EAhuC'_{1,4,2}$, the relative hemolytic potency of fluid phase C′3.

TABLE V

The effect of pH on the formation of EAhuC$'_1$

	EAhuC$'_1$ formed at pH					
	6.5			7.5		
Dilution of human C$'$ in Na$_2$MgEDTA-BBS	OD 540 mμ	OD 540 mμ −blank	Lysis	OD 540 mμ	OD 540 mμ −blank	Lysis
			%			%
Experiment I 1:50	0.440	0.415	36	0.487	0.462	40.7
Blank	0.025	0		0.025	0	
100%	1.153	1.128		1.156	1.131	
Experiment II 1:50	0.592	0.572	50.4	0.665	0.645	58.4
1:100	0.183	0.163	14.4	0.189	0.169	15.3
1:200	0.038	0.018	2	0.032	0.012	1
Blank	0.020	0		0.020	0	
100%	1.153	1.133		1.126	1.106	

not affected by altering the pH from 7.5 to 6.5 (Table III). A summary of the effects of pH alteration from 7.5 to 6.5 on the relative hemolytic potency of human fluid phase C$'$ components at various stages in immune lysis is presented in Table IV.

The effect of pH on the formation of EAC$'$. . . . To confirm the above conclusion that pH alteration largely affects the reaction EAhuC$'_{1,4,2}$ + huC$'$3 → E*, the effect of pH upon the formation of various EAC$'$ . . . intermediates in immune hemolysis was analyzed. We reasoned that if these conclusions were correct, the formation of EAC$'_1$ and EAC$'_{1,4,2}$ (and by implication EAC$'_{1,4}$) should not be enhanced by lowering pH from 7.5 to 6.5. These experiments (Tables V and VI) bear out the conclusions drawn earlier; EAhuC$'_1$ formation is, if anything, slightly inhibited at pH 6.5; EAhuC$'_{1,4,2}$ formation is not affected. In relation to the latter experiment, it is imperative to use an R3 devoid of even small amounts of C$'$3; otherwise there is substantial E* formation at pH 6.5, with the result that much hemolysis occurs during preparation of the cells even though EAhuC$'_{1,4,2}$ formation with the same R3 may proceed satisfactorily at pH 7.5. This observation, although largely of nuisance value in terms of experimental technique, also indicates the primary role of C$'$3 in the enhancement of human C$'$ action at pH 6.5.

The effect of pH on the decay of EAC$'_{1,4,2}$. Although the data so far presented leave little doubt that pH reduction from 7.5 to 6.5 selectively enhances the last stage in immune hemolysis, the same result could be obtained by two different mechanisms. The over-all hemolytic titer of C$'$ is recognized to depend primarily upon the balance achieved between the decay of EAC$'_{1,4,2}$ to EAC$'_{1,4}$ and the conversion of EAC$'_{1,4,2}$ to E* by the action of C$'$3 (19). pH reduction might result in en-

TABLE VI

The effect of pH on the formation of EAhuC$'_{1,4,2}$

	EAhuC$'_{1,4,2}$ formed at pH					
	6.5			7.5		
Dilution of human C$'$ in Na$_3$HEDTA-BBS	OD 540 mμ	OD 540 mμ −blank	Lysis	OD 540 mμ	OD 540 mμ −blank	Lysis
			%			%
1:20	0.794	0.768	68	0.885	0.864	70
1:40	0.770	0.744	66	0.820	0.799	65
1:80	0.587	0.561	50	0.634	0.613	50
1:160	0.318	0.292	26	0.341	0.320	26
1:320	0.104	0.708	7	0.104	0.083	7
1:640	0.043	0.017	1.5	0.031	0.010	1
Blank	0.026			0.021		
100%	1.152	1.126		1.251	1.230	

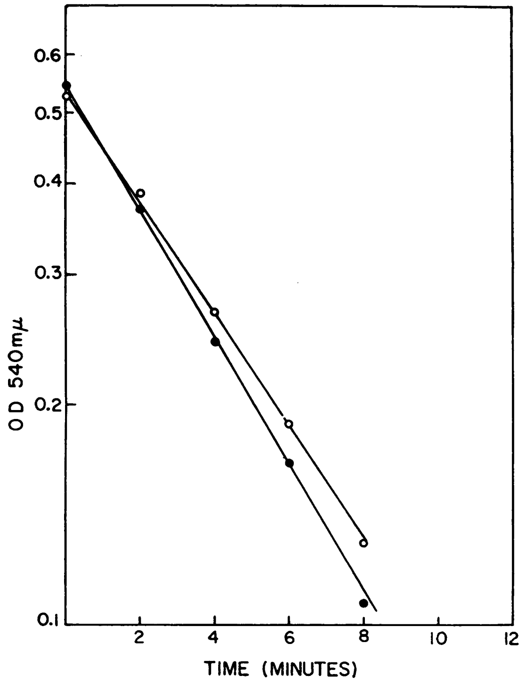

FIG. 5. THE EFFECT OF pH ON THE DECAY OF EAHUC$'_{1,4,2}$ TO EAHUC$'_{1,4}$. ●, EAhuC$'_{1,4,2}$ decayed at pH 7.5; ○, EAhuC$'_{1,4,2}$ decayed at pH 6.5.

