KINETIC STUDIES ON IMMUNE HEMOLYSIS

II. The Reversibility of Red Cell–Antibody Combination and the Resultant Transfer of Antibody from Cell to Cell during Hemolysis*, ‡

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In a quantitative kinetic study of immune hemolysis by Mayer, Croft, and Gray (1) it was found, in confirmation of old, essentially qualitative observations (2),[1] that the kinetic behavior of a hemolytic system containing excess antibody (A) and limited complement (C′) is different from that of a system containing limited amounts of A and excess C′. Thus, in reactions in which C′ is present in low concentration and A is in excess, the lytic process reaches an end within about 1 hour at 37°C., presumably due to exhaustion of the supply of C′. By contrast, in systems in which C′ is in excess and A is limited, no such end-point is reached, *i.e.*, the reaction continues without cessation over long periods of time. The kinetic curves obtained under these conditions are catalytic in character and thus resemble those observed in enzymatic reactions.

On the basis of these results, it was suggested (1) that C′ behaves like a substance, or complex of substances, which is used up or destroyed in the lytic process. Furthermore, it was pointed out, in agreement with Muir (2), that the catalytic character of the kinetic curves obtained with excess C′ and limited A, may be due to reversibility of the red cell–antibody combination; *i.e.*, it was postulated that a molecule of A can combine with a red cell, perform its lytic function in cooperation with C′, and that eventually it can dissociate and thus become available for combination and action, in conjunction with C′, at some other site on the same or another red cell. In this manner, A could transfer

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[1] We wish to thank Professor C. H. Browning for directing our attention to Muir's work on this subject.

from cell to cell over and over again, and C′ would be utilized continuously, resulting in hemolysis as long as a supply of C′ is available. This concept is based on the following picture of the hemolytic process.

It is assumed that A and C′ attack certain substrate regions, S, at the red cell surface, transforming, altering, or damaging them to a form designated by the letter S′. This reaction takes place in two steps, *i.e.*

$$S + A \rightleftharpoons SA \qquad [1]^2$$

and

$$SA + C' \longrightarrow S'AC_i' \qquad [2]^3$$

in which C_i' denotes inactivated C′. A red cell is considered to lyse when a certain critical number, r, of substrate regions has undergone the change from S to S′. (It appears reasonable to postulate that the transformation from S to S′ continues after lysis of the cell.) In terms of this concept the lysis of a cell results from cumulative damage, and it should be noted that this physical picture of the lytic reaction is essentially equivalent to that described by Ponder (4). It has been pointed out by Alberty and Baldwin (5) that at any time during the lytic reaction the number of damaged substrate regions, S′, will not be the same for all the members of the cell population because an element of chance is involved. As shown by Alberty and Baldwin (5) the fraction of cells, $F(x)$, with x substrate regions reacted, may be calculated from the binomial equation, *i.e.*

$$F(x) = c_x^m p^x (1 - p)^{m-x} \qquad [3$$

in which m is the total number of substrate regions, S, per cell, p is the fraction of the total number of substrate regions in the entire cell population which have reacted, and c_x^m is given by the expression

$$\left(\frac{m!}{x!\,(m - x)!}\right)$$

In terms of this formulation, cells having r, $r + 1$, $r + 2$, ... , or m substrate regions reacted are lysed. Therefore, the summation of $F(r) + F(r + 1) + F(r + 2) + \ldots . F(m)$ gives the fraction y of the cells lysed at any given extent of reaction p; *i.e.*,

$$y = \sum_{x=r}^{x=m} c_x^m p^x (1 - p)^{m-x} \qquad [4]$$

[2] The reversibility of reaction [1] was demonstrated long ago by Morgenroth (3) and Muir (2), and has been confirmed in the present studies.

[3] Reaction [2] is written as an irreversible process mainly because the previous kinetic study (1) indicates that C′ is used up or destroyed; therefore, it is postulated that C′ goes to C_i'. Furthermore, Muir (2) failed to detect dissociation of C′ in experiments on the reversibility of reaction [2]. There is no evidence on the conversion of S to S′, but this appears to be a reasonable hypothesis.

If A is considered to act by mediating the reaction between S and C′ (*i.e.*, by "fixing" the C′ in terms of classical ideas, or by catalytic action in terms of the ideas set forth in the preceding paper (1)), and if it is assumed that the extent of the fundamental reaction, p, is directly proportional to C′ used up in the reaction, provided there is no non-specific loss of C′ (an assumption which is not entirely valid[4]), it follows from the treatment of Alberty and Baldwin (5) that plots of the degree of lysis as a function of the amount of C′ used in a titration of this agent will be S-shaped even if all the cells are identical with respect to their reactivity with C′. Thus, the probability effects give rise to an S-shaped plot; any effects resulting from heterogeneity (*i.e.*, variation of r) will be superimposed. In kinetic experiments the same considerations based on probability theory have to be applied.

In hemolytic systems containing limited amounts of A and excess C′, still another probability effect is introduced due to non-uniform distribution of A on the cells in the suspension. Thus, if the complex SA dissociates extremely slowly, the distribution of A on the cell population attained initially on mixing cells with A (this distribution will depend on the manner of mixing) will not change significantly during a few hours of experimental observation; on the other hand, if dissociation of the complex SA is relatively rapid, the distribution of A will not remain constant. Some of the experiments included in the present report were designed to investigate the dissociability of SA, and the experimental procedures were chosen so as to yield information on the rate of dissociation of SA.[2, 5] As in a previous study (7) of the reversibility of antigen-antibody combination, this was done by the method of competitive reactions, *i.e.*, by the use of a kinetic experimental system in which two portions of antigen compete for a limited supply of A. In the present case this involved mixing a small amount of A with red cell stromata, allowing a few minutes for combination to go to completion, and then adding intact red cells to the stromata-antibody complex. If A dissociates from the stromata, the red cells will pick it up, and in the presence of C′, they will hemolyze. This process of equilibrium displacement is referred to as "transfer" of A. The present study includes several experiments of this type as well as others involving transfer of A from red cell to red cell. Our quantitative experiments along these lines have essentially confirmed the old, qualitative observations of Morgenroth (3) and Muir (2).

With respect to the catalytic type of kinetics of hemolysis observed in a system containing limited A and excess C′ (1, 2), the question of the dissociability of A from the reaction product $S'AC_i'$ is of more importance than the disso-

[4] Part of the C′ loss is due to non-specific destruction. This problem is under study at present.

[5] The problem of reversibility is also being studied at present by a thermodynamic approach involving measurement of the equilibrium distribution of hemolytic antibody between the fluid phase and the red cell surface (*cf.* Wurmser *et al.* (6)).

ciation of the complex SA, since if the dissociation of $S'AC_i'$ proceeds at an appreciable rate, A would become available continuously for the formation of new complexes SA.[6] On the other hand, if the rate of dissociation of A from $S'AC_i'$ is exceedingly small, reaction [2] would cease when all of the SA has reacted with C′, and as a result hemolysis would come to a standstill. In this case, the cells lysed at termination of the reaction would be those that on initial admixture of cells and antiserum happened to pick up a number of antibody molecules equal to or greater than the threshold value r. Morgenroth (3) concluded that A is not dissociable from $S'AC_i'$ (the symbolic formulation is our own), but in the light of our experience this conclusion is based on an erroneous interpretation. Indeed, the experiments cited by both Morgenroth (3) and Muir (2) do not clearly answer this crucial question. Therefore, some of the experiments included in the present report were designed for the purpose of investigating the dissociability of A from the complex $S'AC_i'$, as well as the influence of the resultant transfer of A on the kinetics of immune hemolysis.

EXPERIMENTAL

Materials and Methods.—Sheep erythrocytes and guinea pig serum as a source of C′ were collected, stored, and used experimentally in the manner described previously (1). The guinea pig serum was absorbed with sheep erythrocytes as previously described (1). Dilutions of the various reagents were made in the buffer[7] used earlier (1).

Standardization of cell suspensions was performed spectrophotometrically as described in a previous paper (1). In most experiments the concentration was adjusted to an optical density (O.D.) of 0.680 ± 0.003, which is equivalent to 10^9 cells per ml. in terms of our procedure of standardization (1); the statement "adjusted to O.D. = 0.680" means that 1.0 ml. of the cell suspension lysed by addition of 14.0 ml. of 0.1 per cent sodium carbonate, yields a lysate of O.D. = 0.680, read in a Beckman quartz spectrophotometer at a wave length of 541 mμ in a cuvette of 10 mm. light path and with distilled water as a reference standard.

Hemolytic antisera "A" and "B" were pooled bleedings from rabbits inoculated as described in a previous paper (1). Antisera "C," "D," and "E" were bleedings collected from individual rabbits after 2 courses of inoculations with a 10 per cent suspension of washed sheep erythrocytes, and one course of injections with sheep erythrocyte stromata. Antiserum "G" was a pool of bleedings of two rabbits inoculated with sheep erythrocyte stromata. Since antisera "C," "D," "E," and "G" were prepared for teaching purposes 4 to 8 years prior to the present study, detailed injection schedules were unavailable.

