EVALUATION OF AN OPTICAL DEVICE FOR QUANTITATIVE STUDY OF ERYTHROCYTE AGGLUTINATION

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ABSTRACT

EVALUATION OF AN OPTICAL DEVICE FOR QUANTITATIVE
STUDY OF ERYTHROCYTE AGGLUTINATION

by James John MacKenzie

An optical device for measuring settling rates of agglutinating erythrocytes was studied. Its ability to differentiate between agglutinating and non-agglutinating settling blood suspensions was established. Experimental conditions of magnetic field were applied to determine their effect on agglutinating blood suspensions. No effect was found with the ABO blood system under the stated conditions. The D reaction in the Rh blood system was found not to be measurable by this device. A study was made of the quantitative aspects of the graphical record of agglutinating suspensions which proved inconclusive.

EVALUATION OF AN OPTICAL DEVICE FOR QUANTITATIVE STUDY OF ERYTHROCYTE AGGLUTINATION

By

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A THESIS

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INTRODUCTION

In this thesis we consider a problem in biophysics: the possible influence of magnetic fields upon the agglutination of human erythrocytes in the antigen-antibody reaction. Many biological systems involve such a large number of variables that it is impossible to exercise control over them to the point where the laws of physics can be applied and meaningful conclusions drawn. We have chosen to investigate, in the specific area of bio-magnetism, human-erythrocyte agglutination in vitro as a relatively simple process. This agglutination reaction has a high sensitivity that encourages its use in an experiment to detect small effects, as we know to be the case for magnetic fields. This reaction also exhibits a high degree of specificity with respect to its participants, and permits a quantitative determination of the strength of those participants (1).

In previous work in our laboratory, A. E. Smith found with the antibodies of the Rh system that erythrocyte agglutination was enhanced by magnetic fields (2). This effect was observed visually by the method of Race and Sanger. The fields were homogeneous, with strengths from 20 to 5000 oersteds; and inhomogeneous, with gradients of 3,000 oersted/cm. at a level of 4,000 oersteds. With antibodies of the ABO blood system, no effect was found, but the data were not adequate to establish definitely a negative result.

Independently of Smith, Foner at the Boston University School of Medicine confirmed enhancement of agglutination in a magnetic field, again by use of the Race-Sanger visual scoring system (3). The effect was noted for Anti-D and Anti-A, and also for a plant

1

agglutinin lectin. Both homogeneous and inhomogeneous fields were used at strengths between 1200 and 2600 oersteds.

In these two cases, the results were obtained by methods that are to some extent subjective. Smith attempted to use the Coulter Counter as the basis for an objective measure of agglutination, but was unable to detect any response. He speculated that the electronic counter disrupted the aggregates and suggested use of a less disruptive method for evaluating agglutination, for example, sedimentation (2). In our laboratory, H. P. Cole built a device to measure simultaneously the change of optical density with time in up to eight settling blood suspensions. A detailed account of the individual components and the mode of operation is given in his thesis (4). The device consists of a moving boom with light from the source at one end passing through an optical train to the other end. The light beam sweeps a series of tubes containing settling blood suspensions. The tubes, which are silicone-treated Pyrex rectangular spectrophotometer cells, are situated on an arc of a circle concentric with the circle traced out by the light source (see Figure 1). The light, after passing through the sample tubes, falls on the photocell whose output is fed directly into a Sargent recorder to provide a graphical record of transmitted light versus time.

In a settling blood suspension that is agglutinating there will appear over the depth of the liquid a more or less continuous optical-density gradient, which will alter as time goes on. In the absence of agglutination and with a homogenous distribution of erythrocyte sizes, the optical density becomes a step function, constant below the interface of the descending erythrocytes, and very small above it (see Figure 2).

Analyzing the graphs obtained under various concentrations of erythrocytes and antibody should enable us to determine in some
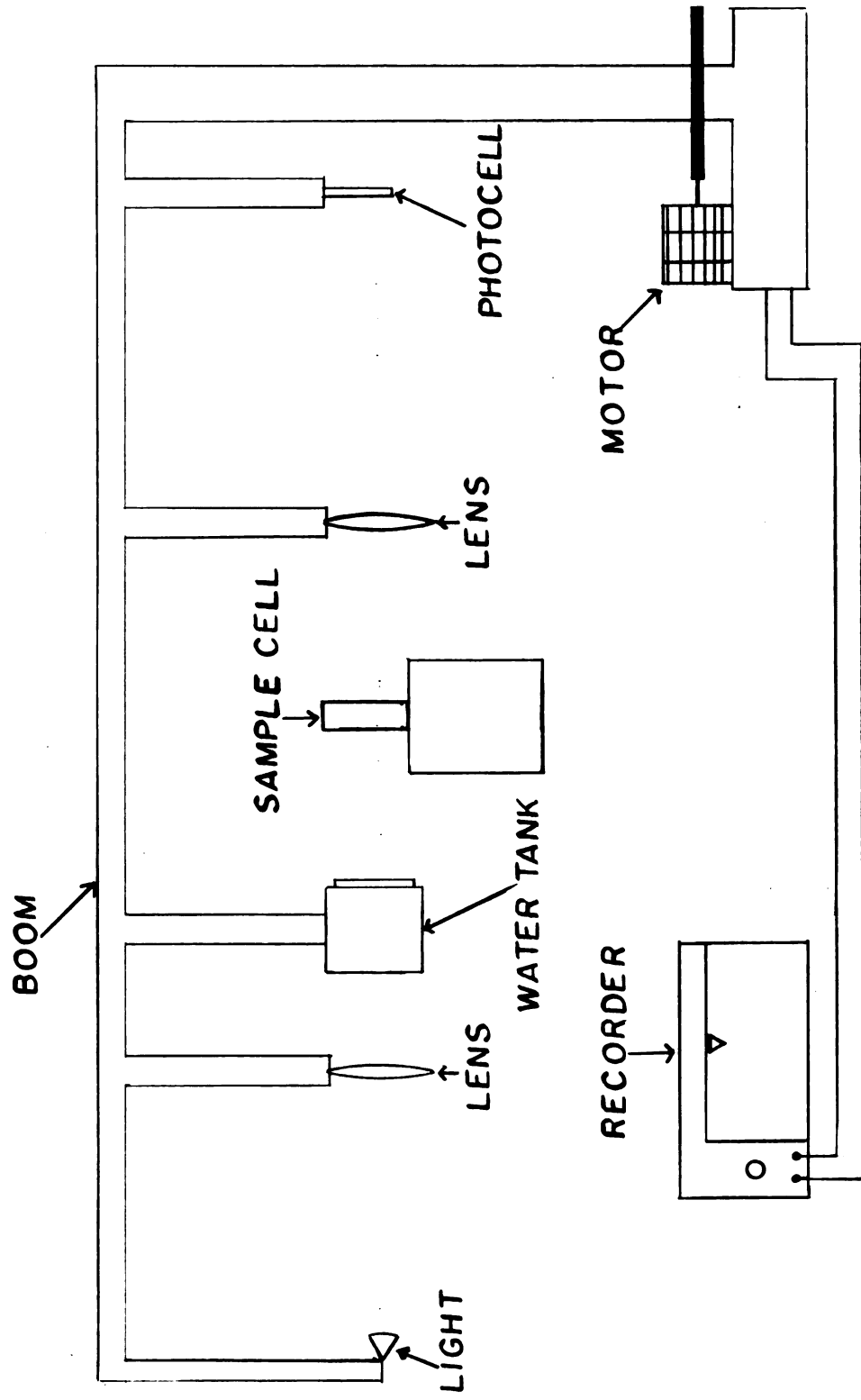
FIGURE 1. Device to Measure Optical Density of Blood Suspension as a Function of Time
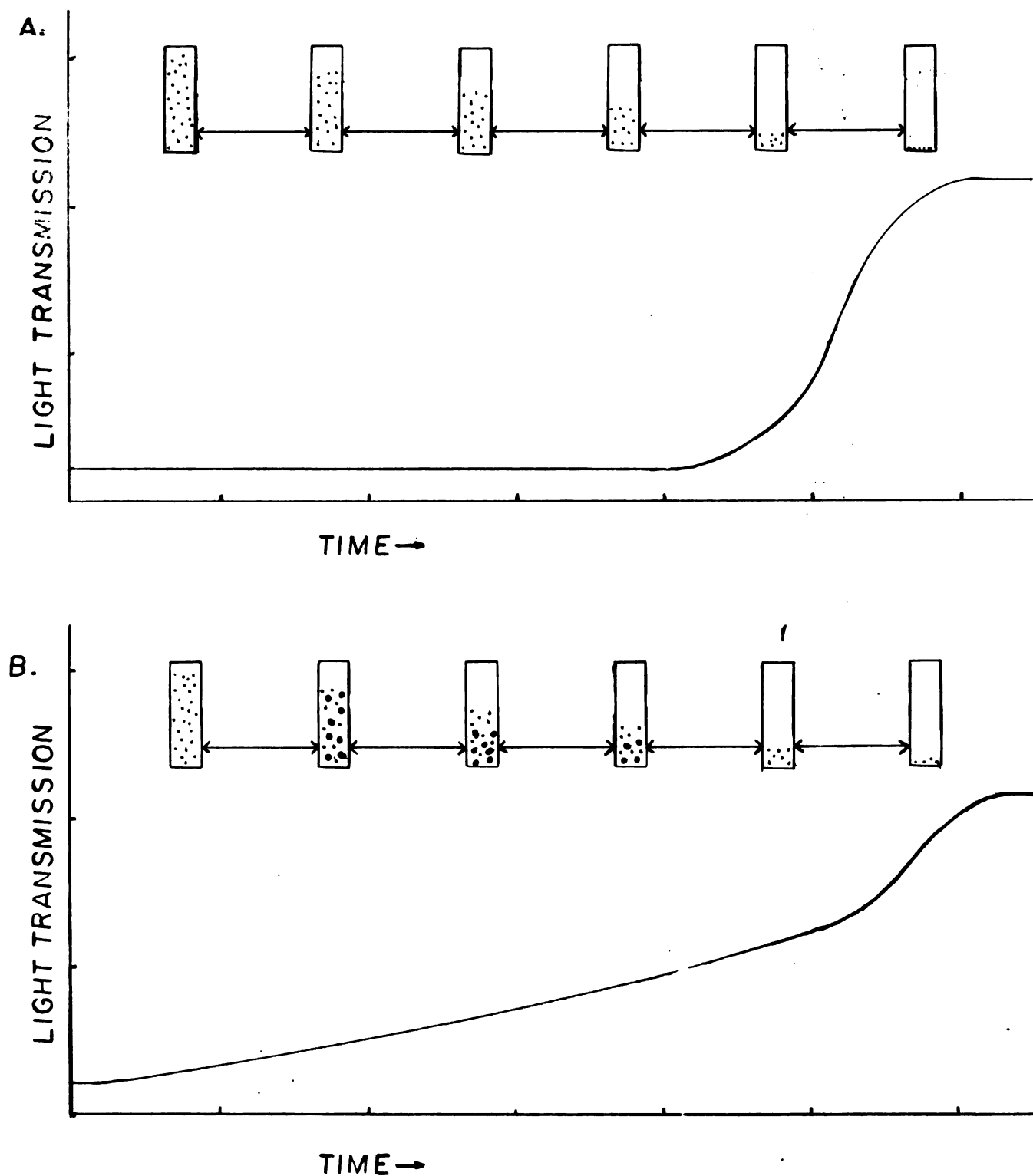
FIGURE 2. A. Graphical record and physical appearance of non-agglutinating settling blood suspension in Beckman cell.
B. Graphical record and physical appearance of agglutinating settling blood suspension in Beckman cell.

quantitative manner the degree of agglutination. We must, of course, determine the reproducibility and the sensitivity of the instrument before any data for magnetic fields can be interpreted meaningfully. Let us examine the nature of agglutination to try to understand the meaning of the records obtained experimentally.

AGGLUTINATION

Human-erythrocyte agglutination is a long-studied reaction, involved in many standard procedures of hospitals, laboratories, and blood banks. Its understanding began in the early part of the twentieth century, when Landsteiner postulated different "types" of blood. He discovered the A and B types with the corresponding antigens on the blood cell, and an O type with neither a B nor A antigen. Next, Von Decastello and Sturli found a fourth type of blood--AB, the red blood cell having both the A and B antigen (5). Many other blood groups have since been discovered and classified, including the A_1 and A_2 antigens into which A antigen can be broken down. One of the most important of these groups is the Rh, discovered in 1939-40 by Landsteiner and Wiener (6). We shall deal with only the ABO and Rh systems. In Table 1 these are tabulated.

