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Analytical Techniques for Cell Fractions

XVIII. Use of Cellulose Wicks to Monitor Agglutination Reactions[1]

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Red blood cell agglutination has played a central role in serology, especially in blood typing for transfusions, in the assay of certain transplantation antigens and antibodies, in the titration of hemagglutinating viruses and their subunits, and in the detection and isolation of hemagglutinins from cells and tissues.

Quantitative measurements based on hemagglutination pose several problems, however, especially when agglutination is incomplete. Aggregates may be held together by such weak forces that agitation easily separates them; the number of cells aggregated is not a linear function of antibody concentration; the degree of aggregation is time, temperature, and suspending medium dependent; and the results are usually scored visually and manually recorded, leaving (except as discussed below) no permanent record of the actual experiment. Centrifugal packing of cells at a relatively low speed enhances aggregation and facilitates subsequent scoring providing centrifugation and resuspension are gently done. Recently agglutinated cells separated from unagglutinated ones by sedimentation in a flowing stream were decanted from below and collected on filter paper strips (1). These may be dried and stored as permanent records, or photographically or electrostatically copied (2).

Sedimentation in a capillary containing both test blood sample and antiserum has also been employed (3). In tubes containing nonlayered mixtures of cells and antisera, sedimentation of aggregates in the presence of large numbers of aggregated cells is difficult to observe. Two techniques may assist objective evaluation. In the first, the cells may be

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centrifuged through a density gradient using rate-zonal techniques (4); in the second, aggregated cells may be caught in a filter of suitable porosity and the retained cell mass measured. In practice, filters which would distinguish sharply between single cells and aggregates of a very few cells have not yet been found. It occurred to me that this lack of selectivity might find partial compensation by use of chromatographic methods employing wicks which would hold larger clumps and which would require unagglutinated cells to move in a tortuous path over and around them, thus increasing the chances for new cells to be added to the aggregations. Agglutination would thus be detected as a decrease in the number of cells moving with the liquid front and as an increase in the mass of cells at the origin. The wicks are also intended to serve as a permanent record of the results. In this paper techniques for using wicks and for scoring results have been examined. A preliminary report of this work has appeared (5).

Stained bacteria in the presence of specific antisera agglutinate and may adhere to filter paper by a process termed "surface fixation" (6). This observation led to the development of a simple blotting-paper method for screening blood donors for high-titer antisera (7). In the latter case, the chromatographic possibilities inherent in filter paper were not exploited.

CHOICE OF WICK MATERIALS

With the proper choice of wick porosity, medium or large aggregates should be retained at the base of the wick, while very small aggregates or unaggregated cells ascend past them. The first question is: Do red blood cells in the absence of antiserum ascend at the same rate as the liquid front, do they tend to develop a concentration gradient along the wick, or do they concentrate at the leading edge because water and not cells penetrate the cellulose fibers rsulting in hemoconcentration? Experiments with a variety of ordinary types of filter paper showed that unaggregated cells rapidly ascend with the advancing front and that with thick filter papers the concentration of cells was generally higher at the advancing front than along its lengths.

ABO System

The initial experiments to determine whether unagglutinated cells would move freely along filter paper while agglutinated cells would not were done with small Lucite blocks containing square holes, as shown in Figure 1. Samples of Whatman Nos. 1, 2, 5, 40, and 42 filter paper were cut in the shape shown. Two drops of antisera[3] and two drops of heparinized

[3] Obtained from Dade Reagents, Inc., Miami, Florida.

