IMMUNOREACTIONS INVOLVING PLATELETS

I. A Steric and Kinetic Model for Formation of a Complex from a Human Antibody, Quinidine as a Haptene, and Platelets; and for Fixation of Complement by the Complex

By N. RAPHAEL SHULMAN,* M.D.

(From the Naval Medical Research Institute and the National Institutes of Health, Bethesda, Maryland)

(Received for publication, December 23, 1957)

Patients who develop thrombocytopenic purpura as a result of sensitivity to some drugs have in their serum an antibody which fixes complement only when the antibody is present together with platelets and the offending drug. This was first demonstrated by Ackroyd in the case of purpura due to sedormid sensitivity (1–5). In addition he determined a number of the qualitative properties of the antibody-drug-platelet complex. Subsequent reports concerning purpura due to other drugs, reviewed by Ackroyd (6), suggested that the immunological mechanism involved was similar. From the observations of Bolton (7) it was evident that the reactions of the antibody formed as a result of quinidine sensitivity had all of the qualitative properties of the reactions of the antibody resulting from sedormid sensitivity which Ackroyd reported. It therefore appeared that study of the reactions involving the antibody of quinidine purpura might provide information concerning a general type of immunologic response which has possible relevance to various diseases of sensitivity.

An interpretation of the mechanism of the molecular associations and the sequence of the reactions involved in formation of the antibody-drug-platelet complex was based on analysis of the effects of varying the initial concentration of each component in the complex on the amount of complement fixed by the complex, and on kinetic aspects of complex formation. A steric model for formation of the complex and fixation of complement by the complex, which was deduced from experimental results, was described mathematically by Dr. Terrell L. Hill. Theoretical results calculated using equations based on the model were consistent in all respects with experimental results.

Materials and Methods

Source of Antibody.—Antibody which was used was in the serum of a patient who developed purpura as a result of sensitivity to quinidine (see Paper IV of this series, patient E.K.S.).

* Present address: National Institutes of Health, Bethesda, Maryland.

The antibody was stable on storage at −20° indefinitely. Serum containing antibody was heated as routine at 56° for 30 minutes immediately before use in complement fixing reactions.

Saline.—0.147 molar sodium chloride.

Quinidine and Other Cinchona Alkaloids.—Highly purified cinchona alkaloids dissolved in saline were used. Quinidine gluconate, MW 524, 62.3 per cent anhydrous quinidine, was obtained from Eli Lilly and Company. Quinine dihydrochloride (neutralized with 1 N sodium hydroxide), MW 397, 81.7 per cent anhydrous quinine; cinchonine sulfate, $(C_{19}H_{22}ON_2)_2 \cdot H_2SO_4 \cdot 2H_2O$, MW 723, 81.4 per cent anhydrous cinchonine; and cinchonidine sulfate, MW 741, 79.4 per cent anhydrous cinchonidine, were obtained from Merck & Company, Inc. Quinidine was measured spectrophotometrically by the method of Josephson *et al.* (8).

Preparation of Platelet Suspension.—All surfaces in contact with platelets during the preparation of suspensions were siliconed. Venous blood was run directly into test tubes containing anticoagulant (one part 1.5 per cent by weight of the disodium salt of ethylenediaminetetraacetic acid, pH 7, to nine parts of blood). Platelet-rich plasma was obtained by centrifuging blood at 200 *g* for 15 minutes and drawing off the supernatant plasma. Packed platelets were obtained by centrifuging platelet-rich plasma at 2500 *g* for 30 minutes. Platelet suspensions were made by washing the platelets once with 1 per cent ammonium oxalate, followed by two washes in saline and resuspension in saline. Platelet counts were performed by the method of Brecher and Cronkite (9), using three pipettes and three chambers. The mean volume of packed platelets in fresh suspensions, determined using a microhematocrit technique (10), was 1.67 volumes per cent when the platelet concentration was 10^6/mm.^{3}, with a standard deviation of 0.032 volumes per cent. In this work the number of platelets/mm.^{3} has the same meaning as a concentration of platelets.

Measurement of Complement Activity.—Complement activity was determined by methods outlined by Kabat and Mayer (11), employing sheep erythrocytes and rabbit antiserum (hemolysin). The concentration of erythrocytes in the presence of 1:2000 hemolysin was adjusted so that 0.5 ml. of sensitized cells produced a density of 0.760 at 540 mμ (Beckman DU spectrophotometer) when completely hemolyzed in a total volume of 3.75 ml. Amounts of complement are expressed in units, one unit being defined as that amount of complement required to hemolyze 50 per cent of the cells in 30 minutes at 37°. With the hemolysin which was used throughout this work, $2.1 \pm 0.1 \times 10^{-3}$ ml. of pooled fresh guinea pig serum was required for 50 per cent hemolysis.

Complement was titrated by determining the amount of solution which produced 50 per cent hemolysis, interpolating on a curve with at least three points in the 20 to 80 per cent hemolysis range. The amount of complement added to a complement-fixing mixture was adjusted whenever possible so that the amount of complement fixed was in the range of 25 to 75 per cent of the total amount of complement added. Complement fixation was carried out as routine in a 0.5 ml. volume for 1 hour at room temperature with frequent agitation of the tubes. Suspended material (platelets) was removed from the fixation mixture by centrifugation before residual complement was titrated.

EXPERIMENTS AND RESULTS

1. Effects of Varying Platelet and Antibody Concentration.—In Table I it can be seen that the amount of complement fixed by a mixture containing antibody, platelets, and quinidine was independent of complement concentration and that up to 82 per cent of the added complement could be consumed during fixation without affecting the total amount fixed. The amount of complement fixed was directly proportional to the concentration of platelets over the range of platelet concentrations used in Table I when antibody con-

centration was kept constant. The curves of Fig. 1 show the effects of wide variations in platelet concentration on complement fixation using three different concentrations of antibody. The points of the initial linear portion of the uppermost curve in Fig. 1 were taken from Table I. Increasing the platelet

TABLE I

Variation in Platelet Concentration, Different Amounts of Complement

Volume in each tube brought to 0.5 ml. with saline. Column 1, patient's serum containing antibody. Column 2, suspension contained 2.5×10^6 platelets/mm.^{3}. Column 3, solution contained 8 mg. quinidine gluconate/ml., final concentration in mixture $10^{-2.91}$ M quinidine. Column 4, fresh pooled guinea pig serum (G.p.s.). After incubating the complement-fixing mixture (columns 1 + 2 + 3 + 4) for one hour at room temperature, platelets were removed by centrifugation. Column 5, the amount of supernatant fluid required to produce 50 per cent hemolysis was determined using appropriate dilutions of supernatant fluid to permit pipetting aliquots greater than 0.1 ml. into hemolysis mixture. Controls, representing full complement activity of 0.16 ml. of G.p.s., required an average of 0.625×10^{-2} ml. of supernatant fluid to produce 50 per cent hemolysis (*i. e.* average of values in column 5 for tubes 10, 11, 12, and 13). The complement activity of 0.16 ml. of G.p.s. was equal to 80 units ($0.5 \div 0.625 \times 10^{-2}$). Column 6, per cent complement fixed calculated from milliliters of supernatant fluid required for 50 per cent hemolysis in experimental tubes compared to control tubes. Column 7, per cent complement fixed times units of complement used.

Tube	1 Antibody	2 Platelet suspension	3 Quinidine solution	4 G.p.s.	5 Supernatant fluid for 50 per cent hemolysis	6 Complement fixed	7 Complement fixed
	ml.	*ml.*	*ml.*	*ml.*	*ml.* $\times 10^{-2}$	*per cent*	*units*
1	0.18	0.01	0.04	0.08	1.538	18.7	7.5
2	0.18	0.02	0.04	0.08	2.190	43.0	17.2
3	0.18	0.04	0.04	0.08	6.865	81.8	32.7
4	0	0	0	0.08	1.254	—	—
5	0.18	0.01	0.04	0.16	0.695	10.0	8.0
6	0.18	0.02	0.04	0.16	0.752	19.2	15.4
7	0.18	0.04	0.04	0.16	1.030	39.3	31.4
8	0.18	0.06	0.04	0.16	1.525	59.1	47.2
9	0.18	0.08	0.04	0.16	3.470	82.0	65.6
10	0.18	0	0.04	0.16	0.623	—	—
11	0.18	0.08	0	0.16	0.629	—	—
12	0	0.08	0.04	0.16	0.625	—	—
13	0	0	0	0.16	0.621	—	—

concentration in the presence of a constant concentration of antibody led to a maximum amount of complement fixation, but further increases in platelet concentration then resulted in a gradual decrease in complement fixation until an asymptotic value much lower than the maximum was reached. The maximum amount of complement fixed and the concentration of platelets necessary for maximum complement fixation were proportional to the amount of antibody used.

When antibody concentration was increased in the presence of a constant concentration of platelets, complement fixation reached an asymptotic maximum directly proportional to the concentration of platelets as shown in Fig. 2. Depression of the initial portion of the curves is due to the high platelet:antibody ratio in this region (*cf.* Fig. 1).