When an antigen enters a higher-order animal, one of the defense reactions enlisted in the organism is the production of antibodies that attack the invader and render it harmless. This reaction is called immunization. Upon subsequent invasion by the same antigen, the organism again produces antibodies, but much more rapidly.

The manner of production of antibodies is imperfectly understood. There are two main theories that have evolved to explain the mechanism of antibody production in response to antigentic stimulus; neither can be reconciled wholly with the evidence. The first theory postulates that the antigenic molecule serves as a template around which the antibody molecule is formed. Pauling, in 1940, suggested that the lock-and-key arrangement of the antigen-antibody combination was due to the folding of the antibody polypeptide chain around the antigen (8). Under this

TABLE 1 (7, 8). ABO and Rh Blood Systems.

Rh System of Blood Factors

Blood Groups	% Population	Agglutinogen	Agglutinin	Reaction with Anti-C
Rh⁺	85%	Rh	none	positive
Rh⁻	15%	none	Anti-Rh	negative

ABO System of Blood Factors

Blood Groups	% Population	Agglutinogen	Agglutinin	Reaction with Anti-Serum	
				Anti-A	Anti-B
O	45%	none	Anti-A Anti-B	negative	negative
A	41%	A	Anti-B	positive	negative
B	10%	B	Anti-A	negative	positive
AB	4%	A,B	none	positive	positive

theory, when the antigen has been removed from the organism the antibody production should stop; but it does not in some cases. The second theory states that the body contains within itself, irrespective of its immunological history, preformed antibody "receptors" of all types. When an antigen unites with one of these receptors, more free receptors (antibodies) are formed. The problem is how to explain the antibody production under the antigenic stimulus of artificial products of the laboratory. Foreknowledge of these artificial products on the part of the organism seems rather doubtful, as well as does a dual type of antibody formation to explain their production under these conditions (8). It might turn out that a combination of the two theories will be necessary.

The antigenic molecules of interest to us are proteins having a molecular weight on the order of 15,000. They reside on or in the surface of the human red blood cell. The antibody molecule is found in the gamma-globulin fraction of the blood serum and has a weight of about 150,000 (9).

As shown on the following page (FIGURE 3), the red blood cell is a bi-concave disk with average characteristics as follows (7): diameter, 7.5 μ; thickness, 2.3 μ; surface area, 150 μ^2; volume, 87 μ^3.

Born in the bone marrow, an erythrocyte has a life span of approximately 120 days as it passes through its various stages of development. Erythrocytes constitute 45% of the volume of whole blood, with plasma, platelets, and white cells making up the rest. The outer membrane of an erythrocyte consists of lipoprotein complex (10). Acidic lipids on the outer surface displace the isolectric point of the erythrocyte towards the acid side. In fact, the pH of red-cell ghosts is approximately 2 (8).

A similar structure of protein, the stroma, exists within the surface structure and acts as a matrix to support the hemoglobin,

which makes up most of the internal part of the erythrocyte. This

structure of the erythrocyte makes it very sturdy and flexible.

The hemoglobin accounts for the ability of the cell to transport oxygen

from the lungs to the tissue of the body.
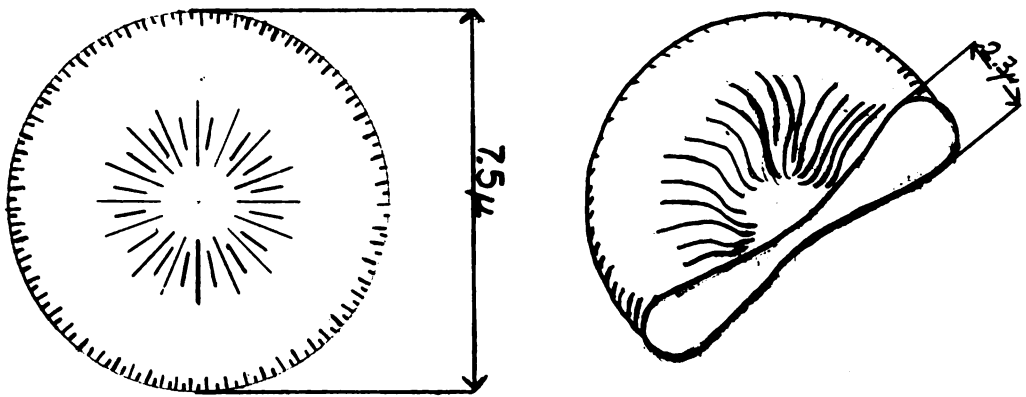
FIGURE 3. Drawing of Red Blood Cell

The phenomenon that our machine records is the sedimentation of

agglutinating red blood cells. The two main theories of agglutination

are the Bordet and the lattice theories. Under the Bordet theory,

agglutination comes about because the antibody molecule merely makes

the cells sensitive to the agglutinating action of the electrolyte in which

they are suspended (11). This theory does not explain, of course, the

specificity of the reaction observed upon mixing two types of erythro-

cytes and antibodies together. Instead of getting clumps made up of

two different types of erythrocytes, one gets instead very specific

aggregates made up of agglutinated erythrocytes of either one type or

the other. The lattice theory, which in a simplified form says that an

antibody molecule "holds hands" with two antigenic molecules, gives

a satisfactory description of the observed specificity. But although the

lattice theory explains the specificity, it does not explain completely

the action of erythrocytes in the presence of excess antibody. Boyd
and Hooker exposed red blood cells to an excess of antibody, so that
all the reactive sites were taken up by antibody molecules (12).
Nevertheless, agglutination did take place. On the other hand, in many
cases there is observed a maximum of agglutination with respect to
serum concentration. The serum concentrations on either side of
this maximum are called the pre-zone in the case of low concentration
and post-zone in the case of high concentration.
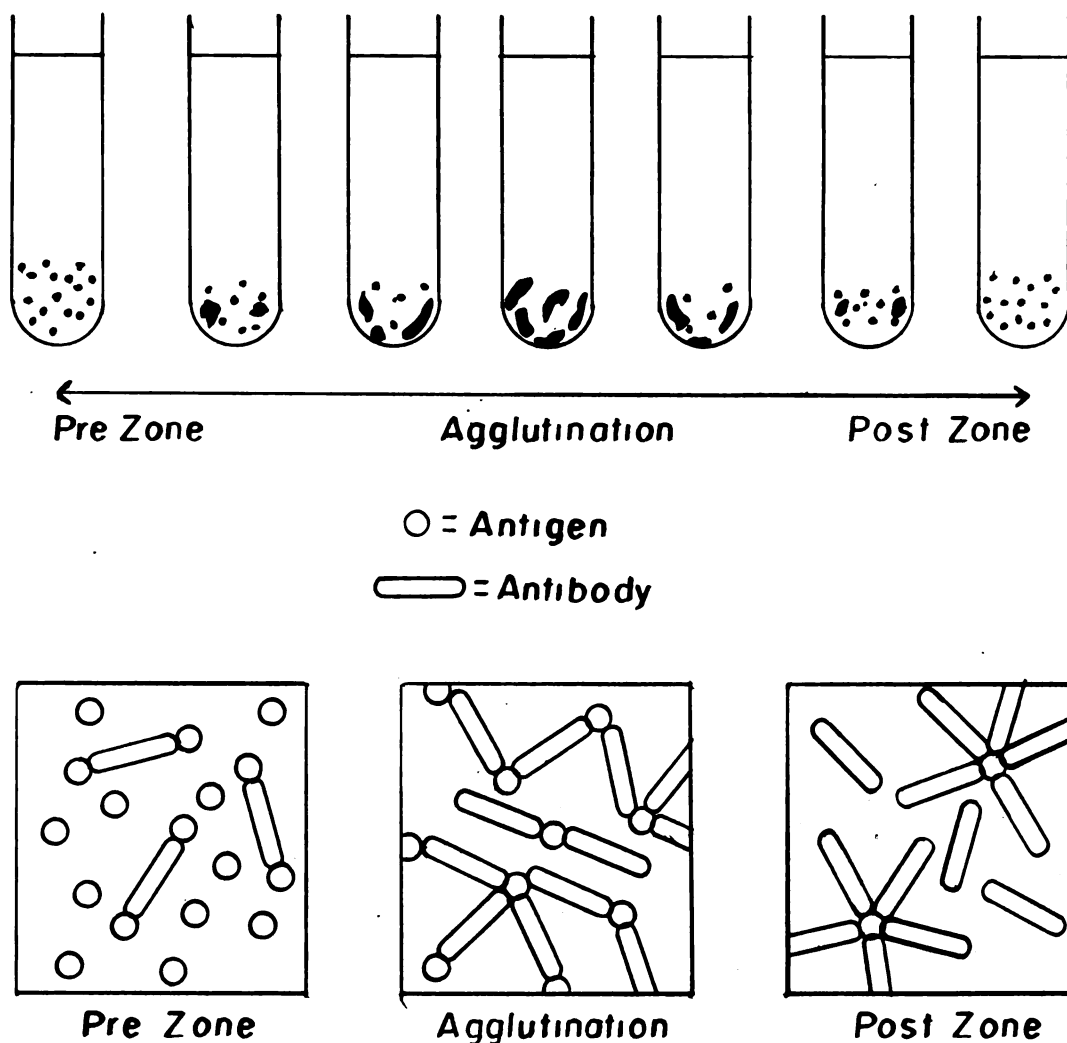
FIGURE 4. Test tube and microscopic observation of pre-zone,
agglutination, and post-zone with bivalent antibody.

The number of combining sites where a B-antibody molecule may combine with a B antigen on a human red blood cell has been calculated by Filitti-Wormser to be 5×10^5. For D (Rho) sites, Boursnell, Coombs, and Rizk found approximately 5.5×10^3 sites per cell (11). Supporting this finding are the results of Belkin and Wiener who prepared A, B, and Rho haptens from the stroma of human erythrocytes (13). Measuring the lowest titer of the A, B, Rho hapten serum that agglutinates the appropriate erythrocytes, they found that the Rho hapten serum titer was consistently lower. They concluded that the number of Rho haptens per red blood cell might be lower than in the case of A or B haptens.

Agglutination can be thought of as taking place in two stages. The first is the simple combining, in some way, of the antibody with the antigen residing on the surface of the blood cell. The second stage is the combining of the antibody-antigen-cell complex with an antigen on another blood cell.

The first stage of the reaction, that of joining the initial antigen with the free antibody molecule, involves three forces, singly or in combination: Coulomb, van der Waals, and hydrogen-bonding. The last two forces will be of short range, increasing markedly as the molecules approach each other. None of these forces in themselves are specific; a charged antigen, for example, cannot differentiate between equal charges on each of two different antibodies. The experimentally exhibited specificity of the reaction would then have to come about from some sort of complementary configuration between the antigen and antibody molecule that would allow these forces to come into play at short distances. If Pauling's theory is correct, the "closeness" of fit of the antigen into the folded antibody would account for the specificity because of strong van der Waals forces, for instance, that bind them together.

Experiments have been done on changing the shape of an antigen combining group (11). A slight modification in shape decreased the strength of the antigen-antibody reaction, in support of the concept that complementary shape is important.

The Coulomb force does not enter into the first stage of the reaction because the blood factors (antigen and antibody) that we deal with do not have any positive or negative reactive groups in them. In the second stage, however, where the erythrocytes come together, agglutinating forces must clearly overcome the Coulomb force of repulsion due to the negative surface charge of the erythrocytes in suspension. We find that this repelling force associated with colloidal particles carrying negative charge is overcome by decreasing the pH of the solution to the point where the particles absorbs H^+ ions and are neutralized. Flocculation can then take place. This change of surface charge was first thought to play an important part in agglutination, with experiments on surface potential of bacteria lending support. Further experiments on reducing the surface charge of erythrocytes by charge-lowering agents, however, showed no increase in sensitivity to agglutination (11). This result indicates that the attachment of the antibody to the antigen of the blood cell does not alter the surface charge of the cell.

When a negatively surface-charged blood cell is placed in an ionic solution, a positive cloud of ions surrounds it. In some way the antibody molecule bridges the gap between blood cells through this ion cloud, without allowing the Coulomb force of repulsion to develop.