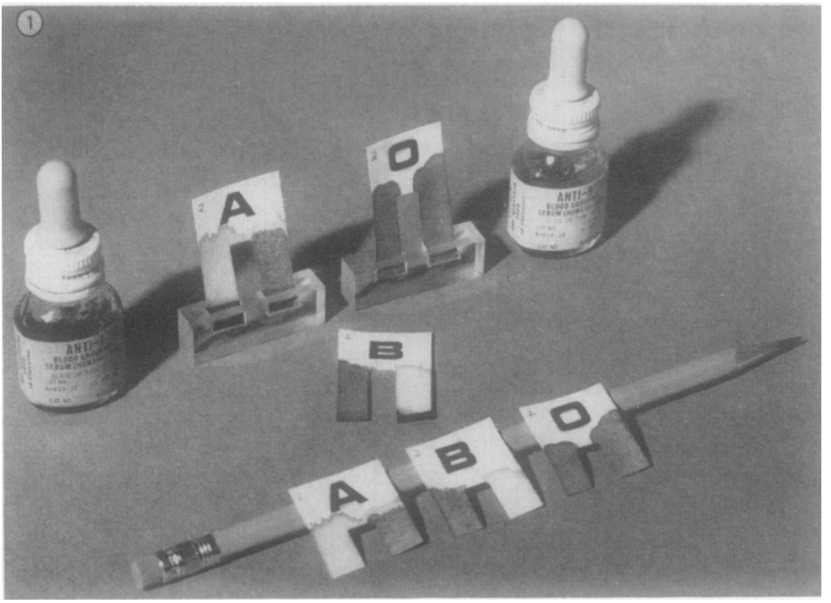

FIG. 1. ABO typing of human blood using wicks of double Whatman No. 2 paper. Upper wicks inserted in small plastic wells used for mixing blood and typing serum. Anti-A serum is used in left well and anti-B in right well in each block. Center dried wick shows cells agglutinated to tip on right (anti-B) side; cells are therefore type B. Lower three wicks with tips cut off illustrate results obtained with A, B, and O blood samples.

whole human blood were added and the wells gently rocked from side to side for 30 sec at room temperature. The wicks were then inserted and the mixture allowed to chromatograph. No marked differences were noted for one type of filter paper when compared with the others. However, in the sample containing agglutinated cells (A blood + anti-A serum, for example), the plasma moved almost twice as far up the wick as did a negative control. This was true of both A and B blood and antiserum. Unagglutinated cells moved very close to the advancing liquid front but generally left a narrow blue or yellow identifying dye zone at the upper edge. Twenty-three different blood samples were examined and the results in all instances agreed with those obtained using conventional typing procedures. One instance was noted, however, in which the unagglutinated cells ascended at the same rate as the serum from agglutinated cells. Examination of the wick showed it to be double. Further studies showed that for the ABO system the time required for chromatography was markedly shortened if double wicks were used. An advantage of this observation is that the results may be obtained in duplicate, should this be required. The

results shown in Figure 1 were obtained with Whatman No. 2 filter paper using double wicks in each case. Note that a clot of agglutinated cells is observed at the tip of the wick, with a large space above clearly colored with the identifying dye (center strip in Fig. 1). In A- or B-negative strips, in contrast, the red blood cells are uniformly distributed with a very narrow band of identifying dye color at the upper edge. The entire wick may serve as a permanent record when dried. In addition, the tips may be cut off as shown in the lower three strips in Figure 1 to yield a record requiring minimal interpretation.

Rh Typing

The differences between blood samples which were positive or negative for three Rh factors, C (rh'), D (Rh_0), and E (rh''), were evident but not marked when double filter paper wicks were used. Thicker wick materials were therefore examined and Schleicher and Schuell No. 470 paper[4] was found to be satisfactory. In addition, the technique was adapted for use in the plastic titration plates used with the microtiter system.[5] To maintain the proper temperature the plastic titration plate was inverted and filled with molten Wood's metal (melting point 73°C). The plastic was then machined away and a heating block attached to the lower surface of the Wood's metal plate to control the temperature. Strips were cut from the wicks as shown in Figure 2. Two drops of antisera and one drop of blood were used and the mixture mixed on a mechanical vibrator[6] for 30 sec using a titration plate preheated to 40°C. After 5 min incubation at that temperature, the wicks were inserted. Approximately 5 min was required before all of the blood-serum mixture was picked up by the wicks.

INTERPRETATION OF RESULTS

Interpretation of the results requires some comments and is best done by comparing the behavior of various mixtures on filter paper with that of whole blood.

When heparinized blood ascends a filter paper wick, the concentration of cells in the advancing front increases relative to that in the remainder of the sample because the cellulose fibers swell and remove water at the advancing edge. When the wick is dried, a dark leading edge is usually seen, while no excess of cells or hemoglobin color is observed at the lower edge of the wick. When only a small amount of aggregation is present,

[4] Obtained from Schleicher and Schuell, Inc., Keene, New Hampshire.
[5] Obtained from Cooke Engineering, Inc.
[6] Available from Arthur H. Thomas, Inc., Philadelphia, Pa.