The antisera designated by numbers were made expressly for studies on the properties of hemolytic antibody, and injection schedules were designed in accord with this purpose.[8]

[6] The fate of C_i' on dissociation of A from $S'AC_i'$ is unknown.

[7] The buffer is prepared as follows: 83.8 gm. NaCl, 2.52 gm. $NaHCO_3$, 3.00 gm. sodium 5,5-diethyl barbiturate, 4.60 gm. 5,5-diethyl barbituric acid, 1.0 gm. $MgCl_2 \cdot 6H_2O$, 0.2 gm. $CaCl_2 \cdot 2H_2O$. Dissolve the last three constituents in about 500 ml. of hot water, add to the solution of the other components, cool, and make up to 2000 ml. with water. Add 0.5 per cent crystallized bovine plasma albumin by weight to a portion sufficient for 1 week's work. Store at 2–5°C. Each day dilute this stock solution accurately 1 part up to 5 with water. The pH of the diluted buffer should be 7.3 to 7.4.

[8] We are indebted to Dr. Charles C. Croft for the preparation of most of the antisera.

Rabbit 46 was injected with a suspension of sheep red cell stromata (0.6 mg. N per ml.), and rabbits 63 and 65 received a 10 per cent suspension of sheep erythrocytes. The injection schedules for both of these antigens were as follows: One intravenous injection of 0.5 ml.; bleeding identified by subscript 1 taken 2 weeks later (not used in the present work); after 2 weeks' rest, the rabbits received a course of 9 injections, spaced over 3 weeks, with doses increasing gradually from 0.5 to 1.75 ml., and totaling 8.75 ml.; 1 week following the last injection the bleeding identified by subscript 2 was taken. After 2 weeks' rest, a second and a third course, each consisting of 9 injections identical with those of the first course, were administered, and bleedings labelled by subscripts 3 and 4, respectively, were taken 1 week following the last injection of each course. Rabbit 46 was bled again 1 and 2 months after the third course (bleedings 46_5 and 46_6). After 5 months' rest, the animal received 5 injections totaling about 5 ml. and was bled again (bleeding 46_7). Antiserum "GPK-43" was a bleeding from a rabbit taken 1 week after the last of three intraperitoneal injections of 10 ml. of guinea pig kidney emulsion, administered at weekly intervals.

The sheep erythrocytes used for immunization were obtained from citrated blood collected aseptically; they were washed three times with sterile physiological saline and suspended in saline to 10 times the volume of the packed cells. Merthiolate (sodium ethylmercurithiosalicylate, Lilly) was added to a final concentration of 0.01 per cent.

The sheep erythrocyte stromata used for immunization were prepared from sheep blood obtained at the abattoir, in proportion of four volumes of blood to one volume of 3.8 per cent aqueous sodium citrate as anticoagulant. The cells were washed twice with saline and lysed with about twelve volumes of cold, CO_2-saturated distilled water to one volume of packed cells. The stromata flocculated and settled out from the solution, thus facilitating collection. The stromata were centrifuged to remove as much fluid as possible, and were washed twice with cold CO_2-saturated 0.2 per cent saline. Washing was continued with 0.85 per cent saline containing 0.01 per cent merthiolate until as much of the hemoglobin as possible had been removed. Stromata suspensions containing about 0.6 mg. of N per ml. were prepared in saline and merthiolate was added to a concentration of 0.01 per cent.

Guinea pig kidney was prepared for injection by grinding the kidney in a Waring blendor and making a suspension of 10 per cent by weight in saline containing 0.01 per cent merthiolate.

Rabbits were bled 1 week after the last injection. After separation from the clot, the serum was centrifuged at 2000 R.P.M. to remove cells, and if necessary at 10,000 R.P.M. for 1/2 hour in the cold to remove any free lipoid material. The clear serum was then heated at 56°C. for 30 minutes to inactivate C′. A portion of serum adequate for a series of experiments was diluted 1/20 in buffer[7] containing 1/10,000 merthiolate and stored either at 4°C. or at −20°C., depending on the frequency of its use as a stock from which further dilutions were made as required, just prior to an experiment. The undiluted serum was stored at −20°C. without preservatives.

Experimental Procedure.—The kinetic measurements used in the present work were made as described in a previous paper (1) and therefore need be outlined only briefly here. The reaction mixtures usually had a total volume of 25 ml. and contained 5 billion red cells, plus hemolytic antiserum and C′ in amounts indicated in the protocols. Reactions were performed in stoppered 125 ml. Erlenmeyer flasks, which were suspended from a rocking mechanism in a water bath maintained at 37°C. ± 0.02°C. During the hemolytic reactions samples of 1.5 ml. were withdrawn at suitable intervals of time and mixed immediately with 3.0 ml. of an ice cold solution containing 0.015 M sodium citrate and 0.12 M sodium chloride to arrest the lytic process. After centrifugation to remove unlysed cells, the samples were analyzed spectrophotometrically for oxyhemoglobin. In conformance with our spectrophotometric procedure of standardization, an O.D. reading of 0.680 corresponded to the lysis of 5 billion cells; *i.e.* the number of cells lysed was calculated by multiplying the O.D. with the factor 7.35×10^9.

Experimental Protocols and Results.—The initial transfer experiments were made with stromata prepared from red cells by immune lysis. 10 ml. of sheep erythrocyte suspension (adjusted to O.D. = 0.340, *i.e.*, 5×10^8 cells per ml.) was mixed with 0.5 ml. of undiluted C′ and 0.5 ml. of antiserum "D" diluted 1/50. After 1 hour at 37°C. the cells had lysed and the mixture was centrifuged at 0°C. for 45 minutes at a speed of 11,500 R.P.M. The sedimented stromata were washed seven times with 11.0 ml. portions of plain veronal-bicarbonate buffer, *i.e.*, the usual buffer[7] except for omission of the albumin. Washes 1, 2, and 3 were made with ice cold buffer and centrifuged immediately at 11,500 R.P.M. for 45 minutes at 0°C. Washes 4 and 5 were in contact with the stromata for 1 hour at 0°C. before centrifugation. Washes 6 and 7 were kept in contact with the stromata for 1 hour at 37°C. before centrifugation. After the 7th wash the stromata were suspended in 11.0 ml. of SA-buffer.

As shown in Protocol 1, 5.0 ml. of this suspension of "immune-lysed stromata" was mixed with 10.0 ml. of red cell suspension adjusted to O.D. = 0.340 (equivalent to about 5×10^8 cells per ml.), and 10.0 ml. of C′ diluted 1/30 (flask 4 in the protocol). In flasks 1, 2, and 3, which served as controls, 10.0 ml. portions of red cells were mixed with 10.0 ml. of C′ 1/30 and 5.0 ml. of buffer, 5.0 ml. of the supernate from the 6th wash, or 5.0 ml. of the supernate from the 7th wash, respectively. Thus, control flask 1 served to evaluate the extent of hemolysis with C′ alone, and flasks 2 and 3 were included to demonstrate the complete removal of free or uncombined A in the process of repeated washing of the stromata. Flask 5 represented an additional control to determine the effect of the stromata suspension on the red cells in the absence of C′, and flask 6 was included as a control to measure spontaneous lysis of the red cells. Reagents were mixed in the order tabulated in Protocol 1, and zero time was taken at the addition of the last reagent.

Protocol 1

Flask	1	2	3	4	5	6
Cells, O.D. = 0.340, *ml.*	10.0	10.0	10.0	10.0	10.0	10.0
C′, 1/30, *ml.*	10.0	10.0	10.0	10.0	0	0
Buffer, *ml.*	5.0	0	0	0	10.0	15.0
Immune-lysed stromata, *ml.*	0	0	0	5.0	5.0	0
Supernate of 6th wash, *ml.*	0	5.0	0	0	0	0
Supernate of 7th wash, *ml.*	0	0	5.0	0	0	0

Results were calculated by subtracting the O.D. readings of the samples of flask 1 from those of the samples of flask 4. In this manner the net lysis was obtained, *i.e.*, the data were corrected for the lytic action exerted by C′ alone. Since the O.D. values of the samples from flasks 2 and 3 were essentially the same as those of flask 1 (O.D. readings of 0.029, 0.029, and 0.032, respectively, after 1 hour's reaction), the net lysis in these controls was zero, indicating absence of measurable quantities of antibody in the 6th and 7th wash fluids. Readings on flasks 5 and 6 were also virtually the same (O.D. readings of 0.015 and 0.012, respectively, after 1 hour's reaction), showing that the stromata did not exert any lytic action.