The antibody molecule is cigar shaped, with major and minor axes in our case of 263 $\overset{o}{A}$ and 37.1 $\overset{o}{A}$ respectively. This length of antibody molecule could provide a link between the blood cells if it attached itself by the end points. The blood cells would then be sufficient distance apart that the Coulomb force would not be a problem.

(See Figure 5). It would follow from this model of end-point attachment that the antibody molecule is bivalent, as is generally agreed (14).
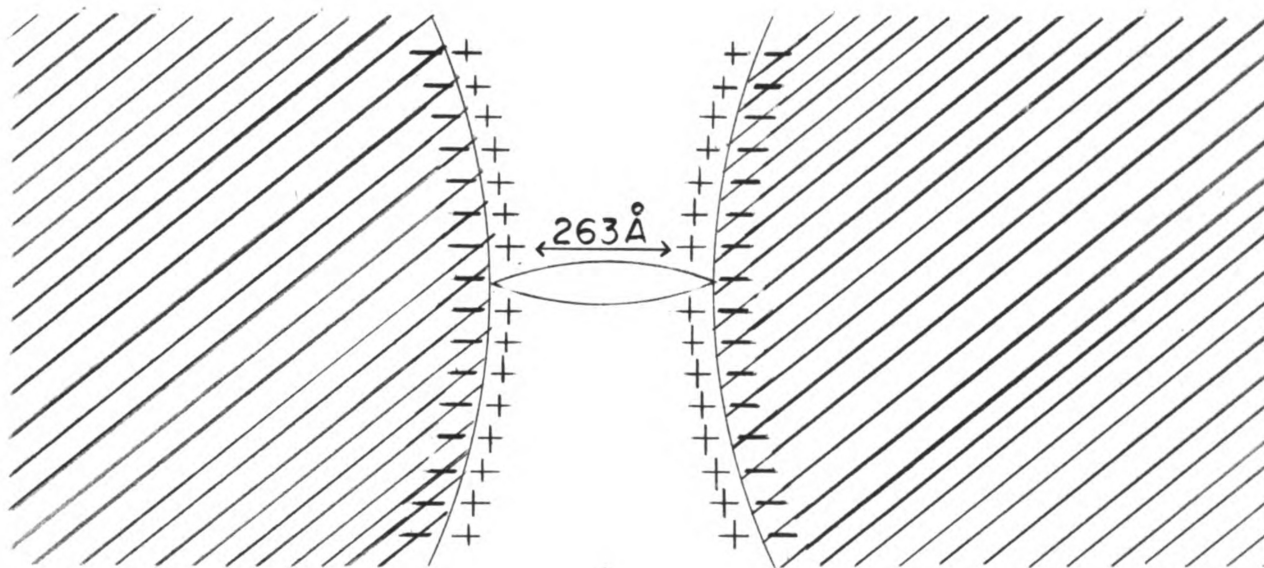
FIGURE 5. Two Blood Cells and Antibody Molecule in Ionic Solution

MATERIALS AND EXPERIMENTAL PROCEDURES

Blood was obtained from the American Red Cross Blood Bank. The Red Cross draws 420 ml. of whole blood intravenously into a glass vessel containing 120 ml. of anticoagulant (A.C.D. mixture: disodium citrate 2.0-2.5 g., dextrose 3 g., distilled water to 120 ml.). The blood is then refrigerated and stored at $4^{\circ}C$. Blood so obtained is classified as outdated 21 days after date of donation. We obtain blood 2-4 days after it has become outdated. Two 5-c.c. portions of whole blood plus A.C.D. mixture were removed from the Red Cross storage bottle by a syringe and transferred to two vacuum containers (evacuated rubber-stoppered test tubes). These containers were stored at $4^{\circ}C$ for up to a week, 1.0-ml. portions being removed from them as needed.

In preparing blood suspensions for the experiment, a 1.0-ml. portion of blood was removed from the vacuum container and placed in a 10 x 75-mm. test tube which was then filled to the top with saline solution. The tube was centrifuged at 3000 rpm for 5 minutes; the supernatant liquid was then removed and fresh saline added. This process was repeated three times so that the A.C.D. mixture and the plasma constitutents of the whole blood were thoroughly washed out.

A suitable volume of packed red blood cells was then removed from the tube with a micropipette. These packed erythrocytes were added to saline in order to make the desired initial suspensions. Depending on the number of samples being measured in the machine, equal amounts of the initial blood suspension were added to equal diluting amounts of saline, or of saline and serum. This procedure resulted in separate blood suspensions with varying amounts of serum but with the same concentration of erythrocytes.

The serum was supplied by Ortho: Anti-A B, Anti-A, Anti-B, Anti-D; and by Dade: Anti-A, Anti-B. The serum was kept refrigerated at 4°C. The Anti-D runs were made at incubation temperatures of approximately 37°C, and the other runs at room temperature.

Two permanent magnets provided fields over the length of the tube. Magnet C1 had a gap of 0.69 inch between identical flat circular pole faces 0.76 inch in diameter. The field at the center of the gap was 4100 oersteds as measured by a Rawson-Lush rotating-coil gaussmeter (Type 723). The sample cells, with outside dimensions 12 x 12 x 48 mm., provided a 10-mm. light path through the liquid. During the run the cell was placed between the pole faces at equal distances from each face, with the center of the volume of the cell at approximately the center of the field as shown in Figure 6. The field strength in the vertical plane through the center of the tube parallel to the pole face is shown in Figure 7.

The other permanent magnet, C2, had one circular pole face 1.5 inches in diameter, and one conical pole piece of base diameter 1.5 inches and height .922 inch. Its apex was .625 inch from the circular pole face. The cell was placed next to the apex as shown in Figure 8. The field mapping in the vertical plane through the center of the tube parallel to the pole face is shown in Figure 9 for magnet C2.

In a typical run the experimental procedure was as follows. The light source was turned on at least 1/2 hour before the start of a run so as to reach a steady state. A heating element was switched on when the samples required incubation above room temperature. In such cases a continuous record of the temperature was kept before and during the run.

The blood suspensions were prepared in individual 20-ml. glass vials. Each of these vials contained 10-ml. of blood suspension, sufficient to fill two sample cells. The sample cells were washed thoroughly
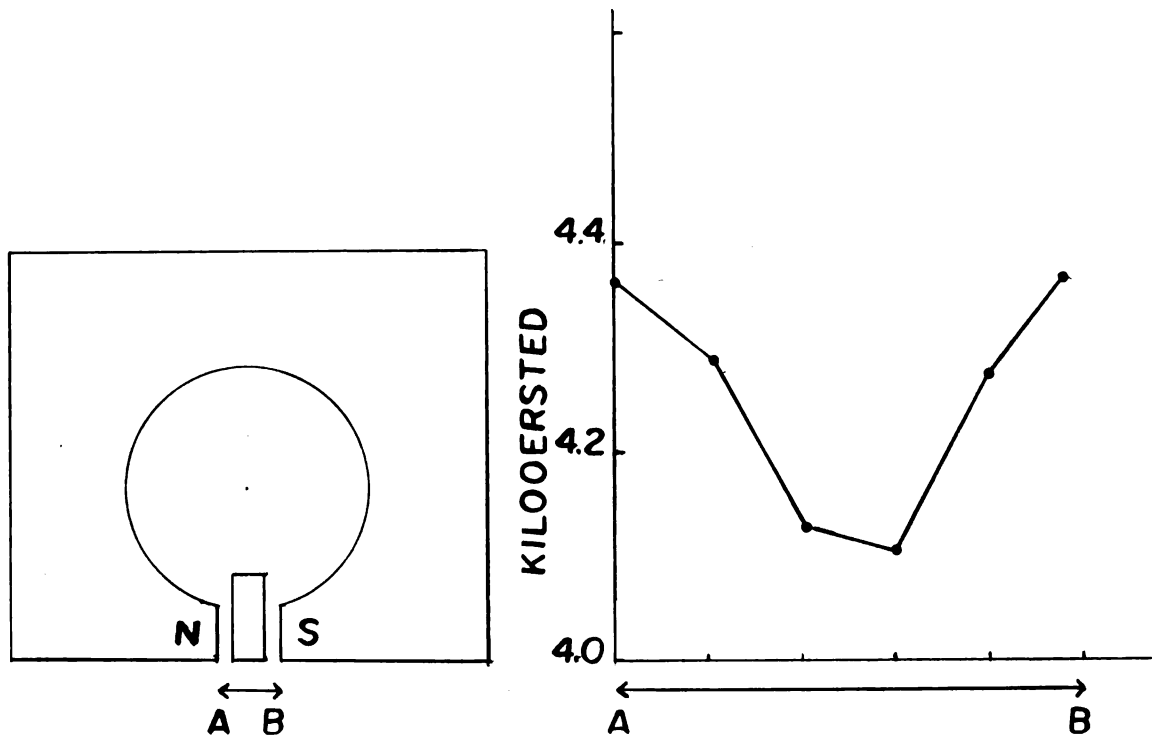
FIGURE 6. Magnet C1 with sample cell and horizontal field measurement from pole to pole at center of field.

after each run, and then dried with cotton swabs and absorbent lens paper. The tubes were wiped inside and outside after drying in order to remove any film deposits. The cells were then placed in the machine, care being taken to keep them perpendicular, since inclination of the tube changes the rate of sedimentation (14).

The windows were adjusted so that approximately the same amount of light energy for each cell would fall on the photocell as indicated by the graphical record. When adjustments were completed, the proper amounts of antibody were added to the separate vials. These vials

12 = 1.2 in.

625	680	760	850	900	935	925	880	815	715	615

10

755	850	980	1110	1200	1235	1225	1150	1025	875	750

8

890	1050	1250	1475	1640	1700	1675	1495	1280	1070	875

6

1050	1260	1600	2000	2340	2475	2350	1975	1600	1275	1025

4

1210	1500	2000	2660	3240	3425	3225	2610	1970	1500	1160

2

1300	1690	2365	3250	3900	4020	3825	3150	2275	1650	1256

10=1 in. 9 8 7 6 5 4 3 2 1 0 1 2 3 4 5 6 7 8 9 10=1 in.

1340	1760	2500	3425	4000	4100	3980	3300	2400	1720	1315

2

1330	1720	2340	3155	3780	3975	3740	3060	2255	1680	1300

4

1250	1560	2000	2560	3070	3250	3030	2500	1970	1550	1250

6

1160	1400	1660	1970	2240	2325	2215	1950	1650	1400	1160

8

1080	1245	1400	1550	1680	1725	1680	1560	1410	1250	1060

10

990	1100	1200	1295	1350	1375	1360	1305	1215	1100	975

12 = 1.2 in.

880	960	1030	1100	1145	1150	1145	1120	1060	1000	880

FIGURE 7. Field mapping in oersteds of magnet C1 (with pole face and tube) in the vertical plane through the center of the tube parallel to the pole face.
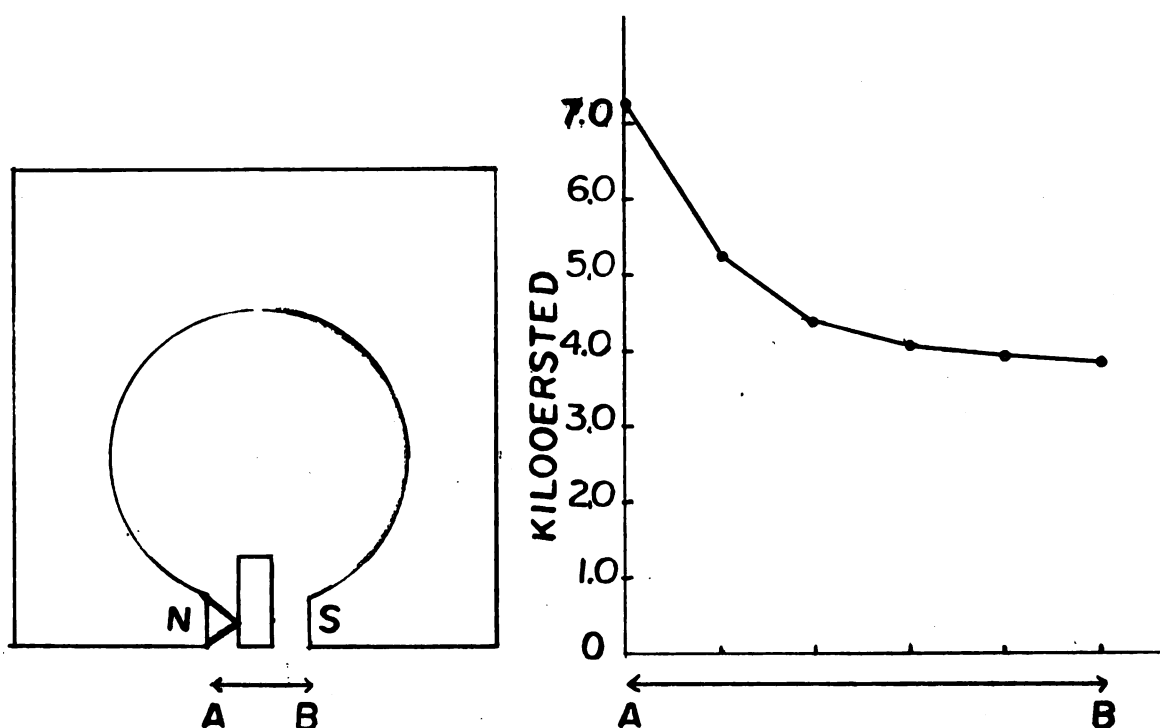
FIGURE 8. Magnet C2 with sample cell and horizontal field measure-
ment from pole to pole at center of field.

were shaken well to ensure complete mixing of suspension and antibody,

and the contents were pipetted into the sample cells. The height of

the bottom of the meniscus above the window was identical for each

cell in order to assure that the light passed through the same portion

of settling suspension in each sample. The windows were placed as

near the bottom of the cell as possible, to provide a long period of

observation before the interface fell across the window. Inasmuch as

the concentration gradient becomes spread out in agglutinating

10=1 in.