Because complement fixation was directly proportional to platelet concentration when antibody concentration was relatively high, under these condi-

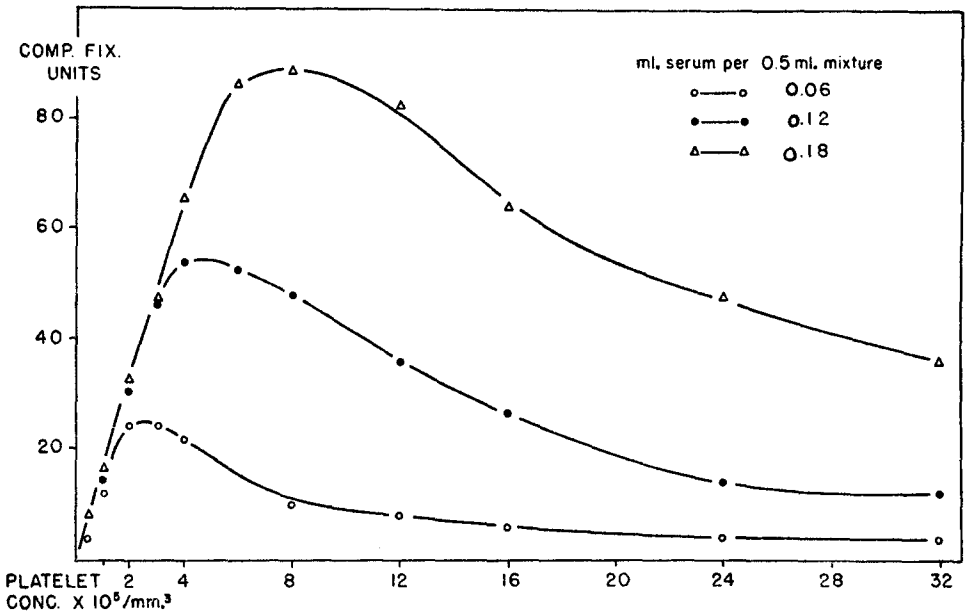

FIG. 1. Effects of wide variations in platelet concentration using three different amounts of antibody. Conditions as in Table I with concentrations of added reagents appropriately altered to maintain the volume of complement-fixing mixture at 0.5 ml.

tions, platelets could be considered to be fully saturated with antibody and the amount of complement fixed related to the absolute amount of antibody adsorbed. The decrease in complement fixation which occurred when platelet concentration was increased above an optimum value suggested that the amount of complement fixed was related not only to the absolute amount of antibody adsorbed but also to the amount adsorbed per platelet.

2. Definition of an Antibody Unit.—

In Fig. 1 it was seen that the maximum amount of complement fixed and the concentration of platelets necessary for maximum complement fixation was dependent on the concentration of antibody used. The concentration of platelets required for maximum complement fixation was found to be directly proportional to antibody concentration with amounts of antibody

which fixed as much as 120 units of complement. As shown in Fig. 3 the maximum amount of complement fixed in the presence of an optimal concentration of platelets was directly proportional to antibody concentration as long as the amount of antibody used in 0.5 ml. of reaction mixture fixed no more than approximately 45 units of complement. Amounts of antibody therefore could be expressed in terms of units based on the concentration of platelets required for maximum complement fixation for any amount of antibody or on the maximum

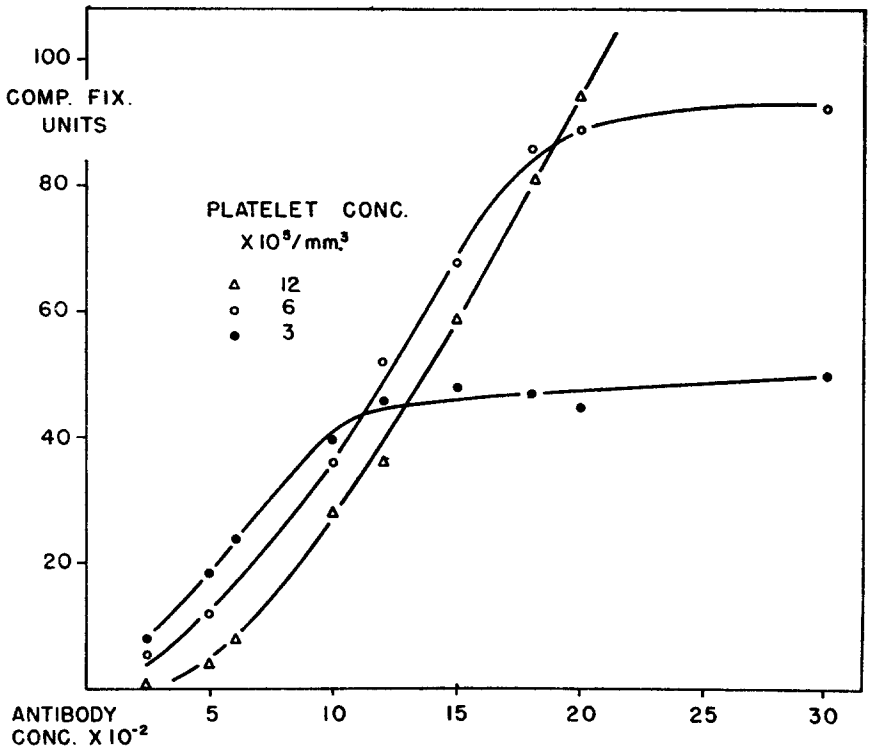

FIG. 2. Effects of varying antibody concentration with platelet concentration constant. Conditions as in Fig. 1.

amount of complement fixed for amounts of antibody which fixed up to 45 units of complement. One unit of antibody was defined as that amount of antibody in a 0.5 ml. volume which gave maximum complement fixation with 10^5 platelets/mm.^{3} or which fixed 10.5 units of complement when the platelet concentration was optimal. A 0.1 ml. aliquot of the serum sample used in Fig. 3 contained sufficient antibody to fix 42 units of complement when the platelet concentration was optimal at 4×10^5/mm.^{3}. Thus 0.1 ml. of this serum contained 4 units of antibody. When measurements were made using an aliquot of 1 serum sample on 10 separate occasions over a period of several months with platelet suspensions obtained from a different donor each time, the concentration of platelets giving maximum complement fixation per unit of antibody varied 21 per cent from the mean of 10^5/mm.^{3} and the number of units of complement fixed varied 9 per cent from the mean of 10.5 units.

3. Effects of Varying Quinidine Concentration.—The amount of complement fixed by a constant concentration of antibody and platelets as a function of quinidine concentration is shown in Fig. 4. Complement fixation was not detectable with up to 10.2 units of antibody and an optimal antibody:platelet

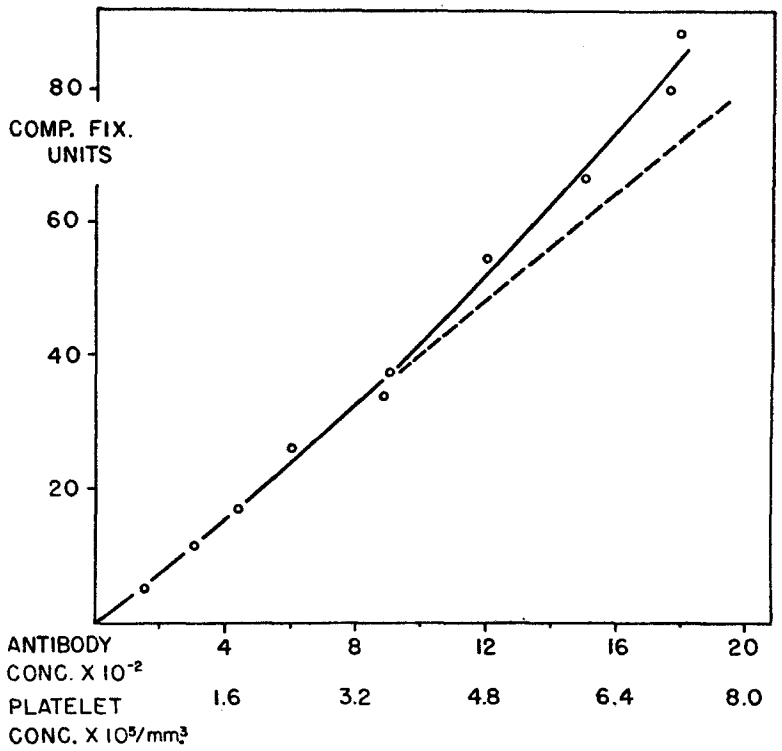

FIG. 3. Effects of increasing the concentration of antibody and platelets while maintaining an optimal antibody:platelet ratio. Conditions as in Fig. 1; same serum sample. A platelet concentration of 4×10^5/mm.^{3} produced maximum complement fixation with 0.1 ml. of patient's serum when determined as in Fig. 1. In other tubes the same ratio of platelet concentration to antibody concentration was maintained. The dashed line is a projection of the linear portion of the experimental curve to show more clearly the deviation with higher antibody and platelet concentration.

ratio when the quinidine concentration was $10^{-6.56}$ M, and was measurable only with high concentrations of antibody when quinidine concentration was 10^{-6} M. The amount of complement fixed increased with logarithmic increases in quinidine concentration and reached a maximum with all concentrations of antibody when the quinidine concentration was in the range of $10^{-3.16}$ to $10^{-2.76}$ M. Further increases in quinidine concentration then resulted in decreasing amounts of complement fixed. When quinidine concentration was greater

than $10^{-2.2}$ M, loss of complement activity occurred in control mixtures and was associated with visible precipitation in the mixtures. Because this may have had some non-specific effect on the fixation of complement by the antibody-quinidine-platelet complex, the portions of the curves in which quinidine was anticomplementary are represented by dashed lines. Complete anticomplementary activity of $10^{-1.66}$ M quinidine prevented use of higher concentrations.