After calculation in terms of the number of cells lysed, the net results for flask 4 were plotted yielding the curve marked transfer in Fig. 1. Since the net results in control flasks 2 and 3 were zero, they appear as points on the abscissa in Fig. 1. The lysis observed in flask 4 was attributed to transfer of antibody from the immune-lysed stromata to red cells.

Successful transfer experiments of this type were done with antisera "A," "C," and "D" with results essentially like those shown in Fig. 1, except for

quantitative differences. With antiserum "B" a similar experiment did not yield evidence of antibody transfer.

It should be pointed out that in none of these experiments (including that

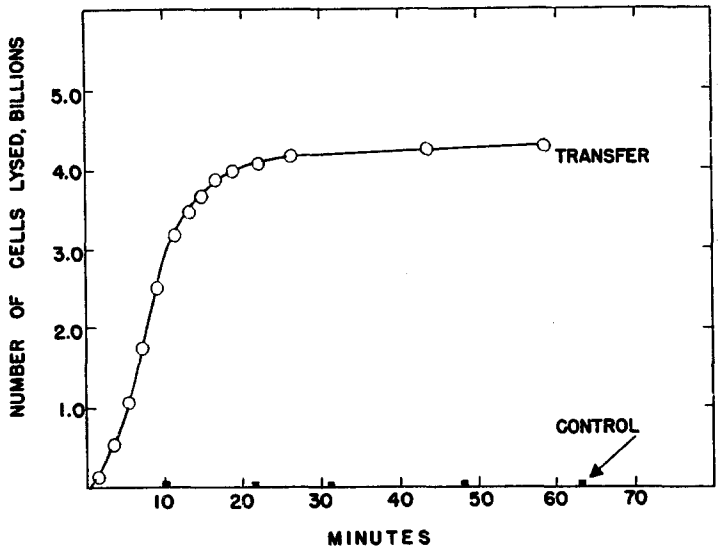

FIG. 1. Kinetics of hemolysis produced by transfer of hemolytic antibody from immune-lysed stromata to intact red cells.

shown in Fig. 1) complete hemolysis was attained. This seemed surprising and called for investigation; accordingly the following experiment was set up:—

Five billion red cells were lysed with 0.5 ml. of antiserum "A," diluted 1/50 and 0.5 ml. undiluted C'. The resulting stromata were washed 8 times and suspended in 16 ml. of buffer. Six reaction mixtures were set up as shown in Protocol 2.

Protocol 2

Flask	1	2	3	4	5	6
Cells, O.D. = 0.340, *ml.*	10.0	10.0	10.0	10.0	10.0	10.0
C', 1/30, *ml.*	0	0	0	10.0	10.0	10.0
Plain veronal buffer,[9] *ml.*	15.0	10.0	5.0	5.0	0	0
Immune-lysed stromata, *ml.*	0	5.0	5.0	0	0	5.0
Antiserum "A," 1/5000, *ml.*	0	0	5.0	0	0	0
Supernate of 8th wash, *ml.*	0	0	0	0	5.0	0

[9] Veronal buffer refers to the buffer described in footnote 7 except for the omission of the albumin. This experiment was done in the early stages of the work when the value of albumin in the buffer was not fully realized.

Flask 1 served as a control to measure cell stability; as shown in Fig. 2 (curve 1), there was appreciable spontaneous hemolysis, probably due in part to the failure to use albumin in the diluent.[9] Flask 2 served to check the absence of lytic action by the immune-lysed stromata. Flask 3 represented a control for the absence of active C′ in the immune-lysed stromata. As shown in Fig. 2, the speed of hemolysis in flasks 2 and 3 was identical with that in flask 1. Flask 4 was included to measure the lytic velocity in a mixture of red cells and C′ (*i.e.*, no antibody, except that remaining in the absorbed guinea pig serum used as C′). Reaction mixture 5 served to check the absence of measurable amounts of A in the final wash fluid obtained in the preparation of the immune-lysed stromata. Flask 6 contained the transfer experiment proper. As shown in Fig. 2, the lytic reaction in flask 6 ceased after about 2 hours, and in order to ascertain whether or not this was due to lack of C′, the reaction mixtures were treated in the following manner: At approximately 230 minutes, as indicated by arrows in Fig. 2, the remaining 13.0 ml. of the reaction mixtures in flasks 4 and 6 was split into equal portions and 0.087 ml. of undiluted C′ and 0.026 ml. of antiserum "A," diluted 1/100, were added to each of the 6.5 ml. portions, respectively. The contents of flask 5 were also split into equal parts, and 0.026 ml. of 1/100 antiserum "A" was added to one of the 6.5 ml. portions in order to ascertain the presence of C′; nothing was added to the other part. The addition of C′ or antibody, respectively, to the reaction mixtures served to indicate whether or not hemolysis had stopped owing to lack of C′. The results of this experiment, plotted in Fig. 2, indeed show that hemolysis in flask 6 (the transfer test proper) came to a standstill owing to the rapid exhaustion of the available supply of C′ by the large quantity of stromata heavily loaded with A.

The interpretation of transfer experiments of this type is not altogether clear since it is not known whether the transferred antibody was dissociated from the complex SA or from the complex $S'AC'_1$. The following experiments were therefore designed to determine whether the complex SA is dissociable.

Stromata were made by lysis of red cells with water; after removal of hemoglobin by thorough repeated washing, the stromata were mixed with a desired quantity of A. Following incubation for 15 minutes at 37°C. for union of stromata with antibody, the stromata-antibody complex was collected by centrifugation and washed once to remove any trace of free or uncombined A. The washed stromata-antibody complex was resuspended in buffer for use in transfer experiments.

Attempts to demonstrate antibody transfer with stromata-antibody complex prepared in this manner were unsuccessful. Eventually the failure was traced to the two centrifugations interposed in the process of washing the stromata-antibody complex. Success in obtaining evidence of antibody transfer was met when only one centrifugation was employed, *i.e.*, when the single washing previously employed was omitted. Even better transfer took place when the stromata-antiserum mixture was not centrifuged at all. Since the small amount of A used in these experiments was taken up completely by the stromata, separation of the stromata-antibody complex was superfluous, and consequently it was omitted in all subsequent experiments.

A typical experiment of this type is shown in Fig. 3. The experimental details were as follows (*cf.* Protocol 3):—

40 ml. of washed sheep erythrocytes, adjusted to O.D. = 0.680 (10^9 cells per ml.) was centrifuged, the supernatant fluid removed, and the cells lysed with 25 ml. of cold distilled water. The resulting stromata were collected by centrifugation at 15,000 R.P.M. for 15 minutes in the cold, and were washed twice with cold water and once with cold buffer to remove hemoglobin. The washed stromata were suspended in 20 ml. of buffer, yielding a suspension twice as concentrated as the initial red cell suspension ("double strength stromata"). 15 ml. of the double strength stromata was mixed with 15 ml. of antiserum "D" diluted 1/6000, and the mixture was rocked at 37°C. At the end of 15 minutes, 10.0 ml. of the mixture was removed and centrifuged immediately at 15,000 R.P.M. for 15 minutes in the cold. The supernatant fluid was poured off with care not to spill over any of the sedimented stromata sludge. 5 ml. of this

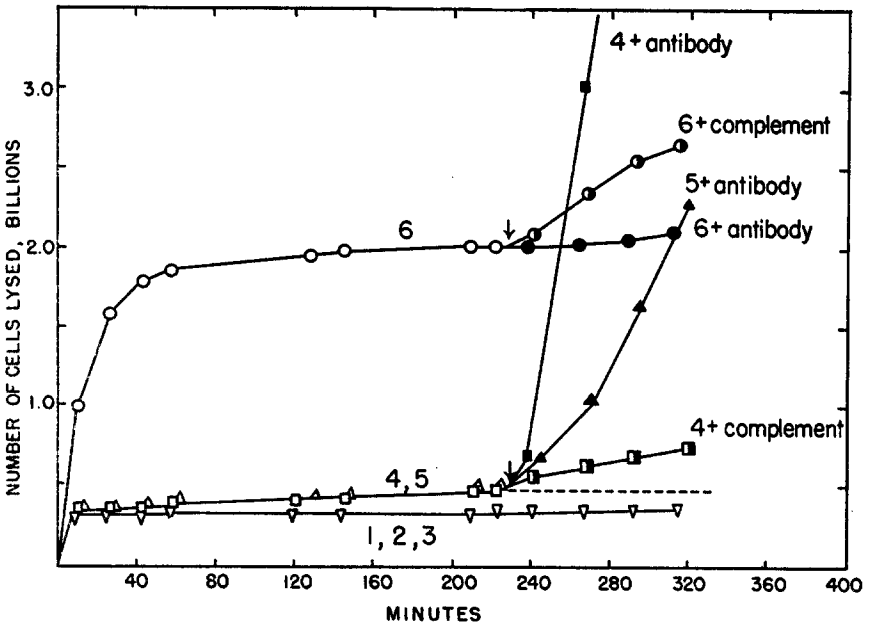

FIG. 2. Kinetics of hemolysis produced by transfer of hemolytic antibody from immune lysed stromata to intact red cells.

supernatant fluid was assayed for A by incubation with red cells and C′ (flask 3 in Protocol 3). The remainder of the stromata-antibody mixture was rocked for a total of 30 minutes at 37°C., and then 5.0 ml. was removed and mixed with red cells and C′ in flask 2 for the transfer experiment proper. Flask 4 received 5.0 ml. of red cells and 5.0 ml. of the stromata-antibody complex as a check on lytic action in the absence of C′. Flask 5 contained red cells and C′ to measure the hemolytic activity of the guinea pig serum used as a source of C′. Finally, flask 6, containing stromata-antibody complex + C′, was included as a colorimetric control.