880	990	1175	1370	1570	1740	1825	1830	1760	1600	1390	1200	1020	860	740
970	1160	1400	1710	2000	2270	2405	2420	2300	2020	1710	1415	1160	975	810
1080	1325	1680	2100	2525	2840	3005	3020	2880	2540	2075	1660	1325	1070	890
1200	1500	1950	2500	2950	3300	3545	3580	3350	2900	2440	1890	1450	1150	925
1275	1625	2150	2740	3200	3640	4050	4060	3750	3275	2675	2075	1560	1220	925
1315	1675	2225	2810	3290	3700	4350	4500	3975	3380	2790	2140	1610	1240	990
1300	1635	2170	2750	3210	3600	4000	4050	3725	3260	2700	2075	1570	1220	900
1225	1510	1990	2530	2900	3300	3500	3525	3350	2975	2450	1910	1475	1155	940
1125	1360	1725	2175	2590	2875	3040	3050	2890	2550	2100	1675	1325	1070	875
1020	1190	1440	1750	2075	2325	2450	2400	2325	2050	1720	1415	1170	990	825
915	1015	1200	1400	1600	1770	1860	1870	1700	1600	1400	1200	1025	880	750

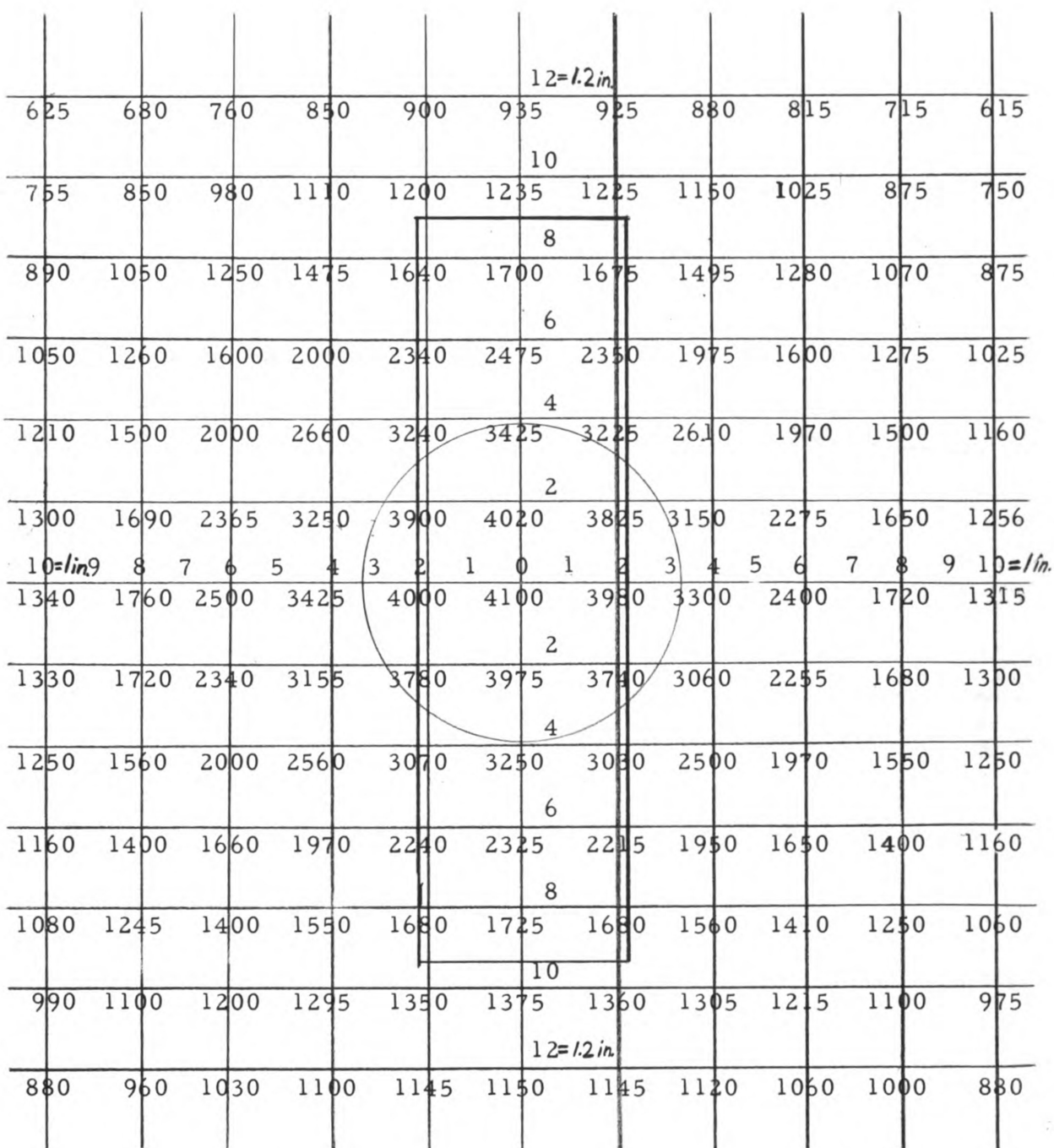14=1.4 in. 12 10 8 6 4 2 0 2 4 6 8 10 12 14 =1.4 in.

10= 1 in.

FIGURE 9. Field mapping in oersteds of magnet C2 (with pole face and tube) in the vertical plane through the center of the tube parallel to the pole face.
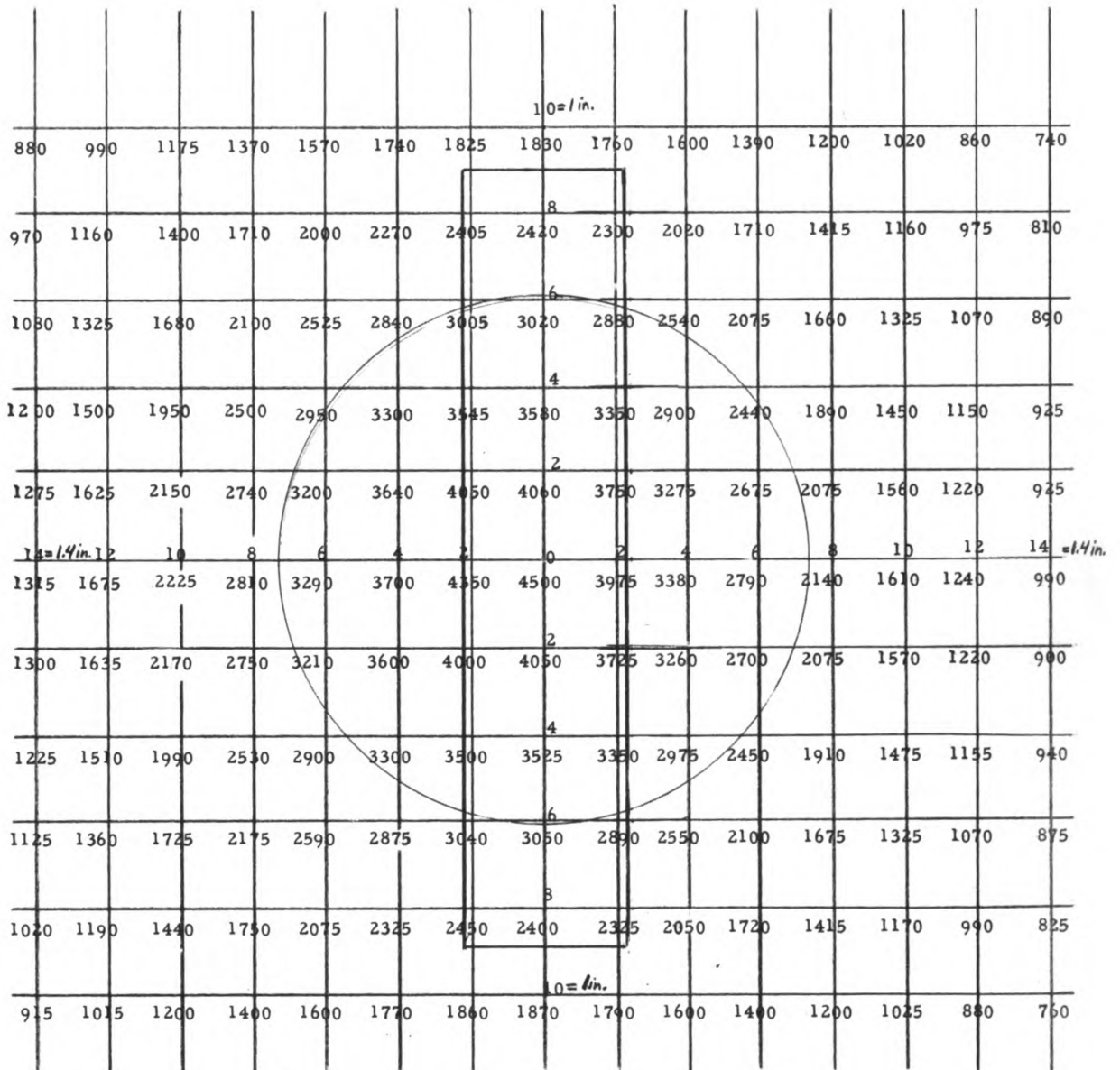
suspensions as time progresses, observation at the lower portion of the tube provides more extensive data. The runs lasted from four to eight hours and were controlled by an automatic timer.

RESULTS

A total of 187 runs were made under various conditions of erythrocyte concentration and type, antibody concentration and type, temperature, and magnetic field. The first fifteen runs were performed to investigate and develop the effectiveness of the machine as an instrument for detecting the difference between agglutinating and non-agglutinating systems. A problem encountered initially was convection currents. These were reduced by placing heat absorbers in the light beam in the form of a 3 x 3 x 3 inch clear plastic tank containing water and a 2 inch square piece of heat-absorbing glass.

Under the proper conditions of erythrocyte and antibody concentration, the graphical record for the agglutinating system was found to be measureably different from that of the non-agglutinating system (see Figure 10).

The separate regions of the graphs are interpreted as follows: (see Figure 11).