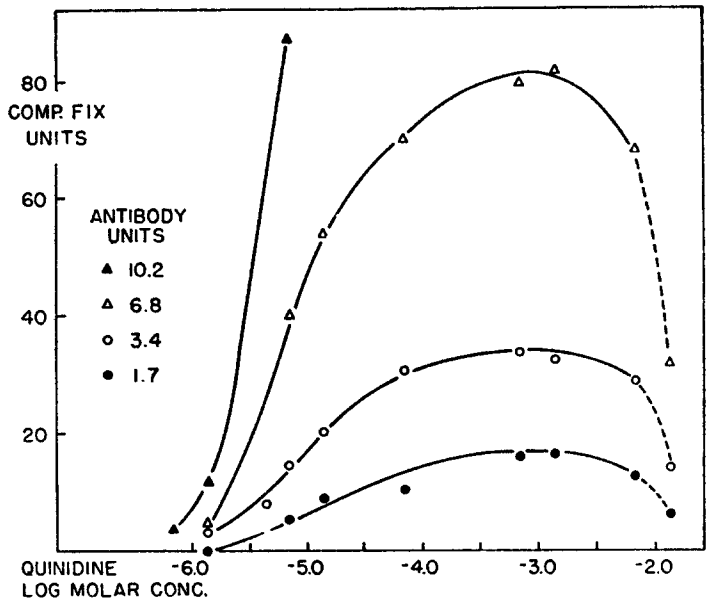

FIG. 4. Effects of varying quinidine concentration. Amounts of antibody are expressed in units as defined in text. The platelet concentration used with each amount of antibody was kept constant at that concentration which gave maximum complement fixation when quinidine concentration was optimal. The amount of complement inactivated by quinidine alone at concentrations greater than $10^{-2.2}$ molar was subtracted from the total amount fixed to obtain the plotted values.

A quinidine concentration of $10^{-2.914}$ M (0.64 mg. quinidine gluconate/ml.) was used as an optimal concentration in the above experiments.

4. Amount of Complement Fixed and Amount of Antibody Adsorbed as a Function of Platelet Concentration at Different Quinidine Concentrations.—Fig. 5 shows complement fixation as a function of platelet concentration at different concentrations of quinidine. One amount of antibody was used (5.14 units). Although the maximum amount of complement fixed was decreased when quinidine concentration was sub-optimal or above optimal, the concentration of platelets required for maximum complement fixation remained approximately the same. In Fig. 5 the maximum amount of complement which could

be fixed by a single amount of antibody was limited by quinidine concentration, whereas in Fig. 1 the maximum amount of complement which could be fixed was limited by antibody concentration. The relationships in Fig. 1 are duplicated in Fig. 5 by the uppermost solid line curve (5.14 units of antibody) and the dashed line curve (2.57 units of antibody), both of which were obtained using an optimal concentration of quinidine.

If decreases in complement fixation at high and low quinidine concentrations were related simply to decreased amounts of antibody attaching to platelets, the relationship between platelet concentration and complement fixation in

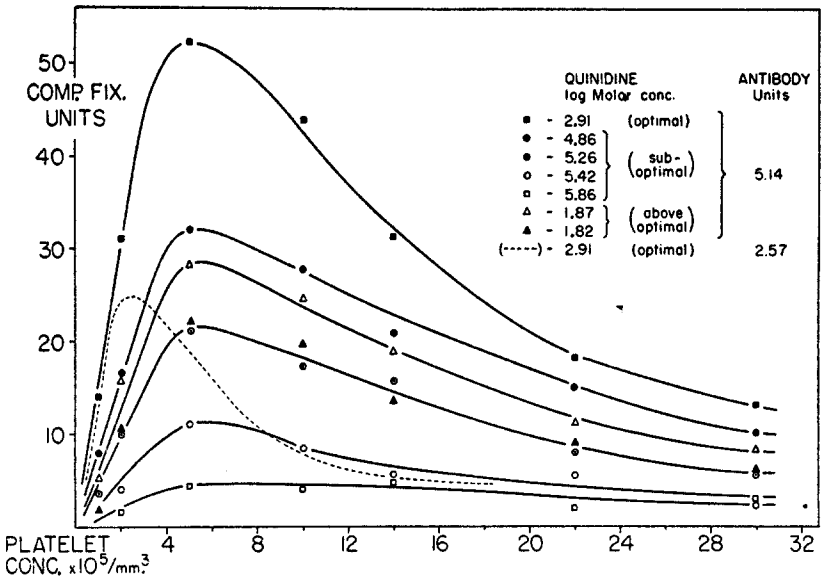

FIG. 5. Complement fixation as a function of platelet concentration at different concentrations of quinidine. See text. Conditions as in Fig. 4.

Fig. 5 might be expected to be more nearly like that in Fig. 1. It seemed therefore that the amount of antibody adsorbed per platelet was not the only factor determining the amount of complement fixed at different quinidine concentrations.

The effect of quinidine concentration on adsorption of antibody by platelets is shown in Fig. 6. The amount of antibody adsorbed by platelets in mixtures of antibody, quinidine, and platelets is compared with the amount of complement fixed by the same mixtures. The uppermost dashed line represents the amount of complement fixed and the uppermost solid line represents the amount of antibody adsorbed when quinidine concentration was optimal at $10^{-2.91}$ M. Maximum complement fixation was obtained when the platelet concentration was approximately 2×10^5/mm.^{3}; and, at this concentration of platelets, ap-

proximately 70 per cent of the antibody was adsorbed. More antibody was adsorbed when platelet concentration was increased but the net result was a decrease in the amount of antibody adsorbed per platelet and in the amount of complement fixed. When quinidine concentration was above optimal at $10^{-1.87}$ M,

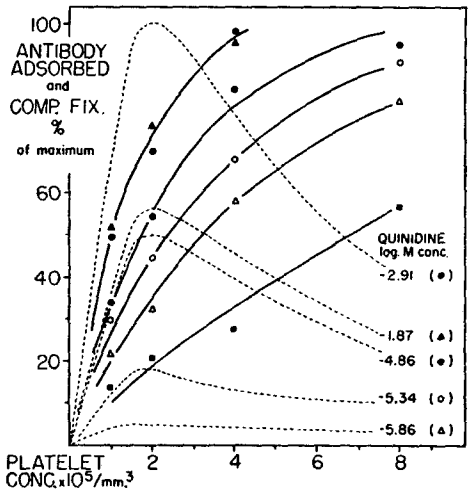

FIG. 6. Effect of quinidine concentration on adsorption of antibody by platelets. Two units of antibody used; 100 per cent complement fixation equivalent to 21 units of complement fixed. Dashed lines are curves of complement fixation obtained as in Fig. 5 at the concentrations of quinidine indicated on the graph. Similar mixtures of antibody, platelets, and quinidine but without complement were used to measure amounts of antibody adsorbed at selected concentrations of platelets. After incubation for 1 hour, platelets with adsorbed antibody (complexes) were removed from the mixtures by centrifugation. Antibody remaining in the supernatant fluid of each mixture was titered as in Fig. 1 using aliquots of the supernatant fluid in the usual complement fixation mixture with various concentrations of fresh platelets and sufficient additional quinidine to give an optimal quinidine concentration. Antibody in the supernatant fluid of mixtures containing above-optimal quinidine was measured after the supernatant fluid had been dialyzed for 48 hours at 5° against saline to remove excessive quinidine. The curve of antibody adsorption represented by (■—■) was obtained with $10^{-6.16}$ M quinidine; there is no corresponding dashed-line curve of complement fixation because no measurable amount of complement was fixed at any concentration of platelets.

the amount of antibody adsorbed per platelet was so similar to that at optimal quinidine concentrations that only a single curve could be drawn through both sets of experimental points. The maximum amount of complement fixed at the higher concentration of quinidine was, however, greatly reduced. When quinidine concentration was progressively decreased below optimal, less antibody was adsorbed per platelet and less complement was fixed. However, the amount of complement fixed was decreased much more than was the amount of antibody adsorbed per platelet. At very low concentrations of

quinidine, antibody was adsorbed on platelets in spite of the fact that no complement fixation could be measured [*e.g.*, $10^{-6.16}$ M].

Although equivalent amounts of antibody were adsorbed per platelet at optimal and above optimal quinidine concentration, the amount of complement fixed was less with above optimal quinidine concentration. With suboptimal concentrations of quinidine, decreases in the amount of complement fixed were relatively greater than decreases in the amount of antibody adsorbed per platelet. These findings could be explained if attachment of antibody to platelets could occur with different numbers of quinidine molecules, and if complement were fixed only when the attachment occurred with an optimal number of quinidine molecules but not with too few or too many quinidine molecules.