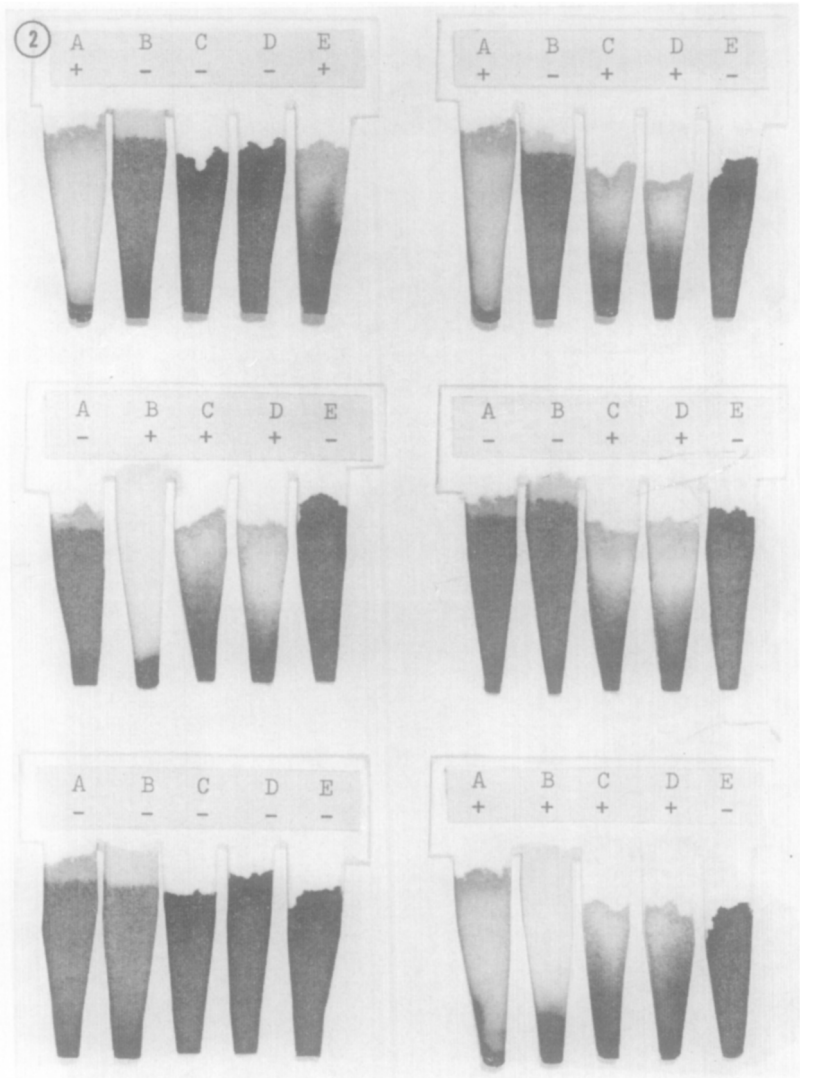

FIG. 2. Typing of six blood samples using microtiter cups and five-pronged wicks. Two drops of typing serum and one drop of blood were used in each instance. Typing sera were anti A, B, C (rh′), D (Rh_0), and E (rh″).

the red cells may not quite follow the advancing edge, and a band of agglutinated cells may be noted at the base.

When complete agglutination occurs (see A-positive and B-positive strips in Fig. 2), the clear plasma plus antiserum mixture rises very rapidly up the wick leaving a dark band of red cells at the base.