In flask 1 a comparison experiment was performed to measure the speed of lysis with the same amount of antibody as in flask 2 (the transfer experiment), but distributed uniformly over stromata and red cells. 5 ml. of double strength stromata suspension was diluted with 5.0 ml. of buffer and 5.0 ml. of this single strength stromata suspension was mixed in flask

1 with 5.0 ml. of red cells, 5.0 ml. of a 1/12,000 dilution of antiserum "D," and 5.0 ml. of a 1/15 dilution of C′. Thus, flasks 1 and 2 were identical in respect to contents and differed only with regard to the order of mixing of the reagents.

Protocol 3

Flask	1	2	3	4	5	6
Buffer, *ml.*	5.0	10.0	10.0	15.0	15.0	15.0
Cells, O.D. = 0.68, *ml.*	5.0	5.0	5.0	5.0	5.0	0
Single-strength stromata, *ml.*	5.0	0	0	0	0	0
C′, 1/15 dilution, *ml.*	5.0	5.0	5.0	0	5.0	5.0
Antiserum "D," 1/12,000, *ml.*	5.0	0	0	0	0	0
Stromata-antibody complex, *ml.*	0	5.0	0	5.0	0	5.0
Supernate from stromata-antibody complex, *ml.*	0	0	5.0	0	0	0

Zero time was taken as the time of addition of antiserum, stromata-antibody complex, or supernatant fluid from the stromata-antibody complex, and the contents of the flasks were sampled for a total time of 408 minutes.[10]

The analyses of flask 4 indicated that the stromata-antibody complex exerted no hemolytic activity in the absence of C′. Furthermore, the results obtained in flask 3 showed that the supernatant fluid was free of detectable antibody. The O.D. readings obtained in flasks 1 and 2 were corrected by subtracting the sum of the O.D. readings obtained in flask 5 (cells and C′) and the color contribution of the stromata (O.D. = 0.004). The corrected O.D. values were multiplied by the factor 7.35×10^9 to yield the number of cells lysed specifically. The results of this experiment are plotted in Fig. 3.

Since the supernatant fluid control (flask 3) showed the absence of free antibody in the suspension of stromata-antibody complex, the extensive hemolysis observed in flask 2 (*cf.* transfer curve in Fig. 3) can be attributed to transfer of antibody from stromata-antibody complex to intact red cells. Therefore, the combination between stromata and antibody is dissociable; however, it is not clear from this experiment whether the release of A is contingent upon C′ action, *i.e.*, whether the transferred A comes from SA or $S'AC_i'$. The speed of transfer, and the resultant equilibration with respect to antibody distribution over the stromata and red cells, can be judged by comparison of the lytic veloc-

[10] Since it was feared that bacterial contamination might influence results in prolonged experiments conducted at 37°C. a number of kinetic control experiments were performed in the presence of penicillin and streptomycin ranging from 7 units of penicillin and 0.07 mg. streptomycin to 285 units of penicillin and 2.9 mg. streptomycin per ml. It was found that quantities exceeding 20 units of penicillin and 0.20 mg. of streptomycin per ml. exerted partial inhibition, but with quantities below this level the results of kinetic experiments conducted with these antibiotics were the same as those conducted without them, even though these lower concentrations of antibiotics sufficed to suppress bacterial growth as ascertained by plating the reaction mixtures on infusion agar at the termination of the experiments. Furthermore, it was noted that very few colonies developed on plates that received the reaction mixtures without the antibiotics after the reaction mixtures had been incubated at 37°C. for 5 to 6 hours, indicating that the hemolytic reaction mixtures are strongly bactericidal probably due to the presence of the large amount of fresh guinea pig serum containing C′ and traces of natural antibody to various microorganisms.

ity of flask 1 (*cf.* velocity control curve in Fig. 3) with that of flask 2 (*cf.* transfer curve in Fig. 3). If transfer were rapid, *i.e.* if equilibration were attained in a matter of minutes, the speed of lysis in flask 2 would have been nearly the same as that in flask 1. Since it was much less, the transfer of antibody must

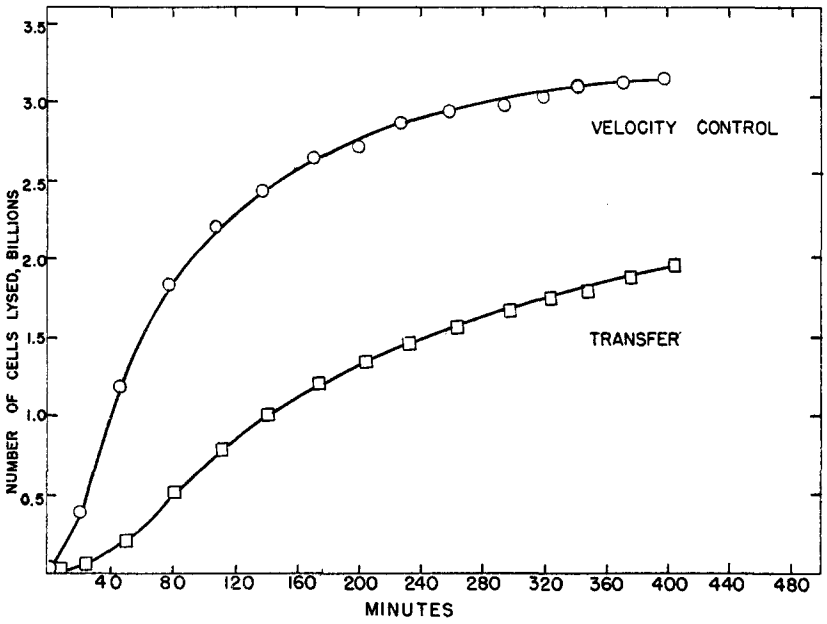

FIG. 3. Kinetic experiment indicating transfer of antibody from water-lysed stromata to intact red cells. Velocity control curve represents the rate of lysis expected if all antibody transferred at start of experiment.

be a slow process, necessitating at least several hours for attainment of equilibrium.

In order to appraise more closely the speed with which antibody dissociates from stromata and transfers to red cells, and in order to investigate more definitively the dissociability of SA, an experiment was performed in which several mixtures of red cells and stromata-antibody complex were shaken at 37°C. for varying periods of time prior to addition of C′ to initiate hemolysis. The resulting lytic velocities should serve as a rough index of the speed with which antibody initially in combination with stromata becomes distributed over the total available antigen, *i.e.*, both stromata and red cells.

Fifty ml. of a suspension of washed red cells, adjusted to O.D. = 0.680, was centrifuged and the sedimented cells were lysed with about 25 ml. of cold, distilled water. The stromata were collected by centrifugation of the lysate in the cold for 15 minutes at 15,000 R.P.M. They were washed twice with water, once with buffer, and once again with water. Since a small amount