5. Rates of Complement Fixation with Different Concentrations of Quinidine and Antibody.—In Fig. 7 the rates of complement fixation are shown when quinidine concentration was sufficient to give maximum complement fixation (curve 1), and when complement fixation was decreased because of too little quinidine (curves 3 and 5) and too much quinidine (curve 6), all with the same concentration of antibody and platelets. The rate of complement fixation was proportional to the concentration of quinidine. This was true even when the final amount of complement fixed was decreased at above optimal concentrations of quinidine (curve 6). All curves approached an asymptotic maximum which did not change significantly after incubation for 1 hour.

That the rate of complement fixation represented the rate of antibody-quinidine-platelet complex formation can be seen by comparing curves 1 and 7. Curve 7 was obtained by permitting the complex to form under the same conditions as for curve 1 for 1 hour before complement was added. The preformed complex immediately fixed most of the complement which was to be fixed. When complement was present before formation of the complex in mixtures which gave rapid complement fixation when quinidine concentration was high (curve 6), there was no delay in fixing the total amount of complement which was to be fixed. It would appear that complement, when present during formation of the complex, is fixed even more rapidly than indicated in curve 7; and that the rate of complement fixation actually indicates the rate of complex formation. It is evident from the above experiment that complement fixation is a reaction subsequent to antibody-quinidine-platelet complex formation.

Curve 2 shows the rate of complement fixation when quinidine concentration was the same as in curve 1 but antibody concentration was half that in curve 1. Similarly, quinidine concentration in curve 4 was the same in curve 3 but antibody concentration was decreased by half. The experimental points permitted drawing curves 1 and 2 and curves 3 and 4 as twofold multiples with the same reaction half-times which are indicated by arrows. The fact that the

half-time of the reaction at any one concentration of quinidine was independent of the concentration of antibody indicated that the rate constant of the reaction was dependent on quinidine concentration alone. It therefore appeared that the amount of quinidine present, even at low quinidine concentrations

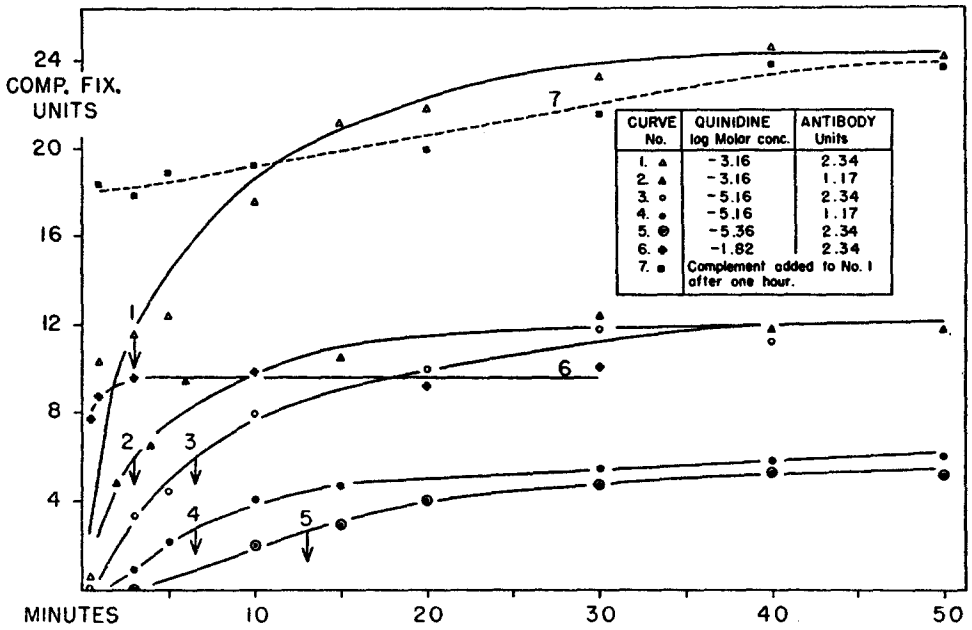

FIG. 7. Rates of complement fixation with different concentrations of quinidine and antibody. The concentration of platelets used with 2.34 and 1.17 units of antibody was the same (2.0×10^5/mm.^{3}). Rate of complement fixation was measured beginning after the addition of quinidine which was the last reagent added to the complement-fixing mixtures. Mixtures were agitated continuously after addition of complement. In curve 7, complement was added 1 hour after other reagents and timing was begun after addition of complement. Complement fixation was stopped at the different time intervals by diluting the complement-fixing mixture 20-fold and immediately removing platelets by centrifugation. With anticomplementary concentrations of quinidine, controls were run at each time interval.

which resulted in decreased complement fixation (*e.g.* curves 3 and 4), was in large excess compared to the amount of antibody.

6. Attempts to Distinguish Steps in the Reaction.—The following experiments were done in an attempt to determine whether a reaction occurred between quinidine and antibody or quinidine and platelets, one of which appeared to be essential as the initial step of the over-all reaction.

(a) Effects of order of addition of reagents:

Antibody and quinidine, platelets and quinidine, and platelets and antibody, were incubated together in the presence of complement before the third component of the complex was

added and the rate of complement fixation determined as shown in Fig. 8. Preincubating any two components of the complex before adding the third produced no difference in the rate of complement fixation. Similar experiments done at 25° with quinidine concentration $10^{-2.9}$ and $10^{-5.36}$ M and with antibody and platelet concentration fourfold that of Fig. 8 also resulted in no detectable difference in rate due to pre-incubating any two components. When platelets and antibody were pre-incubated separately in $10^{-4.46}$ M quinidine and the third component was added along with sufficient saline to produce a fivefold dilution of the incubated reagents, bringing the final quinidine concentration to $10^{-5.16}$ M, the rate of complement fixation was the

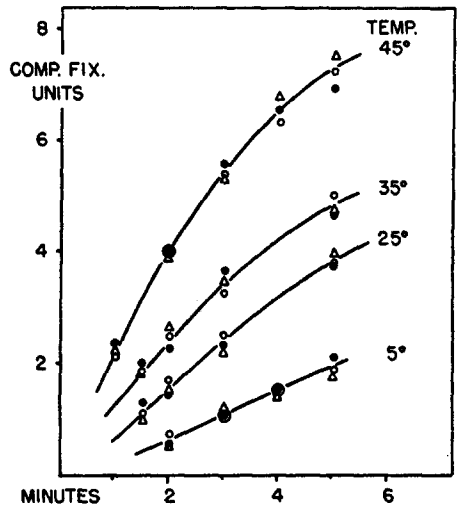

FIG. 8. Effect of order of addition of reagents on rate of complement fixation at different temperatures. An optimal platelet concentration was used with 2.2 units of antibody in the presence of $10^{-5.16}$ M quinidine. (●—●) antibody and quinidine preincubated, (○—○) platelets and quinidine preincubated, (△—△) antibody and platelets preincubated for 15 min. in presence of complement before the missing reagent was added and complement fixation determined as in Fig. 7. Both pre-incubation and complement fixation were carried out at the temperatures indicated on the graph. Each point is the average of two determinations. There was no loss of complement activity at 45° in control tubes during the experimental period.

same in each case and was equal to that obtained when quinidine was added as the third reagent to give a final concentration of $10^{-5.16}$ M. It therefore appeared that association of either quinidine and antibody or quinidine and platelet as well as the reverse reaction occurred very rapidly compared to the rate of formation of the complete complex.

It was considered that an isomer of quinidine might retard equilibrium formation by competing with quinidine for sites of attachment. It was found that cinchonine on a molar basis was as effective as quinidine in complement-fixing reactions whereas quinine and cinchonidine were 10^{-3} times as effective, their small amount of activity being due no doubt to incomplete separation of the isomers. In the one case, antibody was incubated with $10^{-3.16}$ M quinine (or cinchonidine) followed by addition of platelets mixed with quinidine to give a final quinidine concentration of $10^{-5.16}$ M; and in the other case, platelets were incubated with $10^{-3.16}$ M quinine (or cinchonidine) followed by addition of antibody mixed with quinidine to give a

final quinidine concentration of $10^{-5.16}$ M. The rate of complement fixation was the same in each case and was equal to that obtained with $10^{-5.16}$ M quinidine when no quinine or cinchonidine was present. The isomers, quinine and cinchonidine, did not measurably interfere with the rate of complement fixation when present at a concentration 100-fold that of a suboptimal quinidine concentration.