hancement of C′3 activity, but the same result would be achieved if lowering the pH diminished the rate at which EAhuC$'_{1,4,2}$ decayed to EAhuC$'_{1,4}$. This latter possibility was tested by the following experiment: Suspensions of EAhuC$'_{1,4,2}$ (5×10^8 cells per ml) in Na_3-HEDTA-BBS at both pH 6.5 and pH 7.5 were placed in a water bath at 37° C. When the temperature within the suspensions reached 37°, 1-ml samples were removed at measured time intervals and added to 4 ml of a 1:40 dilution of human serum in Na_3HEDTA-BBS, pH 7.5. These tubes were mixed and incubated at 32° for 60 minutes to determine residual EAhuC$'_{1,4,2}$ activity. The results are shown in Figure 5. The rate of the temperature-dependent decay reaction EAhuC$'_{1,4,2} \rightarrow$ EAhuC$'_{1,4}$ is similar at both pH 7.5 and pH 6.5 ($t_{\frac{1}{2}} = 3.5$ and 4.05 minutes, respectively). The slight reduction of the rate of decay seen at pH 6.5 is not sufficient to account for the degree of enhancement seen in EAhuC$'_{1,4,2}$ lysis at this pH. Thus it would seem that most of the enhancement in immune hemolysis at pH 6.5 is attributable to stimulation of C′3 activity.

Discussion

The present data indicate that those human C′-dependent hemolytic systems that function best at pH 6.5 are not different in this respect from classical immune hemolysis. Indeed, it might be said that a hemolytic system involving human C′ that functions better at pH's alkaline to 7, such as the Donath-Landsteiner hemolytic system, is the exception; perhaps in this instance the limiting factor is hemolysin attachment, which is favored by a more alkaline pH. In the present studies, red cell sensitization was always performed at pH 7.5, regardless of the pH at which hemolysis was carried out, and variations in the effect of pH on hemolysin attachment were avoided. However, it might still be argued that lowering of pH diminishes the rate at which hemolysin elutes from EA, and thereby promotes the action of C′ at pH 6.5. Such an argument is difficult to refute on theoretical grounds, since hemolysin is known to elute from EA and to transfer from cell to cell (11*d*), and C′ titer in a limited C′ system can be increased by increasing the number of sensitized sites on the red cell membrane (20). If this were the mechanism operating to increase human C′ effectiveness at pH 6.5, then a similar degree of enhancement would be expected regardless of the C′ source. That guinea pig C′ is in fact not enhanced to the same extent by pH alteration is therefore a strong point in favor of a direct action of pH alteration on human C′ effectiveness. In addition, if pH reduction resulted in tighter binding of A to E, then the enhancing effects of pH reduction on human C′-dependent hemolysis should be obvious during the early phases of immune hemolysis. Formation of EAhuC$'_1$ and EAhuC$'_{1,4,2}$, however, was not increased by pH reduction. The ability of pH reduction to enhance the hemolysis of normal human red cells by high titer cold agglutinins, or of artifically altered red cells by human sera containing heterospecific antibodies, can thus be viewed as a C′-mediated phenomenon, rather than one brought about by increasing hemolysin attachment.

Recognition that classical immune hemolysis produced by human C′ has a pH optimum similar to that of the PNH hemolytic system supports the argument that the C′ system plays the predominant role in *in vitro* PNH red cell lysis. Immune hemolysis and poly I-induced hemolysis of normal red cells have recently been shown to proceed in the absence of Ca^{++}, provided that Mg^{++} is supplied and whole human serum is used as the C′ source (3, 21); PNH hemolysis also displays an absolute requirement for Mg^{++} but not for Ca^{++} (6). Thus the PNH hemolytic system resembles other human C′-dependent hemolytic systems not only in its pH optimum, but also in divalent cation requirements. The enhancement of immune red cell lysis and PNH hemolysis produced by reduction of serum pH to 6.5 are similar in that both phenomena are reversible; restoration of serum pH to 7.5 results in loss of the stimulatory effect and the return of serum hemolytic potency to the original level (22).