of hemoglobin still remained with the stromata, they were frozen and thawed twice, washed once more with water and finally with buffer. While a trace of hemoglobin still remained after this series of treatments, its quantity was not sufficient to cause difficulty in the subsequent transfer experiments. Accordingly, the stromata were suspended smoothly in 25 ml. of buffer and filtered through a tiny wad of absorbent cotton to remove any clumps. 20 ml. of this stromata suspension (double strength with reference to the red cell suspension adjusted to O.D. = 0.680) was mixed with 20.0 ml. of a 1/6000 dilution of antiserum "D," the mixture was shaken at 37°C. and after 20 minutes a portion of 6.5 ml. was withdrawn and centrifuged at once at 15,000 R.P.M. for about 15 minutes. The sediment was resuspended smoothly in 6.5 ml. of buffer, and this suspension of stromata-antibody complex subjected to one centrifugation was used in one of the subsequent transfer experiments (flask 4 in Protocol 4). The supernatant fluid was assayed for antibody (flask 5 in Protocol 4). The bulk of the stromata-antibody mixture (*i.e.*, 33.5 ml.) was kept in the rocker at 37°C. for a total period of 70 minutes,[11] *i.e.*, until it was used in the various transfer experiments. The manner in which these experiments were set up is shown in Protocol 4. Thus, in flask 1, buffer, red cells, and C′ were mixed, followed immediately by stromata-antibody complex; in flasks 2 and 3, buffer, red cells, and stromata-antibody complex were mixed and rocked for 66 and 155 minutes, respectively, prior to addition of C′. In this manner, 0, 66, and 155 minutes were allowed in flasks 1, 2, and 3, respectively, for antibody transfer prior to initiation of the lytic reaction by addition of C′. Flask 6 represented a control for measuring the lytic activity of the guinea pig serum used as a source of C′. The O.D. readings obtained in flask 6, which rose almost linearly from 0.007 to 0.013 in about 300 minutes of reaction, were subtracted from the O.D. readings in flasks 1, 2, 3, 4, and 5. After this correction, there was no lysis whatsoever in flask 5, indicating the absence of measurable amounts of uncombined antibody. Flasks 7 and 10 served as controls to measure the release of traces of colored material from the stromata-antibody complex in the presence and absence of C′, respectively. After correction for C′ color (O.D. = 0.004), the release of colored substance in both flasks was equivalent to an O.D. reading of 0.002 throughout the entire reaction period; although negligible in magnitude, the O.D. readings in the transfer experiments were corrected accordingly. Flasks 8 and 9 were included to measure the hemoglobin release from red cells in the presence and absence of stromata-antibody complex, respectively. After correction for the color released from the stromata-antibody complex (O.D. = 0.002), the release of hemoglobin in both flasks was equivalent to an O.D. reading of 0.003 throughout the entire reaction period of about 300 minutes, indicating that the stromata-antibody complex exerted no lytic action on the red cells.

Protocol 4

Flask	1*	2	3	4	5	6	7	8	9	10
Buffer, *ml.*	10.0	10.0	10.0	10.0	10.0	15.0	15.0	15.0	20.0	20.0
Cells, O.D. = 0.68, *ml.*	5.0	5.0	5.0	5.0	5.0	5.0	0	5 0	5.0	0
Stromata-antibody complex, *ml.*	5.0	5.0	5.0	0	0	0	5.0	5.0	0	5.0
Supernate from stromata-antibody complex, *ml.*	0	0	0	0	5.0	0	0	0	0	0
Stromata-antibody complex centrifuged and resuspended in buffer, *ml.*	0	0	0	5.0	0	0	0	0	0	0
C′, 1/15 dilution, *ml.*	5.0	5.0	5.0	5.0	5.0	5.0	5.0	0	0	0

* C′ added just before stromata-antibody complex.

[11] This was intended to be about 30 minutes, but unforeseen circumstances compelled a delay of 40 minutes.

The results of this experiment, plotted in Fig. 4, indicate that transfer of antibody can take place prior to C′ addition, *i.e.*, it does not depend on C′ action. Therefore, the antibody which underwent transfer arose by dissociation of the complex SA. Furthermore, the rate of transfer of antibody is rather low since hemolysis proceeded appreciably faster in flask 3 than in flask 2, as a result of 155 minutes of preliminary equilibration in flask 3 compared to 66 minutes in flask 2; *i.e.*, complete equilibration was not attained in about 1 hour. It can be estimated on the basis of this and related experiments that at least

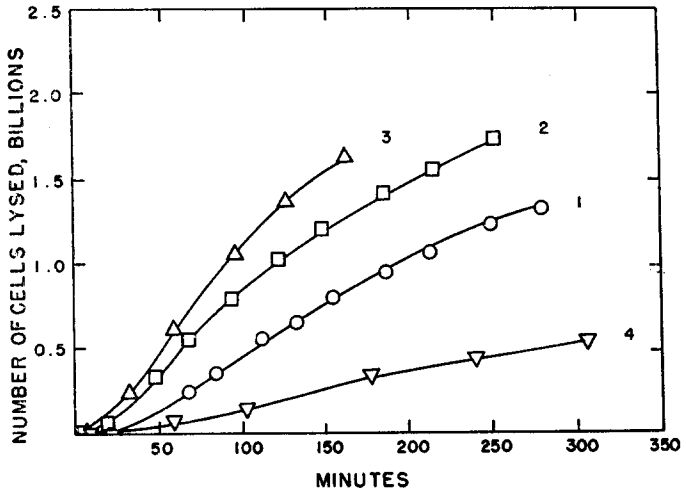

FIG. 4. Transfer of antibody from water-lysed stromata to intact red cells. Curves 1, 2, and 3 represent experiments in which the stromata-antibody complex was incubated with red cells for 0, 66, and 155 minutes, respectively, prior to addition of C′. Curve 4 represents transfer of A from stromata-antibody complex subjected to one centrifugal packing.

4 hours' preliminary rocking of the stromata-antibody complex with red cells would be necessary to approach a state of equilibrium.

In addition, this experiment shows that the rate of antibody transfer is greatly diminished when the stromata-antibody complex is subjected to close packing by a single centrifugation (*cf.* flask 4). Other experiments of this type have shown that antibody transfer becomes indetectable when the stromata-antibody complex is subjected twice to centrifugal packing.

The next type of experiment was designed to show that antibody could transfer from erythrocyte to erythrocyte. A 1/7000 dilution of antiserum "65-4," which had shown good transfer from stromata to erythrocytes in an experiment of the type shown in Fig. 3, was allowed to react with an initial portion of 5 billion erythrocytes for 30 minutes at 37°C. with constant agitation in the mechanical rocker. Following this period of combination, a second portion of 5 billion erythrocytes was added (flask 1, Protocol 5), followed immediately by C′. Control analyses (flasks 2 and 3) containing 5 billion and 10 billion erythrocytes, respectively, were

included for comparison of the kinetic behavior in the transfer experiment with that in direct hemolytic analyses involving 5 billion or 10 billion cells, respectively. If no transfer of antibody from the first to the second portion of 5 billion cells takes place, the course of the hemolytic curve in flask 1 should follow that of the control of 5 billion cells (flask 2); on the other hand, if transfer is complete and rapid, the lytic curve of the transfer experiment should follow that of the control of 10 billion cells (flask 3).

As in the previous experiment, it was imperative to prove that all of the antibody added was in combination with the first portion of cells at the time of addition of the second portion of 5 billion cells. For this purpose, 10.0 ml. of cells (O.D. = 0.680) was allowed to react with 10.0 ml. of antiserum "65-4" diluted 1/7000 for 30 minutes at 37°C. The mixture was then centrifuged for 15 minutes at 15,000 R.P.M. in the cold, and 10.0 ml. of supernatant fluid was added to 5.0 ml. of cells followed immediately by 5.0 ml. of C′ to assay for free or uncombined antibody in the supernatant fluid (flask 4).

Flask 5 contained 5.0 ml. of cells and 5.0 ml. of C′ diluted 1/15, and served as the usual control for the extent of lysis occurring on incubation of cells with fresh guinea pig serum; flask 6 represented a similar control, but contained 10.0 ml. of cells and 5.0 ml. of C′.

Protocol 5

Flask	1	2	3	4	5	6
Buffer, *ml.*	5.0	10.0	5.0	5.0	15. 0	10.0
1st portion of cells (O.D. = 0.68), *ml.*	5.0	5.0	10.0	5.0	5.0	10.0
Antiserum "65-4," diluted 1/7000, *ml.*	5.0	5.0	5 0	0	0	0
Rocked 30 min. at 37°C.						
Supernate from a portion of sensitized cells, *ml.*	0	0	0	10.0	0	0
2nd portion of cells (O.D. = 0.68), *ml.*	5.0	0	0	0	0	0
C′ 1/15 dilution, *ml.*	5.0	5.0	5.0	5.0	5.0	5.0

Samples were collected at suitable intervals for about 160 minutes. Analyses of the supernates showed that the reaction in flask 1 followed a kinetic course intermediate between those of flasks 2 and 3, containing 5 billion and 10 billion cells, respectively (*cf.* Fig. 5). In flask 1 the lytic reaction started more slowly than in the 5 billion cell system, but possibly a little faster than in the 10 billion cell system. At 45 minutes the curve for the transfer experiment (flask 1) crossed the curve for the reaction with 5 billion cells which levelled off because of exhaustion of red cells. In the transfer experiment, on the other hand, sufficient antibody had dissociated from the first portion of cells and combined with the second portion so that lysis continued, although it did not reach the level of the system containing 10 billion cells, at least within the period of observation.

Next, a transfer experiment was designed which differed from the experiment shown in Fig. 5 in respect to the fact that at the time of addition of the second portion of cells about 90 per cent of the first dose of red cells had lysed. Since this experiment was much more complex in its execution, it is necessary to give a detailed description (*cf.* Protocol 6).