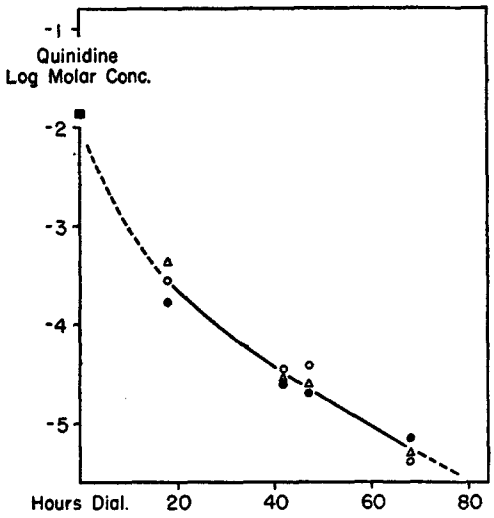

FIG. 9. Rate of removal of quinidine from normal serum and serum containing antibody by dialysis against saline. Quinidine was added to a normal serum sample, to pooled serum from five normal individuals, and to the patient's serum containing 32 units of antibody/ml. to give a final concentration of $10^{-1.86}$ M quinidine. Four 1 ml. aliquots of each serum sample were all dialyzed together in ¼ inch dialysis tubing against 3 l. of saline at 5°. At 8, 18, 42, and 49 hours the dialysis fluid was changed. At 18, 42, 47, and 68 hours quinidine concentration in an aliquot of each serum sample was measured by using 0.2 ml. of the serum sample as the source of quinidine in the usual complement-fixing mixture with optimal platelets and a 0.5 ml. total volume. When measuring quinidine content of normal serum, 0.2 ml. of the patient's plain serum dialyzed against saline in a separate container was used as a source of antibody, and 0.2 ml. of plain normal serum dialyzed against saline was added to the mixture when measuring quinidine content of the patient's serum. Quinidine concentration was determined by the amount of complement fixed using a reference curve prepared as in Fig. 4 with mixtures of dialyzed patient's and normal serum and known concentrations of quinidine. Original quinidine concentration in all samples (■), concentration in single normal serum sample (●—●), in pooled normal serum (○—○), and in serum containing antibody (△—△). It was noted that after the 8 hour change of dialysis fluid the concentration of quinidine in the dialysis fluid was too low to measure; *i.e.*, less than $10^{-6.2}$ M.

(*b*) *Affinity of serum containing antibody and normal serum for quinidine:*

In an attempt to determine whether the presence of antibody increased the non-specific affinity of serum for quinidine, the rates of removal of quinidine from serum containing antibody and from normal serum by dialysis were compared, as shown in Fig. 9. The presence of antibody in amounts up to 32 units/ml. had no measurable effect on the binding of quinidine

TABLE II

Treatment of Platelets to Decrease Their Nitrogen Content and Maintain Their Complement-Fixing Activity

Column 1, all procedures done at 25° unless otherwise indicated. All platelet preparations could be stored at 5° for at least 1 week without loss of activity. Column 2, observations under phase microscopy. Column 3, per cent activity based on relative volume of suspensions required to produce equivalent increments of complement fixation in the presence of excess antibody and optimal quinidine in the usual complement-fixing mixture; 0.01 ml. of suspension 1 fixed 3.8 units of complement. Column 4, 100 per cent nitrogen content equivalent to 0.94 mg. nitrogen/ml. of suspension, determined by semi-micro-Kjeldahl technique. Column 5, ratio of complement-fixing activity to nitrogen content.

1 Platelet preparation	2 Appearance of platelets	3 Complement fixing activity	4 Nitrogen Content	5 Column 3/Column 4
		per cent	*per cent*	
No. 1: Suspension in saline prepared as in Materials and Methods platelet conc. 2×10^6/mm.^{3}	Similar to fresh platelets in plasma	100	100	1.0
No. 2: Platelets sedimented from Prep. 1 (2000 *g* × 15 min.) incubated 1 hr. in each of 3 changes of distilled water 5 times original suspension volume. Final suspension in original volume of saline, platelet conc. 1.5×10^6/mm.^{3}	Discrete, moderately swollen, slightly less refractile with irregular membranes containing numerous spicules. Some unaltered	72	58.7	1.23
No. 3: Prep. 1 quick-frozen and thawed 3 times, then washed 3 times in distilled water. Final suspension in original volume of saline platelet conc. approx. 1.3×10^6/mm.^{3}	Many discrete but numerous small clumps. Almost all greatly swollen, non-refractile and granular, membranous margins indistinct (*i.e.* "ghosts"). Few as in No. 2	63.7	32.1	1.99
No. 4: Prep. 3 centrifuged 1000 *g* for 10 min. ppt. discarded. Supernatant fluid centrifuged 14,000 *g* for 10 min., ppt. resuspended in original volume of saline, platelet conc. 1.8×10^5/mm.^{3}	As in No. 3 but all discrete	10.4	1.75	5.95

by serum. Because normal serum proteins adsorb quinidine so strongly, their presence in such relatively high concentration may have masked possible specific binding of quinidine by antibody.

In the reverse experiment in which one volume of plain normal serum and plain patient's serum (32 units of antibody/ml.) were dialyzed in separate dialysis bags against 1,000 volumes of 10^{-6} M quinidine, the concentration of quinidine in both serum samples reached $10^{-3.5}$ M in 24 hours at 5°, as determined using techniques of Fig. 9. Thus it would appear that antibody had at least the same ability as normal serum proteins to adsorb quinidine from highly dilute solution.

(c) *Affinity of intact platelets and treated platelets for quinidine:*

It was found that platelets could be treated in various ways to decrease their nitrogen content without affecting their activity in complement-fixing reactions (Table II). As shown in Table III, intact platelets (Prep. 1, Table II) adsorbed quinidine in amounts proportional to the concentration of quinidine in which they were incubated, washed platelets (Prep. 2) adsorbed much less quinidine, and platelet "ghosts" (Prep. 3) adsorbed no measurable amount of quinidine. Since the activity per platelet in complement-fixing reactions remained approximately the same in the different preparations, it might appear that adsorption of quinidine by platelet was not the initial step in complex formation. However, because quinidine even at $10^{-5.16}$ M concentration (3.6 μg. quinidine gluconate/ml.) was in large excess compared to

TABLE III

Adsorption of Quinidine by Platelets Treated as in Table II

Sedimented platelets from suspensions prepared as in Table II were resuspended in different concentrations of quinidine, incubated 1 hour at 25°, sedimented again, and quinidine content of the supernatant fluid determined spectrophotometrically. Amounts of quinidine adsorbed were equivalent to decreases in quinidine concentration in the supernatant fluid. Quinidine gluconate, 0.01 mg./ml. equivalent to $10^{-4.72}$ M quinidine.

Conc. of quinidine gluconate	Micrograms quinidine gluconate adsorbed per milliliter by:		
	Prep. 1 2×10^9 platelets/ml.	Prep. 2 2×10^9 platelets/ml.	Prep. 3 approx. 4×10^9 platelets/ml.
mg./ml.			
0.01	3.8	1.3	0
0.03	6.6	2.8	0
0.1	20.0	9.1	0
0.3	33.2	—	—
1.0	104.0	40.0	0
3.0	152.0	93.0	0

the amount of antibody taking part in complement-fixing reactions (see Fig. 7), it is possible that amounts of quinidine required for the reactions could be adsorbed by platelet "ghosts" and yet be insufficient for detection by the method of chemical analysis used. Any quinidine which was adsorbed by 10^9 intact platelets incubated in $10^{-3.72}$ M quinidine was not retained in sufficient amount to cause complement fixation when the platelets were thereupon mixed with 3 ml. of saline, immediately sedimented, and resuspended in the presence of excess antibody. Therefore any reaction between quinidine and platelets which might have occurred was rapidly reversible.

(d) *Affinity of the complete complex for quinidine:*

Reactions involving the removal of quinidine from the antibody-quinidine-platelet complex were slow compared with reactions involving quinidine in the initial step of complex formation.

When a complex, which was formed in 0.5 ml. of $10^{-3.72}$ M quinidine with an optimal antibody:platelet ratio, was sedimented by centrifugation and resuspended in 0.5 ml. of saline containing complement, the amount of complement fixed was approximately 90 per cent of that fixed when the same complex was resuspended in 0.5 ml. of $10^{-3.72}$ M quinidine containing complement. Thus, the rate of complement fixation was more rapid than the rate of dissocia-

tion of quinidine from the complete complex. Complete reversal of the complex by dissociation of quinidine required comparatively long periods of time to take place, as shown in Fig. 10. Dissociation of quinidine from the complex by dialysis also required longer periods of time than removal of quinidine from serum alone. When the complete complex, made by adding an optimal concentration of platelets to the same mixture of patient's serum plus quinidine used in Fig. 9, was dialyzed against saline and tested periodically for quinidine content as in Fig. 9, but without additional platelets, apparent decreases in quinidine concentration took

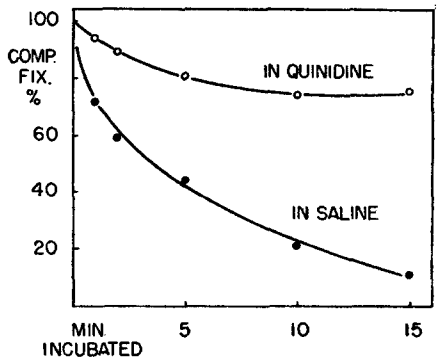

FIG. 10. Dissociation of the complete complex during incubation in saline. A series of tubes containing 2.5 units of antibody, optimal platelet concentration, and $10^{-3.72}$ M quinidine in a 0.5 ml. volume were incubated for 1 hour, centrifuged, and the supernatant fluid removed. Each complex consisting of antibody attached to sedimented platelets was resuspended in 3 ml. of saline, and after periods of incubation at room temperature the complexes were sedimented again and resuspended in 0.5 ml. of $10^{-2.9}$ M quinidine containing complement for the usual measurement of complement fixation. Controls, designed to measure degree of dissociation of the complex caused by an increase in volume alone, consisted of complexes formed at $10^{-3.72}$ M quinidine (in a 0.5 ml. volume) which were resuspended for varied intervals in 3 ml. of $10^{-3.72}$ M quinidine, sedimented, and resuspended again in 0.5 ml. of $10^{-2.9}$ M quinidine containing complement for complement fixation. 100 per cent complement fixation was equivalent to the amount of complement fixed by the complex formed in 0.5 ml. of $10^{-3.72}$ M quinidine, sedimented, and resuspended directly in 0.5 ml. of $10^{-2.9}$ M quinidine containing complement. (O—O) control, complexes incubated in 3 ml. of quinidine; (●—●) complexes incubated in 3 ml. of saline.

two to three times longer to occur than equivalent decreases in quinidine concentration in plain serum. It therefore appeared that quinidine was bound much more strongly in the antibody-quinidine-platelet complex than by antibody or platelets alone.