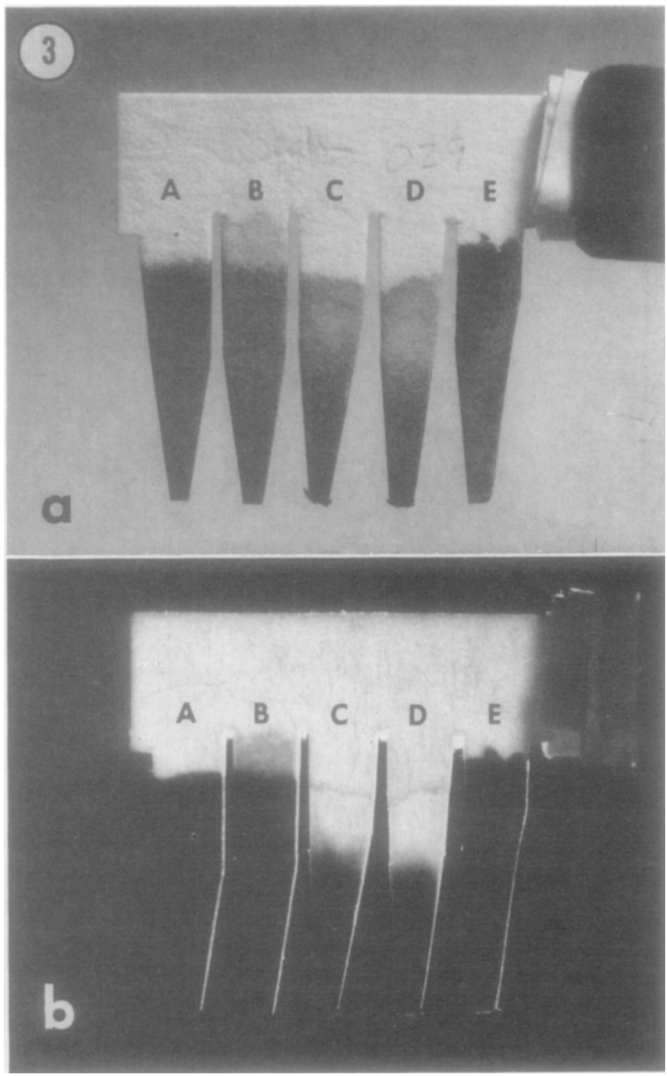

FIG. 3. Illustration of difference between appearance of wicks observed by reflected and transmitted light. Wicks were prepared as described in text and are comparable to those used in Figure 2. (a) Type O, C^+, D^+, E^- blood wick observed by reflected light. (b) Same wick observed by transmitted light. Note very marked translucent zone below boundary in C and D, indicating that transmitted light may be used to score results.

Rh-positive bloods do not give the sharply defined results observed with A or B serum; instead, a definite zone of agglutination is seen at the wick tip and the hemoglobin color falls off gradually over the length of the wick, often leaving a clear zone just below the advancing front (Fig. 2). With transmitted light the gradation is quite obvious (Fig. 3). Thirty-three samples were typed using C, D and E antiserum and complete agreement with the rocking-slide method (8) has been obtained.[7]

The difference in the ease with which positive and negative Rh cells may be distinguished by transmitted light as compared with reflected light suggested that absorbance measurements be made along the strips using a scanning microphotometer.[8] To gain sufficient intensity, no filter was used with the tungsten light source. The results obtained with four wicks are shown in Figure 4. The 100% transmission (zero absorbance) setting was adjusted using the most translucent portion of the strip shown in Figure 4D. The absorbance of the filter paper itself was approximately 0.65 using this setting. B-positive and B-negative blood are readily distinguished (Fig. 4A–B). Type C or rh′ negative blood (Fig. 4C) produces maximum opacity to the edge of the advancing liquid front, while with type E (rh″) positive blood the wick was opaque for the first third and then dropped rapidly to zero absorbance at the advancing front (Fig. 4D). These results suggest simple automatic photometric methods for scoring results. The translucence observed with Rh antiserum and corresponding negative bloods appears to be due to the very high protein content of these antisera.

For definitive typing, presence or absence of antibodies to A, B, or O cells in serum or plasma is also measured. Washed cell suspensions containing 50% packed cells in saline were mixed in equal volume with serum, mixed for 30 sec, and the wicks inserted after 5 min at 40°C. While the results indicated wide variations in the amount of antibody present, they confirmed in each instance the results obtained with whole blood and typing antisera.

The ABO determinations are usually done for different times and temperatures than are used with Rh assays. For routine work antisera might be chosen for both which would work at the same temperature and times,

[7] In two instances discrepancies in Rh typing occurred. On retyping by both the manual method and the wick method it was discovered that errors had been made in recording the results of the manual test. When these errors in recording were corrected, the agreement was complete. This served to emphasize the importance of having a permanent record of the results.

[8] ORTEC microspectrophotometer kindly made available by Dr. R. C. Allen, ORTEC Division of E. G. and G. Corp., Oak Ridge, Tennessee.