The transfer experiment proper (flask 2) contained 5.0 ml. of red cells (O.D. = 0.680) to which was added 5.0 ml. of antiserum "D," diluted 1/9000, 5.0 ml. of buffer, and 5.0 ml. of C′ diluted 1/15. When hemolysis reached about 90 per cent, a second portion of 5.0 ml. of red cells (O.D. = 0.680)was added. Flasks 1 and 7 contained the same reagents as 2, except that they did not receive a second portion of cells. Flask 1 was included as a velocity control to indicate the course of the reaction as it would occur in flask 2 prior to addition of the second portion of cells; it also served to indicate how lysis would progress without further cell addition. Flask 7 served to determine whether the lytic system contained free antibody at the

time of addition of the second cell portion to flask 2. Accordingly, the contents of flask 7 were chilled when lysis reached 90 per cent, and were immediately centrifuged at 15,000 R.P.M. for 10 minutes in the cold. 18 ml. of the supernate was then mixed with cells and C′ (flask 3)

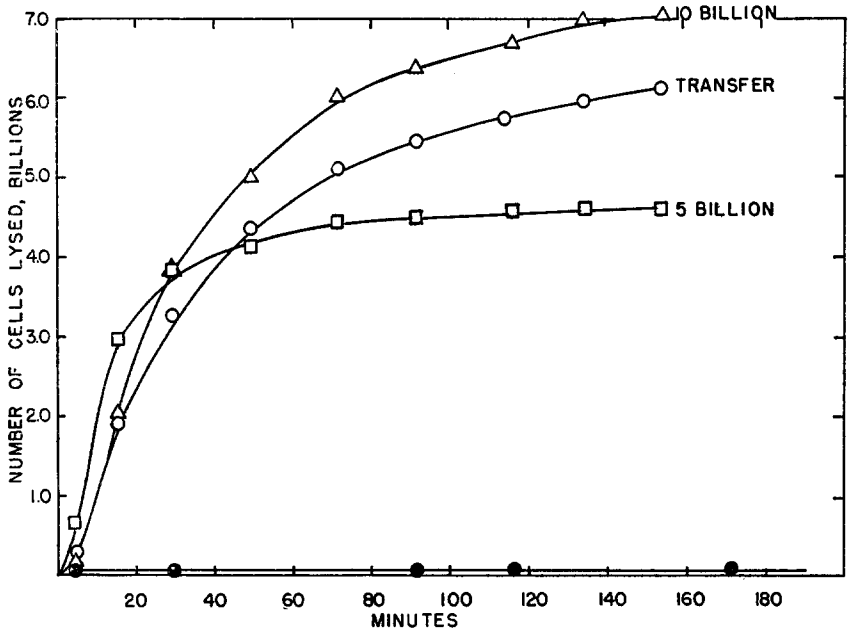

FIG. 5. Kinetic experiment indicating transfer of antibody from red cell to red cell; comparison curves refer to the course of the reaction in systems containing 5 billion and 10 billion cells. The curve designated by solid circles (●), which is almost superimposed on the abscissa, represents the control analysis of supernatant fluid indicating absence of detectable amounts of free antibody.

as assay for free antibody. Flask 4 contained 5.0 ml. cells and 5.0 ml. of C′ as the usual control for the extent of lysis caused by guinea pig serum. Flask 5 was the same as 4 except that it received a second portion of cells at the same time as 2, and flask 6 was the same as 4 except that it contained 9.0 ml. of C′ diluted 1/15 as a control for flask 3 which received additional C′ contained in the 18.0 ml. portion of supernate from flask 7.

Protocol 6

Flask	1	2	3	4	5	6	7
Buffer, *ml.*	5.0	5.0	0	10.0	10.0	11.0	5.0
Cells, O.D. = 0.68, *ml.*	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Antiserum "D" 1/9,000, *ml.*	5.0	5.0	0	0	0	0	5.0
C′, 1/15 dilution, *ml.*	5.0	5.0	0	5.0	5.0	9.0	5.0
Supernate, *ml.*	0	0	18.0	0	0	0	0
Additional cells, *ml.*	0	5.0	0	0	5.0	0	0
C′, 1/6 dilution, *ml.*	0	0	2.0	0	0	0	0

Each flask was sampled at suitable intervals for a period up to 230 minutes and the supernates analyzed spectrophotometrically for hemoglobin. For calculation, O.D. readings on flask 4 were subtracted from those of flask 1, and the corrected values so obtained were taken to represent the course of hemolysis in flask 2 *prior* to addition of the second portion of cells. Since the total reaction volume in flasks 1 and 2 up to the time of the addition of the second cell portion was only 20.0 ml., in contrast to the usual volume of 25.0 ml., and since the conversion of O.D. readings to the number of cells lysed by the factor of 7.35×10^9 is based on a total reaction volume of 25.0 ml., the conversion factor used in this case was $0.80 \times 7.35 \times 10^9$. The O.D. readings for flask 3 were corrected by the subtraction of the O.D. values for

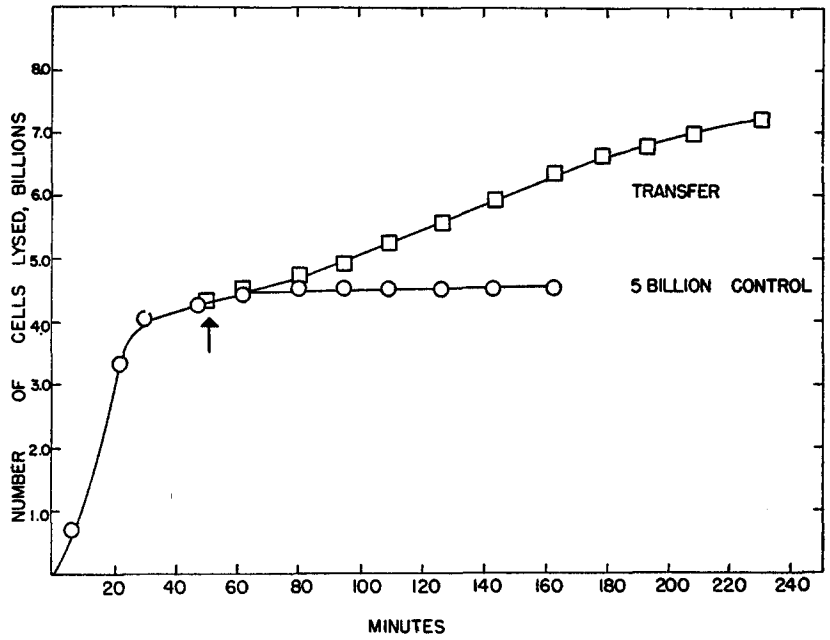

FIG. 6. Kinetic experiment indicating transfer of antibody from one portion of cells which were about 90 per cent lysed when the second portion was added (arrow).

flask 6; since these corrected values showed no increase with time of incubation, it was concluded that the supernate from 7, which was added to flask 3, contained no antibody. Furthermore, the corrected O.D. values found in flask 3 corresponded to the color contribution of the hemoglobin present in the supernate from flask 7. Finally, the O.D. readings in flask 2 *after* addition of the second portion of cells were corrected by subtraction of the O.D. values obtained in flask 5. The results plotted graphically in Fig. 6 show that in flask 2 lysis of the second portion of cells took place, indicating transfer of antibody.

The result of this experiment is crucial in that it is interpreted to indicate the dissociation of A from the hypothetical complex $S'AC_i'$. This conclusion is based on the assumption that at the time of addition of the second cell portion, all, or nearly all, of the available SA had reacted with C′ with the resultant hypothetical conversion to $S'AC_i'$. The assumption of essentially complete

conversion of SA to $S'AC_i'$ is based on the fact that the lytic reaction levelled off slightly above 90 per cent hemolysis within about 1 hour, *i.e.*, by the time the second cell portion was introduced. In terms of the theory of the hemolytic reaction outlined in the introduction, the near cessation of the lytic reaction at the 90 per cent level is considered to be due to the near exhaustion of SA available for reaction with C'. (While dissociation of A from $S'AC_i'$ with resultant transfer would make new SA available continuously, most of the newly formed SA will be on the stromata, rather than on intact red cells, since there is almost complete lysis; therefore, hemolysis nearly ceases, despite transfer of A, when the degree of lysis is high.)

In the initial attempts to demonstrate transfer of A by a method of the type represented in Protocol 1, it was noted that only 1 antiserum of the 4 tested exhibited good transfer, while 2 antisera (antisera "A" and "C") showed transfer to a moderate degree, and one antiserum (antiserum "B") yielded no evidence of transfer. It was therefore apparent that the ability of A to undergo transfer is not shared uniformly by different hemolytic antisera. Accordingly, a total of 18 sera were examined for ability of their A to transfer from stromata to erythrocytes. This was determined by examining each serum as in Protocol 3. The results of this survey are given in Table I, together with data indicating the type of antigen used in the preparation of the various antisera.