We have recently shown that substances usually considered to be anticomplementary, such as polyinosinic acid, streptokinase, and aggregated γ-globulin, can all cause enhancement of PNH red cell hemolysis *in vitro* when added to acidified human serum (3, 22); these three agents are known to function as activators of C′1 (23–26). Furthermore, addition of purified C′1a or C′1 esterase to serum can induce the same stimulation of PNH hemolysis (22). Careful study of all these materials has revealed a striking similarity in the kinetics of their interaction with serum in relation to PNH hemolysis. Therefore, we have suggested that these substances via C′1a, or C′1a itself, activate fluid phase C′2, which in turn is responsible for the generation of fluid phase C′3 hemolytic activity; it is presumably a late-acting C′3 subcomponent(s) that ultimately injures the red cell membrane (3, 27). Since the PNH red cell lacks an antibody coat to localize the process of C′ activation at the cell membrane, its destruction depends upon random hits from activated fluid phase C′3 subcomponents (3). The importance of late-acting C′3 subcomponents in relation to PNH hemolysis is attested to by the recent observations of Rosen and of Jenkins. In preliminary experiments Rosen has observed that purified β_{1C}-globulin can attach to PNH red cells directly without the mediation of other fluid phase C′ components (28). Jenkins, who has found C′ components coating those PNH red cells resistant to *in vitro* acid hemolysis (29), has noted that such cells react strongly and consistently with anti-β_{1C}-globulin (30) (anti-C′3), but weakly and less consistently with anti-β_{1C}-globulin (31) (anti-C′4) (32). We have postulated that PNH hemolysis is, in a sense, a threshold phenomenon that depends upon an intrinsic low grade process of activation of fluid phase late-acting C′ components in serum (3).

In view of the foregoing discussion, it is of great interest to find that pH alterations of serum from 7.5 to 6.5 predominantly enhance the hemolytic function of C′3. If one examines the data in Figures 1 and 3, he is struck by the fact that in immune hemolysis, given the proper conditions, the degree of hemolysis can be altered by pH change to the same extent as is commonly observed in PNH hemolysis. Thus in a 1:160 dilution of human C′3, $EAhuC'_{1,4,2}$ show 9.5% hemolysis at pH 7.5 as compared with 37% hemolysis at pH 6.5 (Figure 3). Similarly, in a 1:640 dilution of whole human C′, EA display 3% lysis at pH 7.5 compared with 29% hemolysis at pH 6.5 (Figure 1). PNH hemolysis *in vitro* results in no diminution of serum C′ titer as subsequently measured by immune hemolysis, and may thus be considered to involve only such small amounts of active C′ material as are represented by serum dilutions in the range mentioned above (33).

Summary

The pH optima of the immune lysis of sensitized sheep red cells by human and guinea pig complement have been investigated. Both human and guinea pig complement function best at pH 6.5, but human complement is more sensitive to pH alteration. Study of the effects of pH alteration upon the formation and lysis of sensitized red cell-complement component intermediates has demonstrated that the enhanced potency of human complement action at pH 6.5 results from a stimulation of the third complement component. The significance of these observations relative to certain "pH-dependent" human hemolytic systems is discussed.

References

1. Dacie, J. V. The haemolytic anemias, congenital and acquired. London, J. and A. Churchill, 1954, p. 250; (*a*) p. 257; (*b*) p. 428; (*c*) p. 251; (*d*) p. 433; (*e*) p. 279.