Agglutinating sample

1. Suspension remains nearly homogeneous in field while agglutination sets in.

2. Agglutinated particles settle out of field.

3. Interface falls across window.

4. All material has fallen past window.

Non-agglutinating sample

1. Suspension remains at constant optical density in field.

2. Interface falls across window.

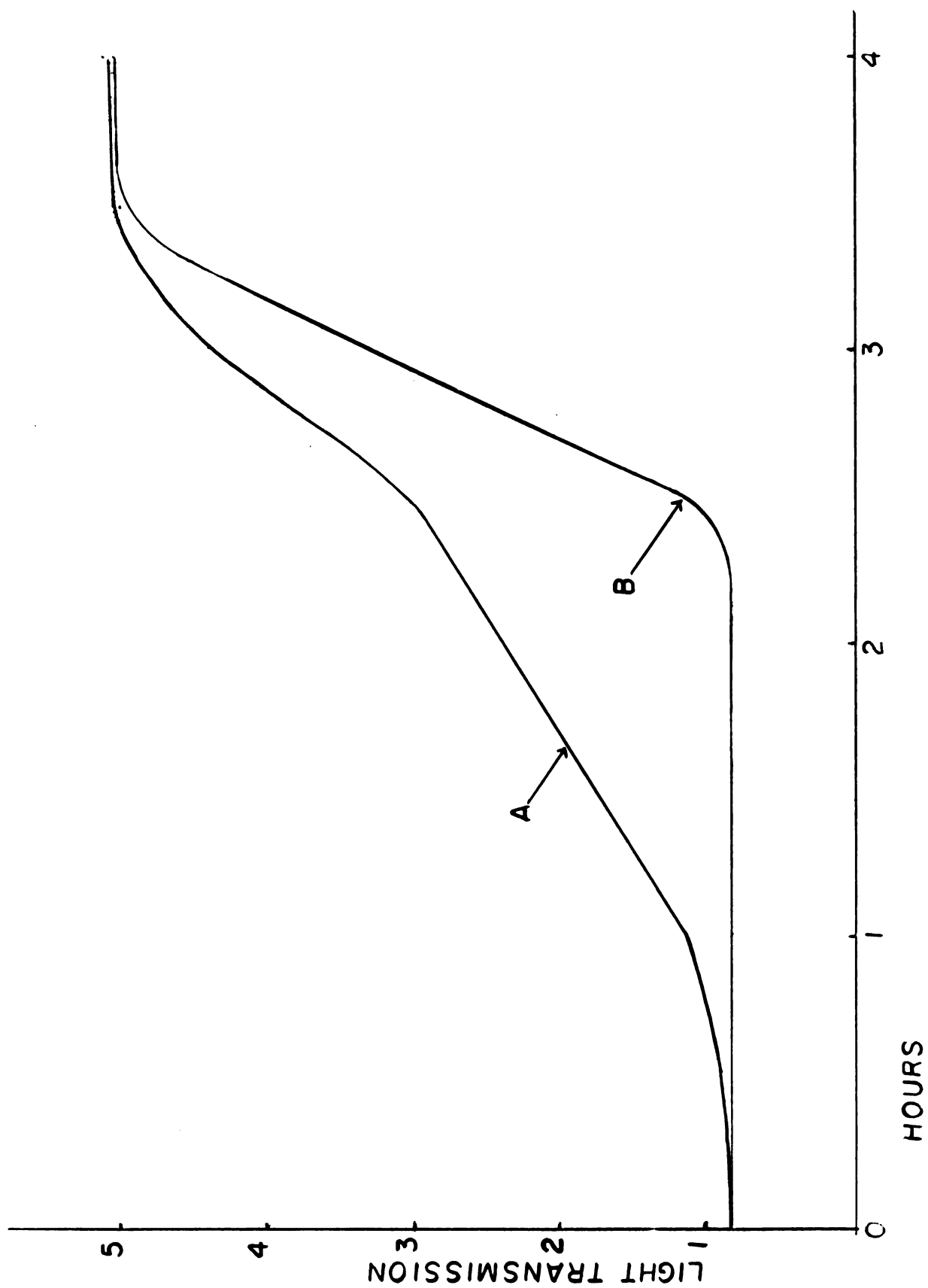3. All material has fallen past window.

FIGURE 10. Plot of run V-15 showing (A) agglutinating and (B) non-agglutinating samples.
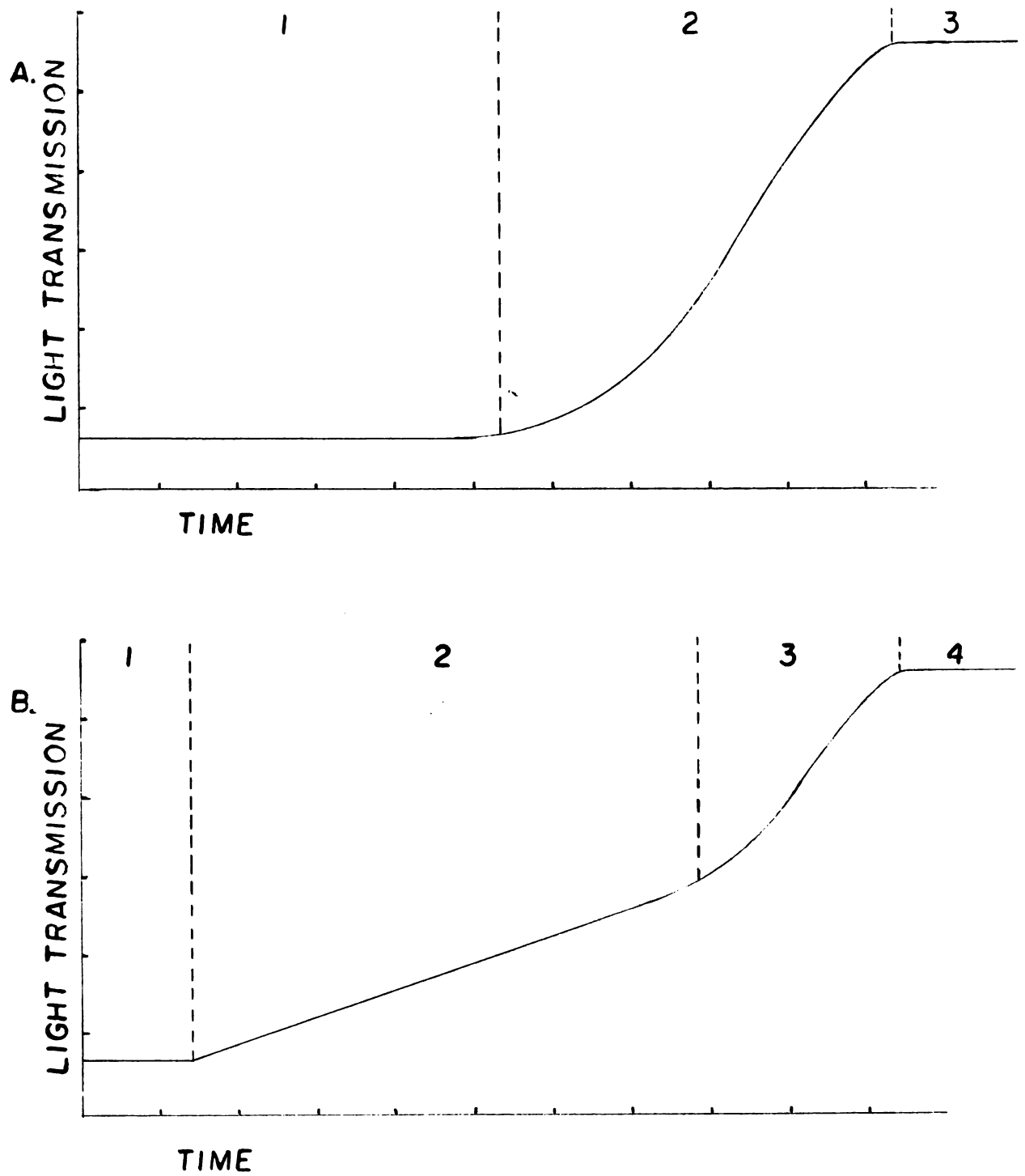
FIGURE 11. Regions of (A) non-agglutinating blood suspension,
(B) agglutinating blood suspension.

The next 61 runs were made with the samples kept in an inhomogeneous magnetic field, to see if any gross effects appeared. Forty-one runs on A, B, and D antibody were made with magnet Cl.

With the ABO blood group, no difference in the graphs was discernible between samples agglutinating in the field and those outside it. Various combinations of concentrations and temperature were tried.

The runs made with the D antibody showed no evidence of agglutination during settling. Concentrations of blood cells and antibody were the same as those used in previous work with the Coulter Counter in which agglutination was detected. Examination of the settled blood on the bottom of the tube showed that the cells had agglutinated. Therefore, although the cells did not agglutinate while settling, they did agglutinate once they had reached the bottom. Such delayed agglutination would indicate an important difference in the conditions necessary for agglutination between the D and A-B antibodies. One explanation suggested for this behavior is that the antibody and antigen molecules in the D reaction need a long time of contact to effect a bond. Therefore, although the cells do not agglutinate when settling, the D reaction does take place during the prolonged contact at the bottom of the sample tube. This difference in behavior between ABO and D systems under comparable erythrocyte concentration could be explained under the complementary-configuration theory of antigen-antibody reaction by saying that the configuration for the D system is more complicated than that for the ABO system. The D participants consequently, require a longer period of time to "fit" together to the point where the short-ranged forces discussed previously could become effective. Very possibly the difference in the number of reactive sites where the D and ABO reactions can take place on the blood cell is a contributing factor to the behavior. In any case, it was found that the D reaction was unsuitable for investigation by this machine.

The next series of runs was made with magnet C2, again on the ABO system. Twenty runs were performed without any discernible differences in the graphical results. One observation made at this time, which may eventually give some insight into the nature of the agglutination reaction, was that of the effect of a time-varying temperature on settling blood suspensions. With a rate of change of temperature of the order of 2-3°C drop per hour, a non-agglutinating settling blood suspension formed no interface, and its graphical record was identical in form to that of a agglutinating sample without the time-varying temperature.

The graphical characteristics of agglutination at a steady temperature appeared when a run was performed with a temperature drop whether the samples contained antibody or not (see Figure 12). The magnitude of the effect on the agglutinating sample was greater because of the combined action of antibody plus temperature drop. Further investigation into the phenomenon might lead to more understanding of the forces involved in agglutination.
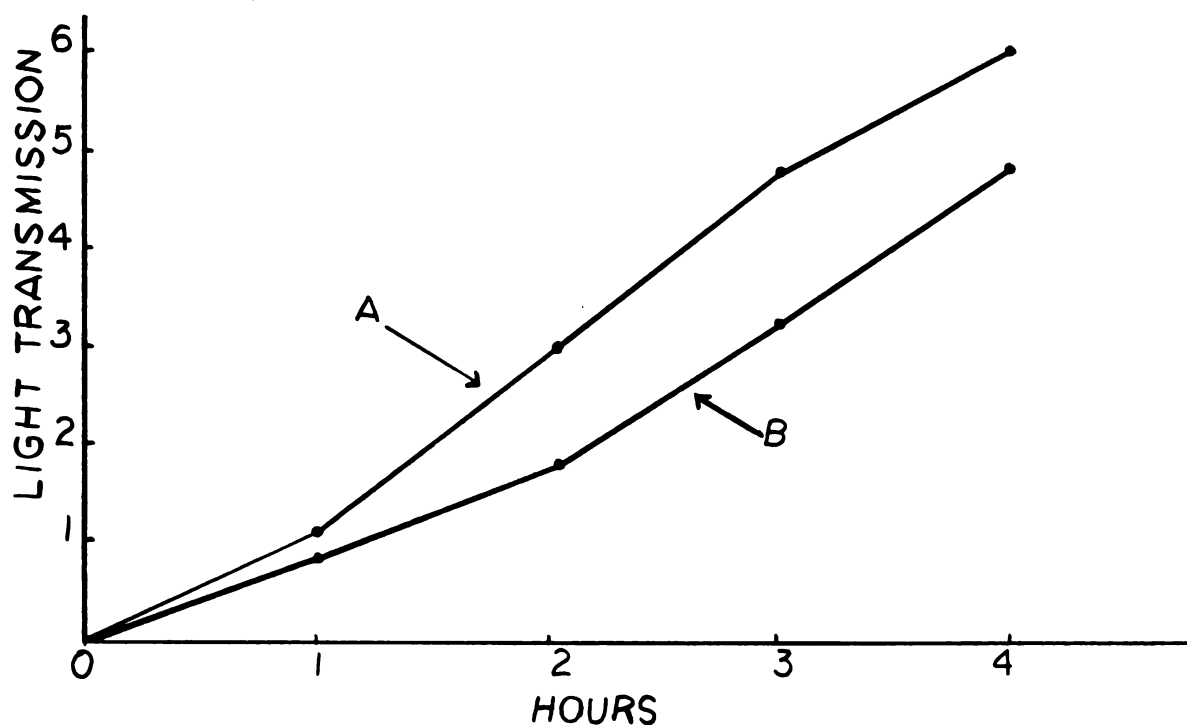

FIGURE 12. Plot of two samples with temperature drop: A. Blood suspension with antibody; B. Blood suspension without antibody.