It was found that of the 18 sera assayed, 6 gave evidence of transfer. Of these 6, one ("A") was a pool of rabbit antisera to intact sheep erythrocytes, another one ("D") a pool of antisera from rabbits injected both with sheep erythrocytes and stromata, and 4 antisera represented single bleedings of rabbits injected with intact sheep erythrocytes. On the other hand, the 12 antisera which failed to show transfer included 6 antisera prepared by injection of sheep cell stromata, 3 prepared with intact cells, 2 made by injection of both intact red cells and stromata, and 1 by administration of guinea pig kidney as antigen.

It should be noted that antiserum "C" showed moderate transfer by the method represented in Fig. 1, while no transfer was observed by the method shown in Fig. 3. This could be expected since conditions in the former type of experiment were more favorable for the demonstration of transfer than those in the latter procedure, due, at least in part, to the use of widely differing amounts of A in these experiments. In general, it should be emphasized that the detection of the transfer tendency depends on the sensitivity of the method chosen for its demonstration.

As pointed out in the introduction, the preceding experiments on transfer of A emerged from the hypothesis that transfer is responsible for the fact that hemolysis with excess C' and limited A proceeds without halt for long periods of time, *i.e.*, that the kinetics are of a catalytic type. If this hypothesis were correct, antisera exhibiting marked transfer tendency should display the catalytic type of kinetic behavior in a more pronounced manner than should antisera showing poor transfer capacity.

This is indeed the case, as may be seen on comparison of the kinetic data for antisera "B" and "A" shown in reference 1, Figs. 1 and 4, respectively. According to the results of the present work, antiserum A displayed good transfer, while antiserum B did not. In order to demonstrate more clearly this correlation between the type of kinetic behavior exhibited by an antiserum and

TABLE I

Description of the Hemolytic Antisera and Their Behavior in Antibody Transfer Experiments

Designation of antiserum	Antigen used in preparation	Comments	Extent of transfer*
"A"	Sheep erythrocytes	2 courses; pool of 4 sera	Good
"B"	Sheep erythrocytes	1 course; pool of 3 sera	None
"C"	Sheep erythrocytes and stromata	2 courses with whole cells	Poor‡
"D"	Sheep erythrocytes and stromata	Plus 1 course with stromata;	Good
"E"	Sheep erythrocytes and stromata	Individual sera	None
"G"	Sheep erythrocyte stromata	Several months' immunization; pool of 2 sera	None
"46-2"	Sheep erythrocyte stromata	1 course	None
"46-3"	Sheep erythrocyte stromata	2 courses	None
"46-4"	Sheep erythrocyte stromata	3 courses	None
"46-5"	Sheep erythrocyte stromata	1 month's rest following 3rd course	None
"46-7"	Sheep erythrocyte stromata	5 additional injection after 5 months' rest	None
"63-2"	Sheep erythrocytes	1 course	None
"63-3"	Sheep erythrocytes	2 courses	Fair
"63-4"	Sheep erythrocytes	3 courses	Good
"65-2"	Sheep erythrocytes	1 course	None
"65-3"	Sheep erythrocytes	2 courses	Good
"65-4"	Sheep erythrocytes	3 courses	Good
"GPK-43"	Guinea pig kidney	3 injections	None

* Estimated by transfer from stromata-antibody complex to red cells, as in Fig. 3.

‡ This antiserum showed moderate transfer of antibody from immune-lysed stromata to red cells, as shown in Fig. 1, but no transfer was observed from stromata-antibody complex, as shown in Fig. 3.

the presence or absence of transferring A, kinetic experiments were performed, on the one hand with antisera yielding evidence of transfer, and on the other hand, with antisera which did not show evidence of transfer. Dilutions were chosen so as to obtain closely similar initial lytic velocities. Experimental conditions were the same as in reference 1.

In one of these experiments, antisera "A" and "65-3" (5.0 ml. diluted 1/7000 and 1/20,000, respectively), which displayed good transfer, were compared with antisera "B" and "G"

(5.0 ml. diluted 1/21,000, respectively), which lacked transferring A. Similarly, in a second experiment, antiserum "65-4" (good transfer) was compared to antiserum "46-3" (no transfer), with dilutions of 1/18,000 for both sera. The resulting kinetic reaction curves are shown in Figs. 7 and 8, and it can be seen that the antisera that possess transferring A, maintained a somewhat higher lytic velocity after prolonged reaction than did those lacking transferring A.

Therefore, the presence of transferring A appears to influence the kinetic behavior of an antiserum in accord with the hypothesis initially advanced. However, other factors also may play a role. For example, it appears possible that the rate at which C′ is used up or destroyed, may vary with different hemolytic antisera, and since the rate of hemolysis depends on the concentration of C′, such variations would lead to divergent kinetic behavior.

DISCUSSION

The experiments included in the present report deal mainly with three questions; *viz.*, (1) the rate of dissociation of the complex SA, (2) the rate of dissociation of A from the postulated complex $S'AC_i'$, and (3) the effect of the dissociation of A from these complexes on the kinetics of immune hemolysis.

The initial transfer experiments shown in Figs. 1 and 2, indicate that A is dissociable at an appreciable rate from stromata prepared by immune lysis. Since a large excess of A was used in these experiments it is possible that the amount of C′ employed even though large, did not suffice to convert all of the complex SA to $S'AC_i'$; therefore it is not clear from this type of experiment whether the dissociated A came from SA or from $S'AC_i'$. For this reason, experiments of the type shown in Figs. 3 and 4 were devised; very small amounts of A were used in these trials in order to approximate more closely conditions existing in a kinetic hemolysis experiment with limited A and excess C′, in which the catalytic type of kinetic behavior, noted previously (1), is observed. The experiment shown in Fig. 3 indicates that A is indeed dissociable from stromata, even though only minimal amounts of A are used, but, since C′ was introduced at the beginning of the transfer process, the type of experiment shown in Fig. 3 fails to show whether the dissociated A came from SA or from $S'AC_i'$. This information, however, is furnished by the observations reported in Fig. 4. Here it was found that the lysis of red cells by C′ and transferred A is accelerated when the red cells are incubated for several hours with the stromata-antibody complex prior to addition of C′. This indicates that transfer of A took place before the introduction of C′ and therefore, the complex SA is dissociable; the action of C′ is not required for the release of A.

These results are essentially in agreement with observations made by Muir (2). This investigator treated red cells with excess A, washed them several times, and produced dissociation of A by raising the temperature; in other experiments Muir observed transfer of A from the washed red cell-antibody complex to another portion of red cells. This is essentially the same as our ex-

periment shown in Fig. 5, except that we used only a small amount of antibody and did not wash the red cell-antibody complex. Washing was omitted in our experiments in order to avoid centrifugal packing, which, as found with stromata-antibody complex (*cf.* Fig. 4), decreases the dissociation tendency of A. We interpret this decrease to be due to interaction of a second (or third?) reactive group on the antibody molecule with a free antigenic site on a neighboring stromata particle brought into intimate contact as a result of centrifugal packing. This difficulty does not arise when the red cells or stromata are heavily loaded with A, as in the experiments shown in Figs. 1 and 2, or as in Muir's experiments, since there are only few free antigenic sites, or none at all, when excess A is used, so that the possibility of multiple bonding of an antibody molecule is minimized.

With respect to the catalytic type of kinetics observed in a system containing limited amounts of A and excess C′ the experiment of Muir as well as our experiments shown in Figs. 1 to 5 is not strictly pertinent, for in respect to this question it is necessary to determine whether A is dissociable after the complex SA has reacted with C′, *i.e.*, whether A can dissociate from the postulated complex $S'AC_i'$. If this reaction product complex is dissociable, transfer of A from cell to cell during hemolysis becomes possible, with resultant formation of more SA, and this would lead to a catalytic type of behavior in hemolytic systems containing limited amounts of A and excess C′. An answer to this question is provided by the experiments shown in Fig. 6. Here a small amount of A was used so that it is reasonable to suppose that most or all the complexes SA have reacted with C′ prior to addition of the second portion of red cells; or to state the proposition in another way, under the conditions employed in this experiment all of the A employed has been performing hemolytic work prior to the addition of the second portion of red cells. Therefore, the antibody molecules, shown to have transferred from the first to the second cell population, were probably dissociated from the complex $S'AC_i'$.

In terms of the postulated similarity between the behavior of hemolytic antibody and enzymes (1), it would be of interest to know whether the participation of C′ enhances dissociation of A, *i.e.*, whether A dissociates more readily from $S'AC_i'$ than from SA. This might be so if S′ forms a weaker bond with A than does S. However, the experiments included in the present report do not indicate whether or not this is the case.