2. Yachnin, S., M. T. Laforet, and F. H. Gardner. pH dependent hemolytic systems. I. Their relationship to paroxysmal nocturnal hemoglobinuria. Blood 1961, 17, 83.
3. Yachnin, S., and J. M. Ruthenberg. The initiation and enhancement of human red cell lysis by an activator of the first component of complement and by first component esterase; studies using normal red cells and red cells from patients with paroxysmal nocturnal hemoglobinuria. J. clin. Invest. 1965, **44**, in press.
4. Ham, T. H. Chronic hemolytic anemia with paroxysmal nocturnal hemoglobinuria: study of the mechanism of hemolysis in relation to acid-base equilibrium. New Engl. J. Med. 1937, **217**, 915.
5. Dacie, J. V., M. C. G. Israëls, and J. F. Wilkinson. Paroxysmal nocturnal hæmoglobinuria of the Marchiafava type. Lancet 1938, 1, 479.
6. Hinz, C. F., Jr., W. S. Jordan, Jr., and L. Pillemer. The properdin system and immunity. IV. The hemolysis of erythrocytes from patients with paroxysmal nocturnal hemoglobinuria. J. clin. Invest. 1956, **35**, 453.
7. Wagley, P. F., and M. D. Hickey. Susceptibility of red cells and serum factor in the mechanism of hemolysis in paroxysmal nocturnal hemoglobinuria (abstract). J. clin. Invest. 1948, **27**, 559.
8. Crosby, W. H. Paroxysmal nocturnal hemoglobinuria. Plasma factors of the hemolytic system. Blood 1953, **8**, 444.
9. Boyd, W. C. Fundamentals of Immunology, 3rd ed. New York, Interscience, 1956, p. 373.
10. Kabat, E. A., and M. M. Mayer. Experimental Immunochemistry, 1st ed. Springfield, Ill., Charles C Thomas, 1948, p. 106.
11. Kabat, E. A., and M. M. Mayer. Experimental Immunochemistry, 2nd ed. Springfield, Ill., Charles C Thomas, 1961, p. 148; (*a*) p. 149; (*b*) p. 163; (*c*) p. 135; (*d*) p. 141.
12. Hinz, C. F., Jr., M. E. Picken, and I. H. Lepow. Studies on immune hemolysis. I. The kinetics of the Donath-Landsteiner reaction and the requirement for complement in the reaction. J. exp. Med. 1961, **113**, 177.
13. Wardlaw, A. C. The complement-dependent bacteriolytic activity of normal human serum. I. The effect of pH and ionic strength and the role of lysozyme. J. exp. Med. 1962, **115**, 1231.
14. Lepow, I. H., O. D. Ratnoff, F. S. Rosen, and L. Pillemer. Observations on a pro-esterase associated with partially purified first component of human complement (C'1). Proc. Soc. exp. Biol. (N. Y.) 1956, **92**, 32.
15. Levy, L. R., and I. H. Lepow. Assay and properties of serum inhibitor of C'1-esterase. Proc. Soc. exp. Biol. (N. Y.) 1959, **101**, 608.
16. DeLooze, L. L., A. Ransby, and M. A. Leon. Activity of C'1 in rabbit antisheep hemolysin. Proc. Soc. exp. Biol. (N. Y.) 1962, **109**, 393.
17. Mayer, M. M., L. Levine, H. J. Rapp, and A. A. Marucci. Kinetic studies on immune hemolysis. VII. Decay of EAC'_{142}, fixation of C'3, and other factors influencing the hemolytic action of complement. J. Immunol. 1954, 73, 443.
18. Lepow, I. H., G. B. Naff, E. W. Todd, J. Pensky, and C. F. Hinz, Jr. Chromatographic resolution of the first component of human complement into three activities. J. exp. Med. 1963, **117**, 983.
19. Austen, K. F., and Z. A. Cohn. Contribution of serum and cellular factors in host defense mechanisms. New Engl. J. Med. 1963, **263**, 933.
20. Yachnin, S. Biologic properties of polynucleotides. I. The anticomplementary activity of polynucleotides. J. clin. Invest. 1963, **42**, 1947.
21. Yachnin, S., and J. M. Ruthenberg. The role of calcium in complement dependent hemolysis. Proc. Soc. exp. Biol. (N. Y.) 1964, **117**, 179.
22. Yachnin, S. Unpublished data.
23. Yachnin, S., D. Rosenblum, and D. Chatman. Biologic properties of polynucleotides. VI. Further studies on the mechanism of complement inactivation by polyinosinic acid: the inactivation of the fourth component of complement. J. Immunol. 1964, **93**, 549.
24. Lepow, I. H., L. Wurz, O. D. Ratnoff, and L. Pillemer. Studies on the mechanism of inactivation of human complement by plasmin and by antigen-antibody aggregates. I. The requirement for a factor resembling C'1 and the role of Ca^{++}. J. Immunol. 1954, 73, 146.
25. Christian, C. L. Studies of aggregated γ-globulin. 1. Sedimentation, electrophoretic and anticomplementary properties. J. Immunol. 1960, **84**, 112.
26. Marcus, D. M. A study of the mechanism of the anticomplementary activity of γ-globulin. J. Immunol. 1960, **84**, 273.
27. Linscott, W. D., and K. Nishioka. Components of guinea pig complement. II. Separation of serum fractions essential for immune hemolysis. J. exp. Med. 1963, **118**, 795.
28. Rosen, F. Personal communication.
29. Jenkins, D. E., Jr., W. N. Christenson, and R. L. Engle, Jr. Erythrocyte complement coating in the acid hemolysin and thrombin tests of paroxysmal nocturnal hemoglobinuria (abstract). Blood 1962, **20**, 788.
30. Müller-Eberhard, H. J., and U. Nilsson. Relation of a β_1-glycoprotein of human serum to the complement system. J. exp. med. 1960, **111**, 217.
31. Müller-Eberhard, H. J., and C. E. Biro. Isolation and description of the fourth component of human complement. J. exp. Med. 1963, **118**, 447.
32. Jenkins, D. E., Jr. Personal communication.
33. Hartman, R. C., and D. E. Jenkins, Jr. Analytical review: paroxysmal nocturnal hemoglobinuria. Blood, in press.