The next series of runs was designed to investigate the repro-
ducibility and the sensitivity of the machine. With runs after V-77,
magnetic fields were not applied to the samples. The test specimens
were four pairs of two samples each, all eight having the same blood
concentration, but each pair having a different antibody concentration.

Various factors influenced the reproducibility from run to run.
The blood samples obtained from the Red Cross were not from the
same donor each time. Since variation in sensitivity to antibody con-
centration is common among individuals, it is not surprising to find
a variation in response with such blood. Aging of the blood is another
factor causing variation. Deterioration takes place even though the
blood is refrigerated. Variations in ambient temperature about the
machine would also affect the agglutination rates. Although each of
these factors in themselves might produce only a slight difference, in
combination they might account for the run-to-run variability noted in
our data.

Before we investigate the sensitivity of the instrument it would
be advisable to consider the mathematical treatment of light trans-
mission as described by a modified form of the Lambert-Beer law (15).
A parallel beam of light with cross sectional area A falls on a sus-
pension of path length $\Delta \ell$ made up of spherical particles of diameters
d_i dispersed in a fluid where i indicates the different diameters. There
are n_i of these particles in a gram of particle material corresponding
to each d_i.

The projected area per gram of material is:

$$\Sigma \; n_i \; k \; d_i^2 \; , \tag{1}$$

where k is a comprehensive constant.

The total projected area of these particles per cm^3 of
suspension is:

$$C \sum_i n_i k d_i^2 , \qquad (2)$$

where C is the concentration of the suspension.

The projected area of particles in light path is

$$A \Delta\ell C \sum_i n_i k d_i^2 . \qquad (3)$$

Expression 3 is equal to the change in effective cross sectional area $(-\Delta A)$ of the beam going through the suspension.

$$-\Delta A = A \Delta\ell C \sum_i n_i k d_k^2 \qquad (4)$$
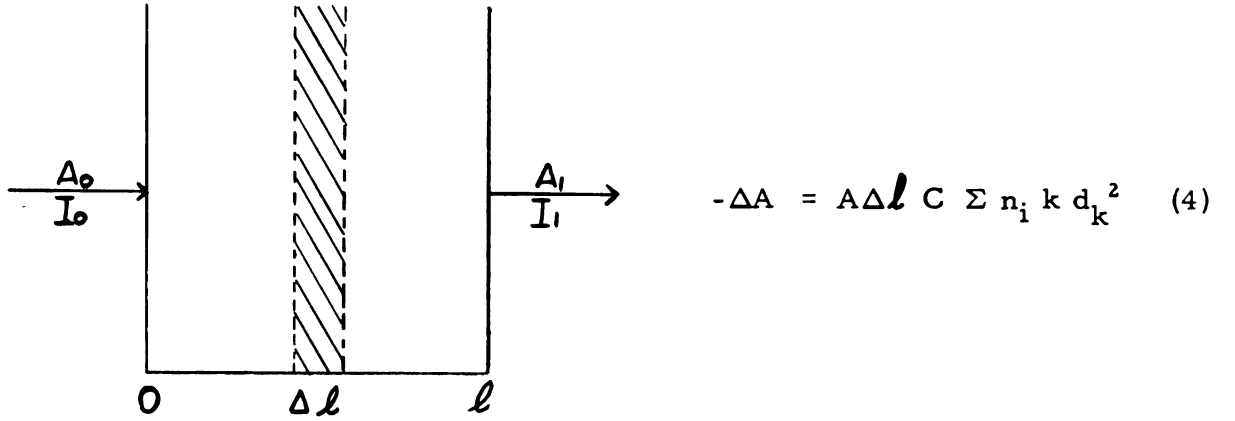
FIGURE 13. Incident light beam of A_0 and intensity I_0 on blood suspension.

$$-\int_{A_0}^{A_i} \frac{\Delta A}{A} = \int_0^\ell \Delta\ell \, C \sum_i n_i k d_i^2 \qquad (5)$$

$$\ell n \frac{A_0}{A_1} = \ell C \sum_i n_i k d_i^2 \qquad (6)$$

Some of the light is blocked by the particles when a beam of light falls on a suspension. The initial beam of area A_0 with intensity I_0 becomes, after passing through the suspension, a beam of area A_1 with same intensity. A photocell will record the light passing through the suspension as being of area A_0 but of lower intensity I_1. The two energies must be the same.

$$I_0 \; A_1 = A_0 \; I_1$$

$$\frac{A_0}{A_1} = \frac{I_0}{I_1} \tag{7}$$

So:

$$\ln \frac{I_0}{I_1} = \ell C \sum_i n_i \, k \, d_i^2 \tag{8}$$

Letting

$$V = C \sum_i n_i \, k \, d_i^2$$

$$\ln \frac{I_0}{I_1} = \ell \; V \; (t) \tag{9}$$

The factor V (t) is the blocking area of material per unit volume in the light beam path. This factor is a function of time because of the change in distribution of particle diameters resulting from agglutination. The major assumptions in the derivation are: (a) multiple scattering is absent; (b) the projected area of the particles determines the amount of light that is blocked; (c) k is a constant not dependent on particle size.

The concentrations of blood suspensions were sufficiently dilute that mutual interference between particles, such as multiple scattering or hindered settling, was not important.

The assumption that this projected area is equal to the light blocked and k is constant is made on the basis that the erythrocyte dimensions are large compared with the wave length of the light.

We have just seen how knowledge of the size distribution of agglomerates would permit calculation of the optical density in a particular stratum of an agglutinating suspension at a particular instant. Analysis of the sedimentation process, along the lines described in Cole's thesis, would in principle permit the determination of the time dependence of the particle distribution at a particular stratum in terms of the initial distribution throughout the suspension. Moreover, we should be able to invert this time dependence to solve the converse problem of inferring the course of the reaction from the observed settling curves. In practice the theory is too complicated to carry out either process. Hence we look for empirical expressions which will let us use the method phenomenologically, and which hopefully will ultimately point to a theoretical scheme for agglutination. Hence we search for parameters to characterize the observed curves, and study their dependence on relevant variables.

Schematically, it is easy to see from Figure 2 how agglutination affects the course of the settling curve. We have seen that in the absence of agglutination the transmission remains low until the interface from a relatively homogeneous distribution of erythrocytes passes before the window. As the concentration of antibody is increased, Figure 14 shows that stronger agglutination produces a nonuniform time-varying distribution which results in an increase in transmission at very early times.

Furthermore, the point at which the transmission shoots up rapidly (corresponding to the passage of the interface in the absence of antibody) moves to earlier times with increasing concentration, as does the place where the transmission levels off at I_∞. It is not clear what parameters will be most useful, and much of our effort has been expended in searching for those.

From Figure 14 it can be seen that each plot of log of the transmitted light intensity against time can be represented by a combination of two different curves as indicated in Figure 15. The first curve (A) indicates the agglutination process and intersects the second curve (B) which represents the interface falling across the path of the light beam. Several different types of quantitative analysis were applied to the experimental graphs without leading to consistent results. Among these analyses were the slopes of various portions, height and distance along the time axis at various percentages of final value, and intersection of agglutinating and interface curves.

The first part of the total curve was finally chosen as the portion best indicating the difference in antibody concentration between samples and the one being most reliable for analysis. This portion would correspond to region 2 of the agglutinating sample in Figure 11. The equation for this portion is given by:

$$\ell n \ I \ = \ \ell n \ I_\infty \ - \ ae^{-bt}$$

$$a, \ b, \quad \ell n \ I_\infty \ \text{are constants}$$

This curve goes to $\ell n \ I = \ell n \ I_\infty - a$ at $t = 0$

and approaches the line $\ell n \ I = \ell n \ I_\infty$ as $t \longrightarrow \infty$

$$\ell n \ I_\infty \ - \ \ell n \ I = ae^{-bt}$$

$$\ell n \ (\ell n \ I_\infty \ - \ \ell n \ I) \ = \ \ell n \ a \ - \ bt$$

Choosing $\ell n I_\infty$ and plotting $\ell n \ (\ell n I_\infty \ - \ \ell n \ I)$ against time, we can obtain b and a.