It is worthy of note that once complement was fixed by the complex, dissociation of the complex by incubation in saline or by dialysis did not result in recovery of complement activity in spite of the fact that antibody and platelets could be recovered from the dissociated complex with unaltered activity. Complement fixation in all of the above experiments appeared to be a stoichiometric reaction, and any equilibrium existing between the complex and complement appeared to be far to the right (*e.g.* Table I). Failure to recover complement following reversal of the complex therefore implied that complement fixed by the complex was altered, either when bound or when dissociated, in such a way that its activity could no longer be measured by usual techniques.

7. Nature of the Platelet Component Involved in Complex Formation.—Platelet "ghosts", the recognizable remnants of individual platelets containing as little as 20 per cent of the original platelet nitrogen (Prep. 4, Table II), were as active in complement-fixing reaction as a similar number of intact platelets. This suggested that the membranous portion of platelets contained the component necessary for complex formation. When a platelet suspension was triturated in a glass tissue homogenizer to produce particulate fragments showing Brownian motion, complement-fixing activity was present in the slightly opalescent filtrate of the suspension obtained with Whatman No. 1 filter

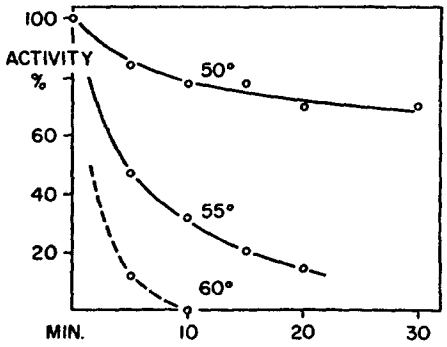

FIG. 11. Effect of heat on the complement-fixing activity of platelets. Aliquots of a saline suspension of platelets were heated for varying periods at different temperatures as indicated on the graph. Per cent activity of heated suspensions was based on the relative volume of suspension required to produce equivalent increments of complement fixation in the presence of excess antibody and an optimal quinidine concentration in the usual complement-fixing mixture. 100 per cent activity was that obtained with the unheated aliquot. There was no loss in activity when platelets were heated for 1 hour at 45°. Platelets heated at 60° for 15 minutes showed morphological changes no more marked than in Prep. 2, Table II.

paper, but no activity remained in the clear filtrate obtained with a fritted-glass filter. Particulate platelet material was essential for complement fixation in all platelet fractions tested. Complement-fixing activity of platelets persisted in strongly acid but not in strongly alkaline solution and was only moderately decreased by dehydrating platelets in air. The manner in which complement-fixing activity decreased when platelets were heated (Fig. 11) suggested that the platelet material involved in the reaction might be proteinaceous in nature. Platelets heated at 60° for 15 minutes had no complement-fixing activity and also did not adsorb antibody, indicating that the platelet component involved in complex formation had been destroyed.

DISCUSSION

A mechanism for the molecular associations involved in formation of the antibody-quinidine-platelet complex and in fixation of complement by the

complex could be deduced from the experimental results. There was evidence only of stoichiometric combination between the four reactants and the reactions were most likely bimolecular and sequential. The sequence appeared to be, first, combination of quinidine with either antibody or platelets, next, formation of the complete antibody-quinidine-platelet complex, and finally, binding of complement by the complex. Whether quinidine combined with antibody or platelets in the initial step could not be determined on the basis of data obtained. The rate-limiting step was formation of the complete complex. The rate constant of the reaction was dependent on quinidine con-

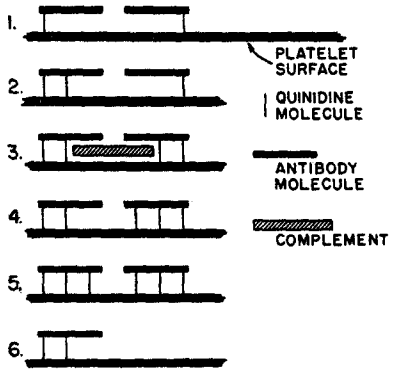

FIG. 12. Schematic representation of the model. Complex 3 is the type which binds complement; others are examples of types which do not bind complement. The distribution of the number of quinidine molecules in the complex would be determined by quinidine concentration, complexes 1 and 2 being more frequent at low quinidine concentrations, and complexes 4 and 5 more frequent at high quinidine concentrations. The frequency with which antibodies would be attached as nearest neighbor pairs would be determined by the antibody: platelet ratio.

centration alone and quinidine was always in large excess compared to the amount of antibody. Some conclusions could be drawn concerning the spatial and stoichiometric relationship between the reactants. In view of their large size, platelets (or particulate platelet fractions) could be considered to provide surfaces containing numerous sites for antibody attachment. The dependence of complement fixation on the number of antibodies attached per platelet suggested that proximity of attached antibodies was essential for complement to be bound. Compared to the findings at optimal quinidine concentration, complement fixation at above-optimal quinidine concentration was decreased with no change in the amount of antibody adsorbed per platelet, and complement fixation at suboptimal quinidine concentration was decreased much more than the amount of antibody adsorbed per platelet. Assuming that the number of quinidine molecules effecting attachment of antibody to platelet varied with quinidine concentrations, it was possible that

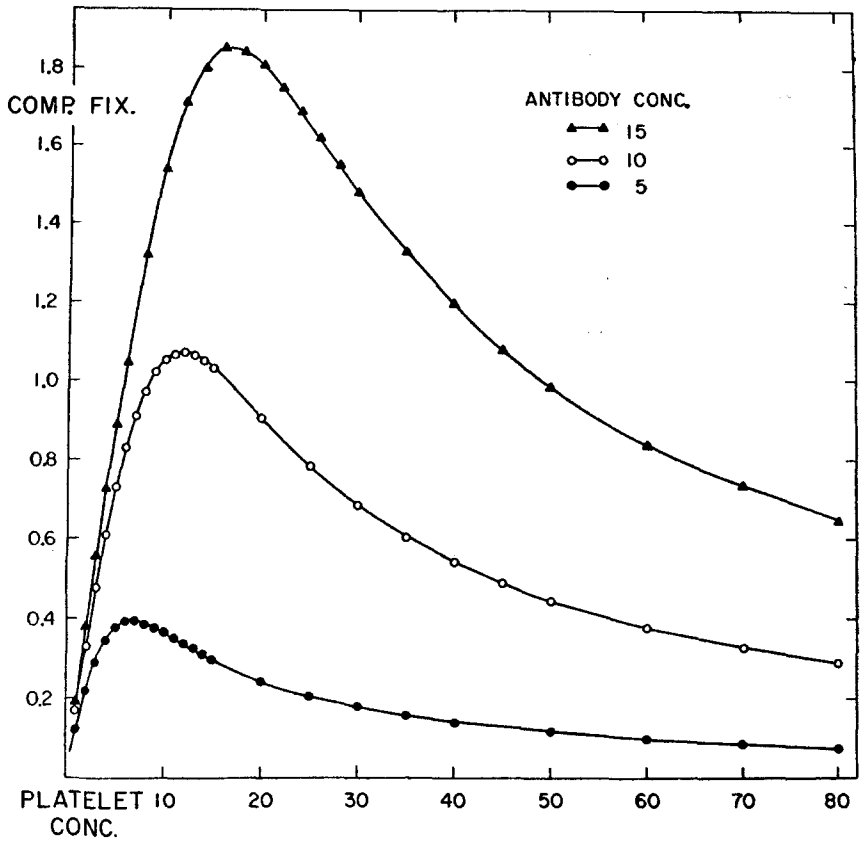

FIG. 13. Theoretical results; platelet concentration varied, antibody concentration constant. Calculations were made using Equations 21, 23, and 24 of Paper II. Arbitrary values, found to be convenient for computation, were assigned to terms proportional to the concentration of quinidine (q in Equation 21), of platelets (p in Equation 23), and of antibody (A in Equation 23). The term θ'_q was calculated using Equation 21, and the term θ_a was calculated using Equation 23. Values proportional to the amount of complement fixed, C, were then calculated using Equation 24. A single quinidine concentration ($q = 1.3$, $\log q = 0.114$), which was found to be optimal (see Fig. 16), was used throughout. Relationships between antibody and platelet concentration and the amount of complement bound were proportionately similar when calculations were made using antibody values from 2.5 to 25.

complement was fixed only when antibodies were attached by an optimal number of quinidine molecules. Complement was considered to have the properties of a molecule. Because the apparent equilibrium between complement and the complex was far to the right, it could be assumed that a "complement molecule" would be bound whenever an appropriate site for its attachment was available.