It is noteworthy that only 6 out of 18 antisera examined in the present study exhibited appreciable transfer by the procedure shown in Fig. 3; with more sensitive transfer techniques a higher proportion of antisera would probably have yielded positive results, but in any event it is evident that the ability of antisera to exhibit antibody transfer is highly variable. While it is not known what factors are responsible for these differences, it appears reasonable to attribute them to variations in bond strength between antibody and red cell

antigens. Such variations are probably not restricted to differences among various antisera but are also manifest in respect to the population of antibody molecules in any single antiserum. Thus, an antiserum which exhibits good antibody transfer probably contains a large proportion of antibody capable of forming relatively weak bonds with red cell antigens, whereas an antiserum exhibiting poor transfer would likely contain antibody capable of strong bonding.

It should also be pointed out that the chance of antibody dissociation to an extent in which it would produce measurable transfer, would be greater with heavily sensitized cells or stromata than with minimal sensitization. For this reason experiments such as those shown in Figs. 1 and 2 in which relatively large amounts of A were used, are more likely to yield evidence of transfer than the other experiments which were conducted with small quantities of A (*cf.* antiserum "C," Table I). On the other hand, the use of large amounts of A as in the experiments shown in Figs. 1 and 2, does not furnish a test for transfer which is strictly valid in respect to the evaluation of the effect of antibody transfer on the kinetics of hemolysis in systems containing only very small amounts of A.

In view of the variable dissociation rates exhibited by different hemolytic antisera as reflected in their variable transfer rates, it would be expected that such different hemolytic antisera exhibit diverse kinetic behavior. This is indeed the case, and, as shown in Figs. 7 and 8, such variable kinetic behavior can be correlated with the ability of the antibody to undergo transfer. Thus, 3 of the 6 antisera examined exhibited transfer and 3 did not. In designing these experiments dilutions were chosen which would yield closely similar initial velocities, and in the comparison of antisera "46-3" and "65-4" shown in Fig. 8, this goal was achieved so that it was possible to demonstrate that antiserum "65-4," which exhibited transfer, maintained better hemolytic activity over a longer period of reaction than did antiserum "46-3," which did not show transfer. In the experiment represented in Fig. 7, the dilutions of the 4 antisera were not as well matched in respect to their initial velocities of hemolysis, since it was found that antisera "A" and "65-3," which contained transferring antibody, yielded somewhat lower initial velocities than did antisera "B" and "G." Despite their lower initial velocities, the transferring antisera ("A" and "65-3") eventually performed more lytic work, *i.e.*, they overtook the other two antisera in about 1 hour's reaction.

The observations of Mayer, Croft, and Gray regarding the kinetics of immune hemolysis have been confirmed by Morris (8), but this author has challenged the suggestion that antibody transfer is responsible for the catalytic type of hemolytic kinetics. Instead she has proposed that heterogeneity of the red cells in respect to their susceptibility to lysis is responsible. While it cannot be doubted that red cells are inhomogeneous, and that this factor influences the kinetics of hemolysis to some extent, the experimental results of Morris were

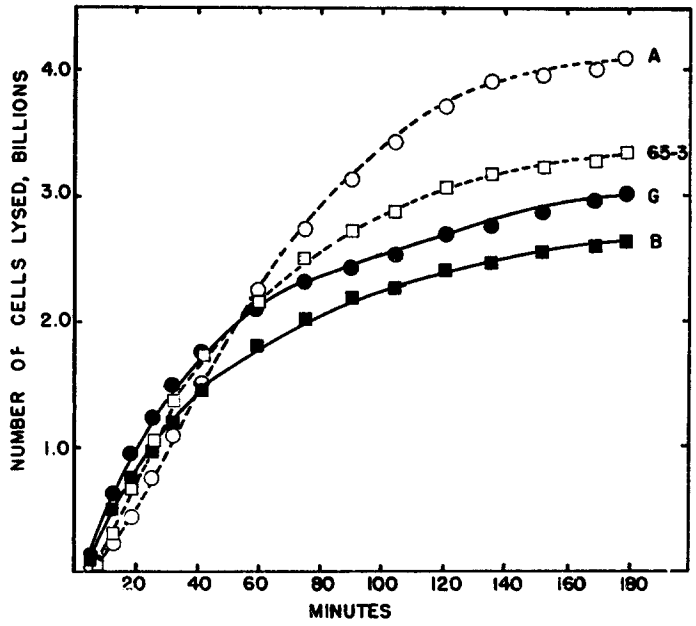

FIG. 7. Kinetic curves of antisera "A," "65-3," "B," and "G," with a limited amount of antibody and excess C′. Open symbols represent sera which exhibited transfer; closed symbols, antisera in which transfer was not detectable.

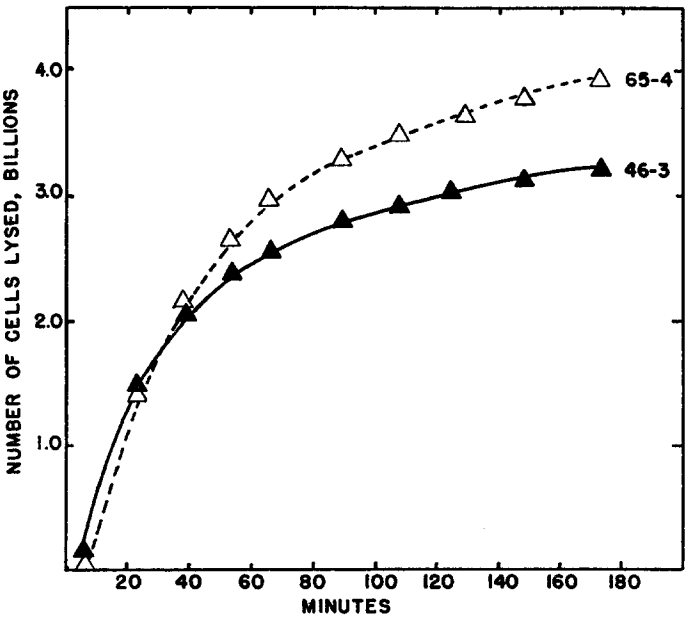

FIG. 8. Kinetic curves of antisera "65-4" and "46-3" matched so that initial velocities are closely similar. Antiserum "65-4" exhibited good transfer, but transfer was not detectable with antiserum "46-3."

not controlled in an entirely satisfactory manner and therefore convey an exaggerated impression of the role played by heterogeneity of the red cells. However, the main point at issue, *i.e.* the suggestion of antibody transfer made in a previous study (1), is resolved by the direct demonstration of this effect in the present work.

As to the role which transfer of A plays in influencing the kinetics of hemolysis, it is important to recognize that any A which dissociates can form new complexes SA either on the intact red cell or on the stromata present in a partially lysed reaction mixture. Thus, red cells and stromata compete for dissociated A, and the higher the degree of lysis, the greater the proportion of stromata to intact cells. Accordingly, as lysis progresses the intact cells obtain less and less of the dissociated A, while the stromata get an increasing proportion. Therefore, the effect of the transfer of A on the kinetics of lysis diminishes as the lytic process goes on, and eventually it becomes negligible as complete lysis is approached.[12] This effect can be seen in Figs. 1 and 4 of reference 1.

It is of interest to speculate on the significance of the present findings in respect to the hypothesis that immune hemolysis is an enzymatic process. This concept has been postulated by numerous investigators, but it has been difficult to secure convincing experimental support. When it was found a few years ago that Mg^{++} in trace amounts is an essential cofactor in the hemolytic reaction (9) the idea of an enzymic mechanism was again brought to the foreground. The kinetic observations presented previously (1) have lent further support to the concept that hemolytic antibody behaves like an enzyme while the complement appears to be a reactant since it is used up in the lytic process. The results of the present study further serve to substantiate this hypothetical mechanism. It appears likely that a definitive answer concerning the enzymic mechanism of the hemolytic reaction will be obtained when and if the nature of the fundamental reaction, *i.e.* the change from S to S′, can be elucidated. It is mainly for this reason that investigations are under way in this laboratory toward the isolation and chemical characterization of the postulated substrate, S.

SUMMARY

The reversibility of the red cell–antibody combination has been demonstrated by means of experiments showing the transfer of antibody, either from red cell stromata to intact red cells, or from red cell to red cell.

The extent of reversibility is variable with respect to different antisera.

The transfer of antibody from stromata to intact red cells is not dependent upon the action of complement.

[12] It has been found that if red blood cell stromata in large excess are added to a hemolytic system shortly after initiation of the reaction, the lytic process comes to a halt gradually due to transfer of A from the red cells to the stromata; control titrations indicated that the cessation of lysis was not due to loss of C′.

Transfer of antibody can also take place after interaction of the stromata-antibody complex with complement. As a result, antibody molecules can perform hemolytic work over and over again, provided complement is available, and this leads to kinetic behavior of a catalytic character.

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