The value of b so obtained was plotted against concentration of antibody in the sample. In Figure 16 we have the slope of $\ell n \ (\ell n I_\infty - \ell n I) = \ell n \ a \ - \ bt$ plotted against serum concentration for 6 different runs. There is indicated some sort of general relationship between b and concentration which seems to hold for lower concentrations of antibody
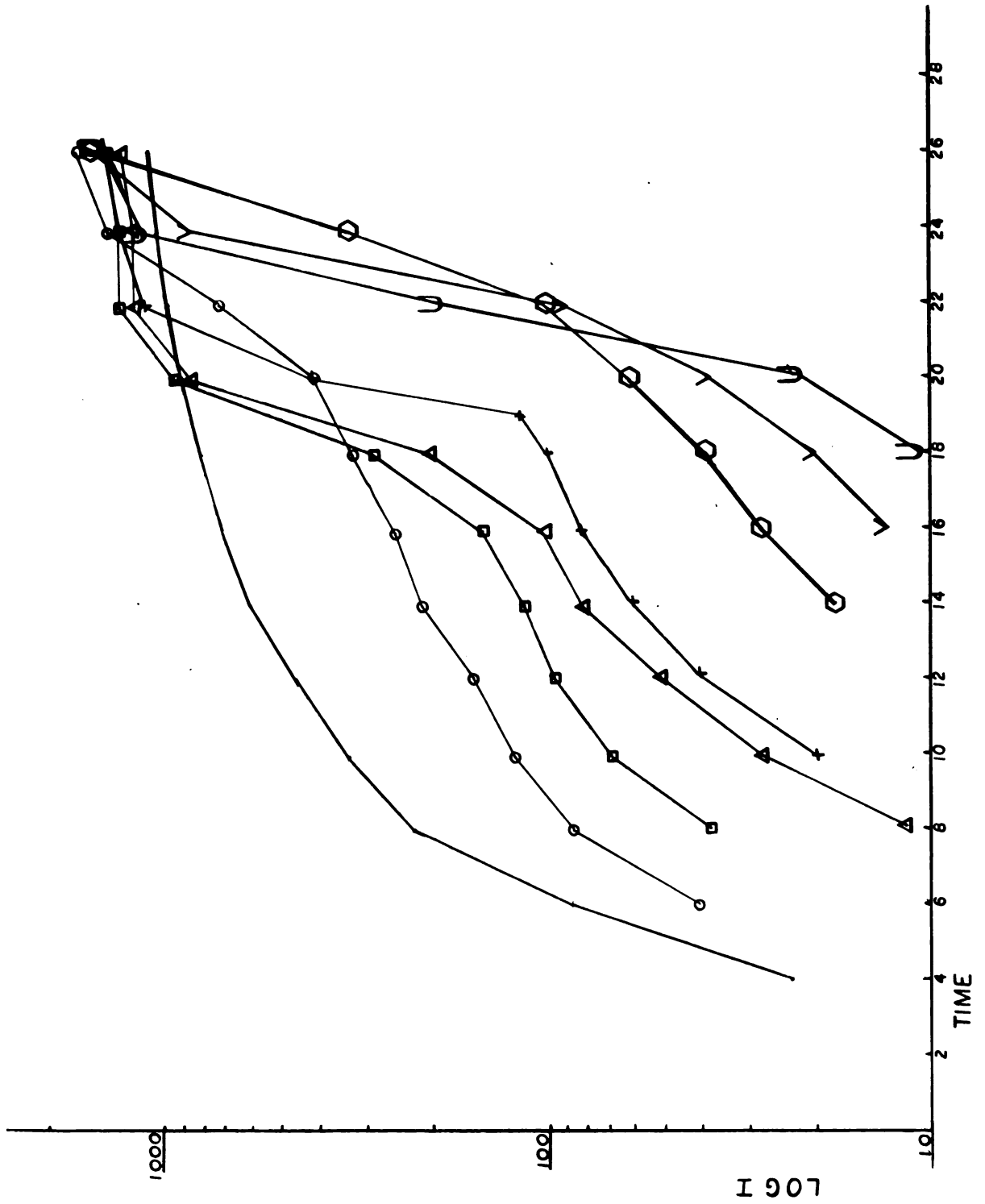
FIGURE 14. Plot of the log of the intensity of transmitted light against time for 8 different serum concentrations in V-187.
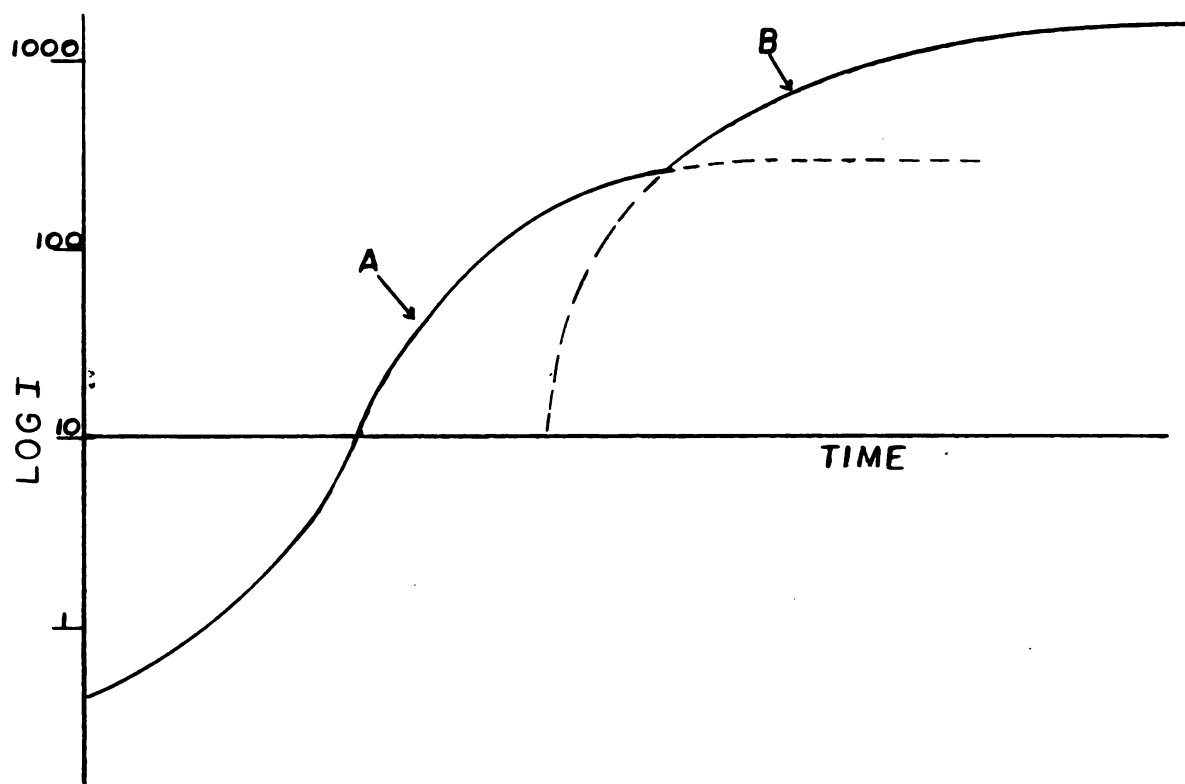
FIGURE 15. Interpretation of settling curves which were obtained by
plotting log of transmitted light intensity against time.
A. Agglutination curve. B. Interface curve.
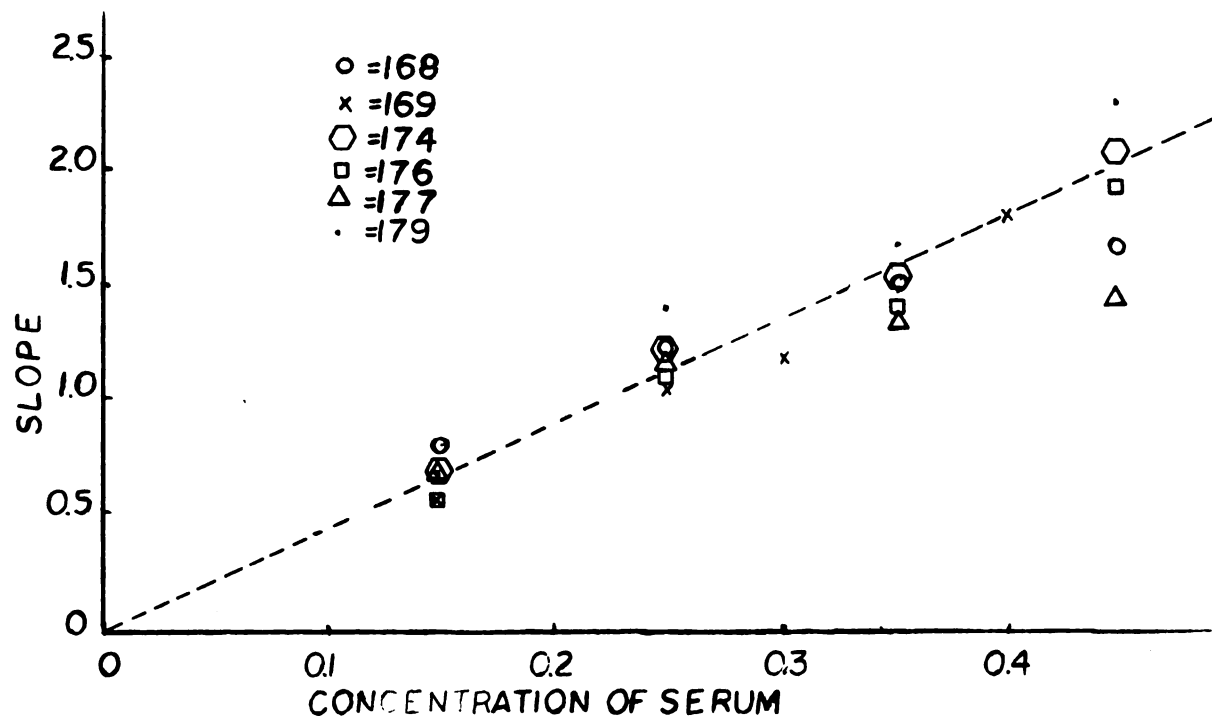
FIGURE 16. Plot of the slope of $\ell n(\ell nI_\infty - \ell n I) = \ell n\, a - bt$ against
concentration of serum for six runs.

and which breaks down as the concentration increases. Further analysis showed, that although this equation somewhat predicted the behavior of a family of curves with concentration of serum as the parameter there were other variables not under complete control which caused this analysis to break down.

CONCLUSIONS

1. For certain immune systems, antibody concentrations may be assessed by observing the sedimentation rate of agglutinating erythrocytes. The sedimentation rate is determined by observing the time dependence of the optical density at a given stratum of agglutinating erythrocytes suspended in a plasma-saline medium.

    A. The sedimentation rate in the ABO agglutination process may be used to assess antibody concentration quantitatively, though not very precisely.

    B. The Anti-D reaction in the Rh system was found unsuitable for study by the sedimentation process with the apparatus described herein.

2. No effect on ABO system agglutination was detected by the sedimentation process as observed by this machine under the stated condition of magnetic field.

3. A search for useful parameters characterizing the strength of the agglutination reaction as determined by the sedimentation curves was undertaken. The earliest portion of the settling curve was established as holding the most promise for quantative understanding of the agglutination process. The analysis remains unsatisfactory, probably because of imperfect control of all variables in the experiment.

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BIBLIOGRAPHY

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APPENDIX

DATA

APPENDIX

DATA

Run No.	Blood Type and Conc.	No. Samp. in Mag	No. Samp. Out Mag	Temp.	Conc. Serum	Type
V-1	O+ (150 λ/100 ml)	0	4c*	room	0	-
V-2	O+ (150 λ/100 ml)	0	4c	room	0	-
V-3	O+ (150 λ/100 ml)	0	4c	room	0	-
V-4	O+ (150 λ/100 ml)	0	4c	room	0	-
V-5	O+ (150 λ/100 ml)	0	$4c_2$ (2 with caps, 2 without)	room	0	-
V-6	O+ (150 λ/100 ml)	0	$4c_2$ (2 with caps, 2 without)	room	0	-
V-7	A (50 λ/33.3 ml)	0	4c	room	0	-
V-8	A (50 λ/18 cc)	0	2c, 2s**	room	0.036	A
V-9	A (50 λ/18 cc)	0	2c, 2s	room	0.0071	A
V-10	A (50 λ/18 cc)	0	2c, 2s	room	0.021	A
V-11	A (50 λ/18 cc)	0	2c, 2s	room	0.036	A
V-12	A (50 λ/18 cc)	0	2c, 2s	room	0.036	A
V-13	A (50 λ/18 cc)	0	2c, 2s	room	0.036	A
V-14^a	B (50 λ/15 cc)	0	2c, 2s	room	0.036	B
V-14^b	B (50 λ/15 cc)	0	2c, 2s	room	0.071	B
V-15	B (50 λ/15 cc)	0	2c, 2s	room	0.036	B
V-16	B (50 λ/15 cc)	1s	1c, 2s	room	0.036	B
V-17	B (50 λ/15 cc)	1s	1c, 2s	room	0.036	B
V-18	B (50 λ/15 cc)	1s	1c, 2s	room	0.036	B
V-19	B (50 λ/15 cc)	1s	1c, 2s	room	0.036	B
V-20	O+ (50 λ/15 cc)	1s	1c, 2s	room	0.036	D
V-21	A (50 λ/15 cc)	1s	2s	room	0.036	A
V-22	A (20 λ/15 cc)	1s	2s	room	0.022	A
V-23	A (20 λ/15 cc)	1s	2s	room	0.021	A
V-24	A (20 λ/15 cc)	1s	2s	room	0.043	D
V-25	O+ (20 λ/15 cc)	1s	2s	37°	0.043	D
V-26	O+ (50 λ/15 cc)	1s	2s	39°	0.125	D
V-27	O+ (50 λ/15 cc)	1s	2s	38°	0.125	D
V-28	O+ (50 λ/10 cc)	1s	2s	37°	0.125	D

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*C = without antibody
**S = with antibody

Run No.	Blood Type and Conc.	No. Samp. in Mag	No. Samp. Out Mag	Temp.	Conc. Serum	Type
V-29	A+ (50 λ/15 cc)	1c	1c, 1s	37°	0.125	D
V-30	A+ (50 λ/15 cc)	1c	1c, 1s	37°	0.25	D
V-31	O+ (50 λ/10 cc)	1c	1c, 1s	37°	0.25	D
V-32	O+ (100 λ/10 cc)	1c	1c, 1s	37°	0.25	D
V-33	O+ (50 λ/10 cc)	1c	1c, 1s	37°	0.25	D
V-34	O+ (50 λ/15 cc)	1c	1c, 1s	37°	0.25	D
V-35	O+ (50 λ/15 cc)	1c	1c, 1s	37°	0.25	D
V-36	O+ (50 λ/15 cc)	1c	1c, 1s	37°	0.25	D
V-37	O+ (50 λ/15 cc)	1c	2c	37°	0	-
V-38	O+ (50 λ/15 cc)	1c	2c	37°	0	-
V-39	AB+ (50 λ/15 cc)	1s	2s	37°	0.036	B
V-40	AB+ (50 λ/15 cc)	1s	2s	room	0.036	B
V-41	AB+ (50 λ/15 cc)	1s	2s	33° fall- ing	0.036	B
V-42	AB+ (50 λ/15 cc)	1s	2s	room ing	0.036	B
V-44	AB+ (50 λ/15 cc)	1s	2s	33° fall- ing	0.036	B
V-45	AB+ (50 λ/15 cc)	1s	2s	32°	0.036	B
V-46	AB+ (50 λ/15 cc)	1s	2s	30°	0.036	B
V-47	AB+ (50 λ/15 cc)	1s	2s	38°	0.036	B
V-48	AB+ (50 λ/15 cc)	1s	2s	33° fell to 33°	0.036	B
V-49	AB+ (50 λ/15 cc)	1s	2s	35° falling	0.036	B
V-50	AB+ (50 λ/15 cc)	0 (1 in dummy)	3s	35° falling	0.036	B
V-51	AB+ (50 λ/15 cc)	0 (1 in dummy)	3s	35° falling	0.036	B
V-52	AB+ (50 λ/15 cc)	1s	2s	35°	0.036	B
V-53	AB+ (50 λ/15 cc)	1s	2s	35° fell and then rose	0.036	B
V-54	AB+ (50 λ/15 cc)	1s	2s	37° falling	0.036	B
V-55	AB+ (50 λ/15 cc)	1s	2s	39° falling	0.036	B
V-56	AB+ (50 λ/15 cc)	1s	2s	37° falling	0.036	B
V-57	AB+ (50 λ/15 cc)	1s	2s	38° falling	0.036	A
*new mag. V-58	AB+ (50 λ/15 cc)	1s	2s	room	0.036	B
V-59	AB+ (50 λ/15 cc)	1s	2s	34.5° falling	0.036	B
V-60	AB+ (50 λ/15 cc)	1s	2s	37° falling	0.036	B
V-61	AB+ (50 λ/15 cc)	1s	1c, 1s	37° falling	0.018	B
V-62	AB+ (50 λ/15 cc)	1s	1c, 1s	36.5°	0.036	B
V-63	AB+ (50 λ/15 cc)	1s	2s	36°	0.036	A
V-64	AB+ (50 λ/15 cc)	1s	2s	room	0.036	A
V-65	A+ (50 λ/15 cc)	1s	2s	27° room	0.036	A
V-66	A+ (100 λ/15 cc)	1s	2s	room	0.036	A
V-67	A+ (100 λ/15 cc)	1s	2s	room	0.036	A
V-68	A+ (100 λ/15 cc)	1s	2s	room	0.014	A