The following model for the reaction incorporates the above conclusions

in what appears to be the simplest form. Platelets provide surfaces containing a distribution of independent equivalent sites for antibody attachment. Each site can be occupied by one antibody and as many as three quinidine molecules. An antibody will not occupy a site when there is no quinidine molecule

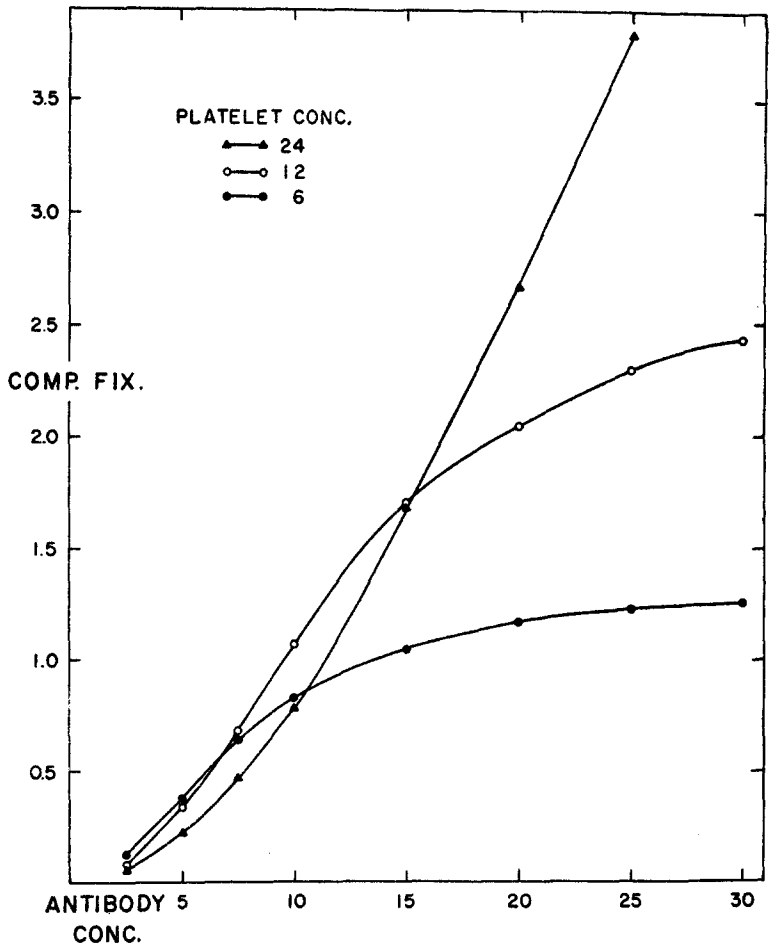

FIG. 14. Theoretical results; antibody concentration varied, platelet concentration constant. See Fig. 13.

associated but can occupy a site along with one, two, or three quinidine molecules. It is possible that quinidine attaches first to an antibody which then occupies a platelet site or attaches first to a platelet site which is then occupied by an antibody. Complement can be bound between antibodies whenever each of two neighboring platelet sites is occupied with one antibody

and two quinidine molecules. Complement will not be bound if the sites occupied by antibodies are not nearest neighbors or do not each contain exactly two quinidine molecules. The model is represented schematically in Fig. 12.

This model was amenable to mathematical description. Dr. Terrell L. Hill, considering that the steps of the reaction could be treated mathematically in the same manner as association between molecules in dilute solutions, derived formulae describing the properties of the assumed system using methods of statistical mechanics. He incorporated the principles of the model into equations in such a way that theoretical results could be calculated

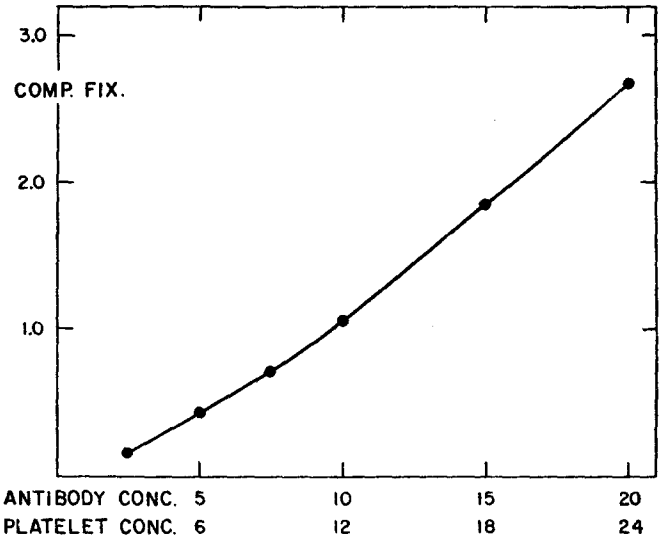

FIG. 15. Theoretical results; concentration of antibody and platelets varied maintaining an optimal antibody:platelet ratio. See Fig. 13.

by assigning values for concentration of reactants as they were used experimentally. Dr. Hill's derivations are in Paper II of this series. In one derivation it was assumed that quinidine was bound first by antibody and in the other that quinidine was bound first by platelets. Both derivations gave the same final equation for use in theoretical calculations. Arbitrary values, found to be convenient for computation, were assigned to terms in the equation representing antibody, platelet, and quinidine concentrations to calculate the theoretical amount of complement fixed under various conditions.

Theoretical results in Figs. 13, 14, and 15 correspond to experimental results in Figs. 1, 2, and 3, respectively. These figures show effects of varying antibody and platelet concentrations on the amount of complement fixed in the presence of a constant, optimal concentration of quinidine. Theoretical curves of complement fixation with varied quinidine concentration are shown in Fig. 16 which

correspond to experimental results in Fig. 4. The theoretical amount of antibody bound by platelets and the platelet concentration required for maximum complement fixation when quinidine concentration was varied are shown in Fig. 17 which corresponds to experimental results in Figs. 5 and 6. Because there was no way of relating assigned values to actual quantities of reagents

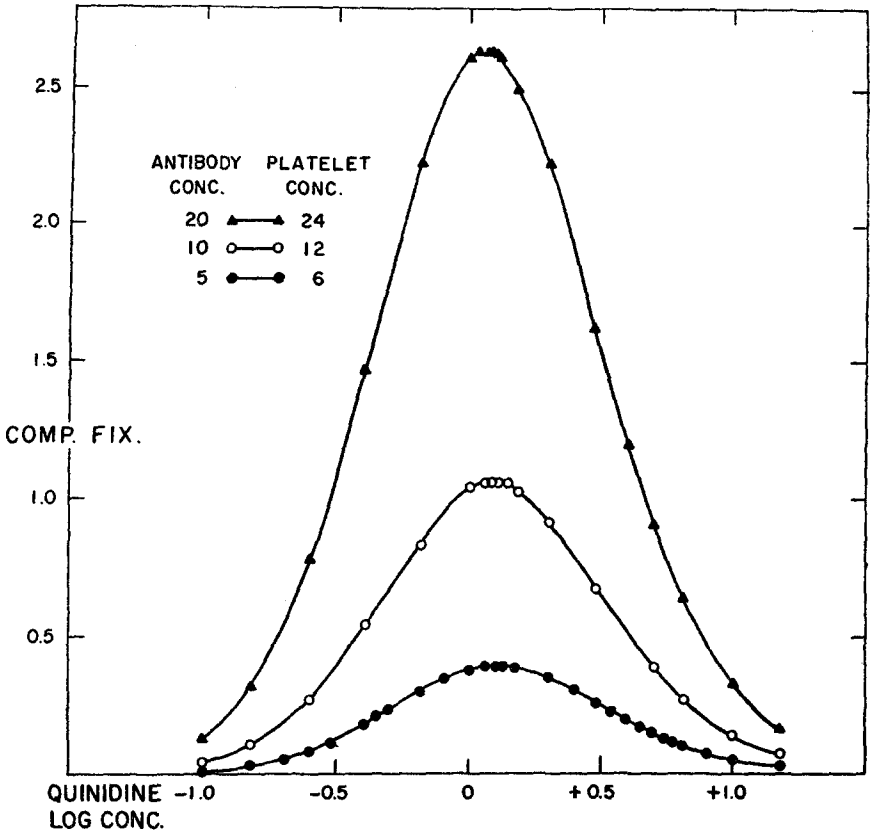

FIG. 16. Theoretical results; quinidine concentration varied, antibody:platelet ratio constant. Calculations made as in Fig. 13 but quinidine concentration varied.

used experimentally, only qualitative comparisons of experimental and theoretical results were possible. In each case experimental and theoretical results were quite similar.

The most evident difference between experimental and theoretical results involved the amount of complement fixed and the amount of antibody adsorbed with changes in quinidine concentration. The theoretical amount of antibody adsorbed (Fig. 17) changed more abruptly with changes in quinidine concentration than did the measured amount of antibody adsorbed (Fig. 6),

and the quinidine concentrations required for theoretical curves of complement fixation covered half as many decades on a log scale as the quinidine concentrations required for experimental curves of complement fixation (*cf.* Figs. 16 and 4). In addition the experimental curves of Fig. 4 had a skewed configuration, but complications involved in using high quinidine concentrations experimentally (anti-complementary effect) may account for this.