Run No.	Blood Type and Conc.	No. Samp. in Mag	No. Samp. Out Mag	Temp.	Conc. Serum	Type
V-69	A+ (100 λ/15 cc)	1s	2s	room	0.0071	A
V-70	A+ (100 λ/15 cc)	1s	2s	room	0.0036	A
V-71	A+ (100 λ/15 cc)	1s	2s	room	0.0043	A
V-72	A+ (100 λ/15 cc)	1s	2s	room	0.071	A
V-73	A+ (100 λ/15 cc)	1s	2s	room	0.071	A
V-74	A+ (100 λ/15 cc)	1s	2s	37°-35°	0.036	D
V-75	A+ (50 λ/15 cc)	1s	2s	37°-40°	0.071	D
V-76	A+ (50 λ/15 cc)	1s	2s	37°	0.071	D
V-77	A+ (300 λ/15 cc)	1s	2s	room	0.044	D
V-78	A+ (200 λ/30 cc)	0	8s	room	0.005-0.020	A
V-79	A+ (200 λ/30 cc)	0	8s	room	0.006-0.009	A
V-80	A+ (200 λ/30 cc)	0	8s	room	0.011-0.014	A
V-81	A+ (200 λ/30 cc)	0	8s	room	0.005-0.021	A
V-82	A+ (200 λ/30 cc)	0	8s	room	0.011-0.014	A
V-83	A+ (200 λ/30 cc)	0	8s	room	0.010-0.016	A
V-84	A+ (200 λ/30 cc)	0	8s	room	0.010-0.016	AB
V-85	A+ (300 λ/30 cc)	0	8s	room	0.010-0.016	AB
V-86	A+ (200 λ/30 cc)	0	8s	room	0.010-0.016	AB
V-87	A+ (200 λ/30 cc)	0	8s	room	0.012-0.018	AB
V-88	A+ (200 λ/30 cc)	0	8s	room	0.012-0.018	AB
V-89	A+ (200 λ/30 cc)	0	8s	room	0.012-0.018	A
V-90	A+ (200 λ/30 cc)	0	8s	room	0.005-0.020	A
V-91	A+ (200 λ/30 cc)	0	8s	room	0.005-0.020	A
V-92	A+ (200 λ/30 cc)	0	8s	room	0.005-0.020	A
V-93	A+ (200 λ/30 cc)	0	8s	room	0.005-0.020	A
V-94	A+ (200 λ/30 cc)	0	8s	room	0.005-0.020	A
V-95	B+ (200 λ/30 cc)	0	8s	room	0.010-0.04	B
V-96	B+ (200 λ/30 cc)	0	8s	room	0.00-0.030	B
V-97	B+ (200 λ/30 cc)	0	8s	room	0.00-0.030	B
V-98	B+ (200 λ/30 cc)	0	8s	room	0.0-0.020	B
V-99	B+ (200 λ/30 cc)	0	8s	room	0.0-0.0120	AB
V-100	B+ (200 λ/30 cc)	0	8s	room	0.0-0.012	AB
V-101	B+ (200 λ/30 cc)	0	8s	room	0.004-0.01	AB
V-102	B+ (200 λ/30 cc)	0	8s	room	0.0-0.012	AB
V-103	B+ (200 λ/30 cc)	0	8s	room	0.0-0.015	AB
V-104	B+ (200 λ/30 cc)	0	8s	room	0.02-0.05	AB
V-105	B- (200 λ/30 cc)	0	8s	room	0.01-0.025	AB
V-106	B- (200 λ/30 cc)	0	8s	room	0.0-0.015	AB
V-107	B- (200 λ/30 cc)	0	8s	room	0.0-0.012	AB
V-108	B+ (200 λ/30 cc)	0	8s	room	0.0-0.012	AB
V-109	AB+ (200 λ/30 cc)	0	8s	room	0.012	AB

Run No.	Blood Type and Conc.	No. Samp. in Mag	No. Samp. Out Mag	Temp.	Conc. Serum	Type
V-110	B+ (200 λ/30 cc)	0	8 s	room	0.0-0.012	AB
V-111	B+ (150 λ/30 cc)	0	8 s	room	0.0-0.012	AB
V-112	B+ (150 λ/30 cc)	0	8 s	room	0.0-0.012	AB
V-113	B+ (200 λ/30 cc)	0	8 s	room	0.0-0.012	AB
V-114	B+ (200 λ/30 cc)	0	4 s	room	0.0-0.012	AB
V-115	AB+ (150 λ/30 cc)	0	8 s	room	00-0.012	AB
V-116	AB+ (200 λ/30 cc)	0	8 s	room	00-0.016	AB
V-117	AB+ (150 λ/30 cc)	0	8 s	room	00-0.016	AB
V-118	AB+ (200 λ/30 cc)	0	8 s	room	00-0.016	AB
V-119	AB+ (150 λ/30 cc)	0	8 s	room	00-0.03	AB
V-120	AB+ (150 λ/30 cc)	0	8 s	room	00-0.03	AB
V-121	AB+ (150 λ/30 cc)	0	8 s	room	00-0.03	AB
V-122	AB+ (200 λ/30 cc)	0	8 s	room	00-0.03	AB
V-123	AB+ (200 λ/30 cc)	0	8 s	room	00-0.03	AB
V-124	AB+ (200 λ/30 cc)	0	8 s	room	00-0.03	AB
V-125	AB+ (200 λ/30 cc)	0	8 s	room	00-0.03	AB
V-126	AB+ (200 λ/30 cc)	0	8 s	room	00-0.03	AB
V-127	AB+ (200 λ/30 cc)	0	8 s	room	00-0.03	AB
V-128	AB+ (200 λ/30 cc)	0	8 s	room	00-0.03	AB
V-129	AB+ (200 λ/30 cc)	0	8 s	room	00-0.03	AB
V-130	AB+ (200 λ/30 cc)	0	8 s	room	0.005-0.02	AB
V-131	AB+ (200 λ/30 cc)	0	8 s	room	0.005-0.02	AB
V-132	AB+ (200 λ/30 cc)	0	8 s	room	0.005-0.02	AB
V-133	AB+ (200 λ/30 cc)	0	8 s	room	0.005-0.02	AB
V-134	AB+ (200 λ/30 cc)	0	8 s	room	0.005-0.02	AB
V-135	AB+ (200 λ/30 cc)	0	8 s	room	0.005-0.02	AB
V-136	AB+ (200 λ/30 cc)	0	8 s	room	0.005-0.02	AB
V-137	AB+ (200 λ/30 cc)	0	8 s	room	0.005-0.02	AB
V-138	AB+ (200 λ/30 cc)	0	8 s	room	0.005-0.02	AB
V-139	AB+ (200 λ/30 cc)	0	8 s	room	0.005-0.02	AB
V-140	AB+ (200 λ/30 cc)	0	8 s	room	0.005-0.02	AB
V-141	AB+ (200 λ/30 cc)	0	8 s	room	0.005-0.02	AB
V-142	AB+ (200 λ/30 cc)	0	8 s	room	0.005-0.02	AB
V-143	AB+ (200 λ/30 cc)	0	8 s	room	0.005-0.02	AB
V-144	AB+ (200 λ/30 cc)	0	8 s	room	0.005-0.02	AB
V-145	AB+ (200 λ/30 cc)	0	8 s	room	0.005-0.02	AB
V-146	AB+ (200 λ/30 cc)	0	8 s	room	0.005-0.02	AB
V-147	AB+ (200 λ/30 cc)	0	8 s	room	0.005-0.02	AB
V-148	AB+ (200 λ/30 cc)	0	8 s	room	0.005-0.02	AB
V-149	AB+ (200 λ/30 cc)	0	8 s	room	0.005-0.02	AB
V-150	AB+ (200 λ/30 cc)	0	8 s	room	0.01-0.04	AB
V-151	AB+ (200 λ/30 cc)	0	8 s	room	0.01-0.04	AB
V-152	AB- (200 λ/30 cc)	0	8 s	room	0.005-0.02	AB

Run No.	Blood Type and Conc.	No. Samp. in Mag	No. Samp. Out Mag	Temp.	Conc. Serum	Type
V-153	AB- (200 λ/30 cc)	0	8s	room	0.005-0.02	AB
V-154	AB- (200 λ/30 cc)	0	8s	room	0.005-0.02	AB
V-155	AB- (200 λ/30 cc)	0	8s	room	0.005-0.02	AB
V-156	AB- (200 λ/30 cc)	0	8s	room	0.01-0.04	AB
V-157	AB+ (200 λ/30 cc)	0	8s	room	0.015-0.03	AB
V-158	AB+ (200 λ/30 cc)	0	8s	room	0.005-0.02	AB
V-159	AB+ (200 λ/30 cc)	0	8s	room	0.015-0.03	AB
V-160	AB+ (200 λ/30 cc)	0	8s	room	0.015-0.03	AB
V-161	AB+ (300 λ/30 cc)	0	8s	room	0.000-0.02	AB
V-162	AB+ (400 λ/30 cc)	0	8s	room	0.007-0.025	AB
V-163	AB+ (400 λ/30 cc)	0	8s	room	0.007-0.026	AB
V-164	AB+ (500 λ/30 cc)	0	8s	room	0.007-0.028	AB
V-165	AB+ (400 λ/30 cc)	0	8s	room	0.015-0.039	AB
V-166	AB+ (500 λ/30 cc)	0	8s	room	0.02-0.05	AB
V-167	AB+ (500 λ/30 cc)	0	8s	room	0.02-0.05	AB
V-168	AB+ (475 λ/30 cc)	0	8s	room	0.01-0.04	AB
V-169	AB+ (400 λ/30 cc)	0	8s	room	0.015-0.04	AB
V-170	AB+ (500 λ/30 cc)	0	8s	room	0.01-0.04	AB
V-171	AB+ (500 λ/30 cc)	0	8s	room	0.02-0.05	AB
V-172	AB+ (500 λ/30 cc)	0	8s	room	0.01-0.04	AB
V-173	B+ (400 λ/30 cc)	0	8s	room	0.01-0.04	AB
V-174	B+ (400 λ/30 cc)	0	8s	room	0.015-0.045	AB
V-175	B+ (400 λ/30 cc)	0	8s	room	0.015-0.045	AB
V-176	B+ (400 λ/30 cc)	0	8s	room	0.015-0.045	AB
V-177	B+ (400 λ/30 cc)	0	8s	room	0.015-0.045	AB
V-178	B+ (400 λ/30 cc)	0	8s	room	0.035	AB
V-179	B+ (400 λ/30 cc)	0	8s	room	0.015-0.045	AB
V-180	B+ (400 λ/30 cc)	0	8s	room	0.011-0.045	
V-181	B+ (400 λ/30 cc)	0	8s	room	0.010-0.045	
V-182	B+ (400 λ/30 cc)	0	8s	room	0.01-0.045	AB
V-183	B+ (400 λ/30 cc)	0	8s	room	0.005-0.090	AB
V-184	AB+ (400 λ/30 cc)	0	8s	room	0.005-0.040	AB
V-185	AB+ (400 λ/30 cc)	0	8s	room	0.005-0.040	AB
V-186	AB+ (400 λ/30 cc)	0	8s	room	0.005-0.040	AB
V-187	AB+ (400 λ/30 cc)	0	8s	room	0.005-0.040	AB