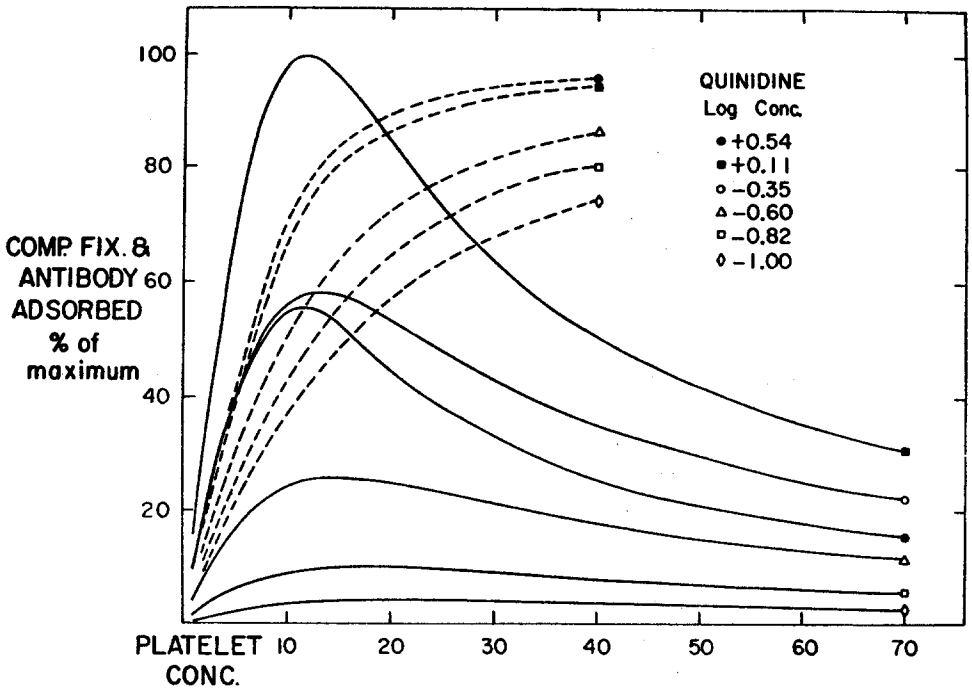

FIG. 17. Theoretical results; effects of quinidine concentration on platelet concentration required for maximum complement fixation and on amount of antibody bound by platelets. Solid line curves of complement fixation calculated as in Fig. 13 with different values for quinidine concentration (see Fig. 16). Dashed line curves of amount of antibody bound calculated using Equation 25 of Paper II. Antibody concentration constant at $A = 10$. Maximum amount of complement fixed = 1.07.

The model on which the equations were based represented an attempt to describe the reactions as simply as possible. Thus it was assumed that all quinidine and antibody molecules attached with equal ease and that all platelet sites or molecules of antibody were equivalent and had the same valence for quinidine. The actual reactions were no doubt complicated by a number of factors such as: a specific distribution of heterogeneity of valence for quinidine including the possibility that more than three quinidine molecules are involved per antibody (*e.g.* 12), changes in association constants with succeeding at-

tachments of quinidine or antibody molecules, the possibility of sterically determined order of addition of quinidine molecules, and possible favored adsorption of antibody with a specific number of quinidine linkages. All of these possible complications would not fundamentally change the concepts of the model but could account for the observed differences between theoretical and experimental results. The fact that experimental and theoretical results differed so little suggests that the model resembles approximately the true mechanism and sequence of reactions involved in complex formation and fixation of complement by the complex.

It has generally been considered that antibodies which attach to platelets or other cells have been formed either against some specific cellular substance or against some common antigen shared by the cells. If it had been demonstrated that quinidine combined with platelets in the initial step of antibody-quinidine-platelet complex formation it could have been concluded that a platelet substance in combination with quinidine was the antigenic component of the reaction. But it was not possible to determine experimentally whether antibody or platelets combined first with quinidine, and mathematical derivations based on both possibilities resulted in the same final equations for theoretical calculations. It therefore appeared equally possible that platelets were not part of the antigen.

The assumption that platelets in combination with quinidine form the antigen necessitates making other assumptions which are not in accord with previous immunologic observations. For instance, it would be necessary to assume that individuals who develop sensitivity have a peculiar ability to form antibodies against very weakly adsorbed haptene, as was suggested by Ackroyd (5). In addition, if platelet-protein in combination with quinidine were the antigen, inhibition of antibody attachment by haptene might be expected (13), but was not observed even at the highest possible quinidine concentrations used experimentally. There may have been sufficient competition by the protein portion of the antigen with antibody for haptene to prevent such inhibition; but this would be a unique immunologic reaction in contrast with the usual circumstances in which a pre-formed protein-haptene antigen may compete more strongly than haptene alone for antibody (13).

An alternate possibility is that the antibody may be formed, not against platelets with weakly adsorbed haptene, but against some non-specific protein combined with haptene. In this case the antigen could be a stable complex, possibly with an oxidative product of the haptene (13), which would be more likely to promote antibody formation. Combination between antibody and haptene could be considered to take place in the classical manner (13). Antibody, which was unable to be adsorbed by platelets in the absence of haptene, would acquire that ability when combined with haptene. Platelets would enter the reaction non-specifically by virtue of having sites on their membranes

with appropriate charges and configuration to permit attachment of antibodies containing haptene molecules. Such adsorption of antibody would be similar to the non-specific adsorption of various proteins on membranes of platelets and other cells (14, 15). Haptene inhibition of antibody attachment would not be expected to occur. The idiosyncrasy would not necessarily involve an unusual ability to form antibodies against a weakly adsorbed haptene, but might involve some other peculiarity such as the formation of a stable antigenic protein complex with an abnormal metabolite of a certain drug. Similar diseases of sensitivity associated with attachment of antibodies to cells would not have to be considered the result of antibody formation against a specific cellular antigen. It is possible that certain antibodies may interact non-specifically with cellular membranes conducive to their adsorption; and in some instances the adsorbed antibody may be injurious to the cell.

Conclusions drawn in the present study concerning the nature of complement and complement-fixing reactions should be as valid as those concerning antibody-quinidine-platelet complex formation. These concepts may have application in further elucidating the controversial nature of complement activity (16–18).

SUMMARY

A steric and kinetic model for the sequence and mechanism of reactions leading to formation of a complex from an antibody, a haptene (quinidine), and a cell membrane (platelets), and to fixation of complement by the complex was deduced from the effects of varying the initial concentration of each component of the complex on the amount of complement fixed, from kinetic aspects of the sequential reactions, and from other chemical and physical properties of the various components involved. Theoretical results calculated using equations based on the model, which were derived by Dr. Terrell L. Hill, were similar in all respects to experimental results. Results of this study were consistent with the possibilities that the protein moiety of a haptenic antigen involved in development of an antibody which attaches to a cell is not necessarily a component of the cell, and that the cell reacts with the antibody by virtue of having a surface favorable for non-specific adsorption of certain haptene-antibody complexes.

I wish to express my appreciation to William G. Clutter, Hospital Corpsman First Class, United States Navy, Miss Mildred L. Garrison, Frederick R. Henry, Chief Hospital Corpsman, United States Navy, and Paul K. Schork, Chief Hospital Corpsman, United States Navy, for their assistance in this work.

BIBLIOGRAPHY

1. Ackroyd, J. F., *Clin. Sc. Inc. Heart*, 1949, **7**, 249.
2. Ackroyd, J. F., *Cl. Sc. Inc. Heart*, 1949, **8**, 235.
3. Ackroyd, J. F. *Cl. Sc. Inc. Heart*, 1949, **8**, 269.

4. Ackroyd, J. F., *Cl. Sc. Inc. Heart*, 1951, **10**, 185.
5. Ackroyd, J. F., *Cl. Sc. Inc. Heart*, 1954, **13**, 409.
6. Ackroyd, J. F., *Brit. Med. Bull.*, 1955, **11**, 28.
7. Bolton, F. G., *Blood*, 1956, **11**, 547.
8. Josephson, E. S., Undenfriend, S., and Brodie, B. B., *J. Biol. Chem.*, 1947, **168**, 341.
9. Brecher, G., and Cronkite, E. P., *J. Appl. Physiol.*, 1950, **3**, 365.
10. Strumia, M. M., Sample, A. B., and Hart, E. D., *Am. J. Clin. Path.*, 1954, **24**, 1016.
11. Kabat, E. A., and Mayer, M. M., Experimental Immunochemistry, Springfield, Charles C. Thomas, 1948.
12. Pauling L., Pressman, D., and Campbell, D. H., *J. Am. Chem. Soc.*, 1944, **66**, 330.
13. Landsteiner, K., The Specificity of Serological Reactions, Cambridge, Harvard University Press, 1946.
14. Abramson, H. A., Moyer, L. S., Gorin, M. H., Electrophoresis of Proteins and the Chemistry of Cell Surfaces, New York, Reinhold Publishing Corporation, 1942.
15. Ponder, E., Hemolysis and Related Phenomena, New York, Grune and Stratton, 1948.
16. Heidelberger, M., and Mayer, M. M., *Advances* in *Enzymol.* 1948, **8**, 71.
17. Mayer, M. M., *Ann. Rev. Biochem.*, 1951, **20**, 415.
18. Marrack, J., *Ann. Rev. Microbiol.*, 1955, **9**, 369.