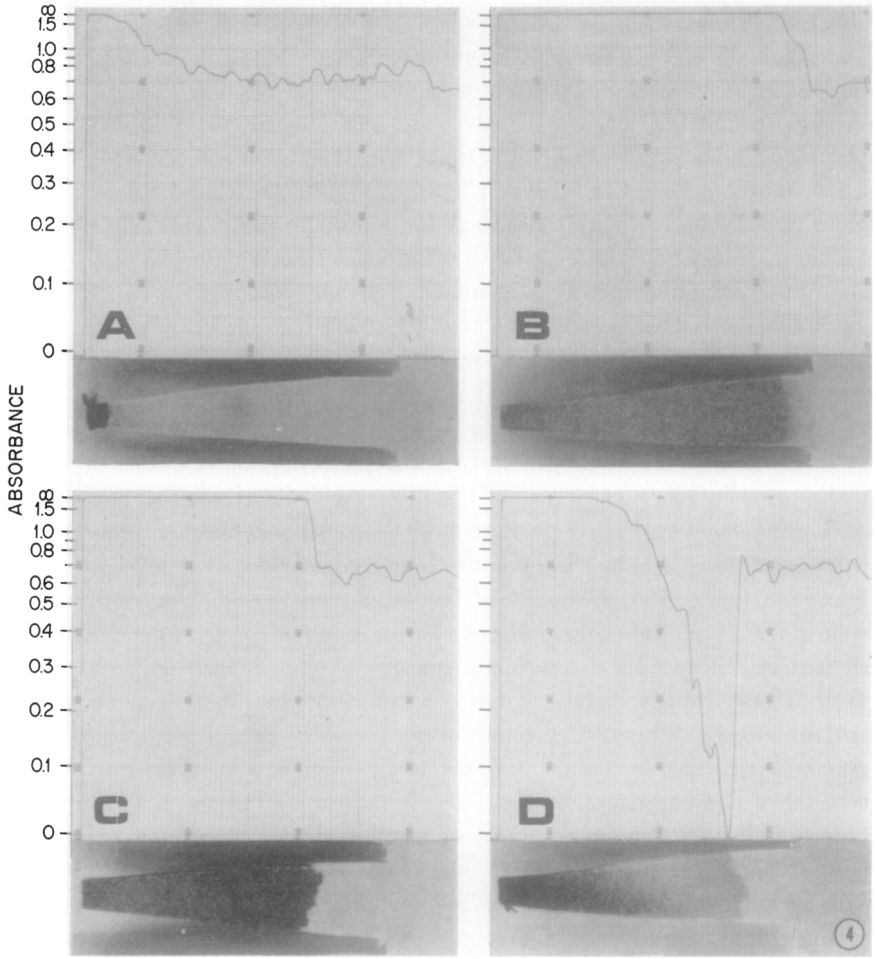

FIG. 4. Microabsorptiometric scanning of individual wicks using an unfiltered tungsten light source. Zero absorbance setting adjusted for most translucent portion of wick D, and all scans run at the same setting. Direction of migration on wicks is from left to right. (A) Anti-B serum with type B whole blood. (B) Anti-B serum with type A blood. (C) Anti-C (rh′) serum and C-negative blood. Note that cells appear most concentrated at leading (right) edge. (D) Anti-E (rh″) serum with E-positive blood. Portion of wick next to advancing boundary is much more translucent than the wick material alone.

or the microtiter plates may be divided into sections maintained at different temperatures. The whole blood may be added to the A and B wells after incubation of the Rh wells is complete, or the wick strip may be divided into two portions and the AB portion run before incubation at a higher temperature for the Rh determination.

Titrations

Filter paper wicks may be used to provide a convenient method of scoring the results of titrations done either in tubes or with microtitration plates.

Twofold dilutions or anti-A typing serum were prepared in tubes using 0.9% NaCl as the diluent. Two drops of the undiluted antiserum followed by the various dilutions were placed in microtitration cups using a standard 0.025 ml dropping pipet. One drop of type A whole blood was then added to each cup using the same size pipet. The mixtures were shaken 30 sec at room temperature and the wicks inserted 90 sec later. The results using three different blood samples are shown in Figure 5. Whole blood was used in these titrations. A higher titer is observed if fewer red cells are used. For best results, the red blood cell suspension used should contain at least 5% cells and preferably more.

Elution of Hemoglobin from Wicks

Hemoglobin may be eluted from dried wicks using 0.1 *N* NaOH for 4–16 hr. The absorption spectrum showed a maximum at 393 nm. For convenience, 395 nm was chosen for further experimental studies. The results obtained when the wicks were cut $\frac{3}{16}$ inch from the tip and the tips eluted in 10 ml of 0.1 *N* NaOH and read in the 1 cm cells of a GeMSAEC G-IIC fast analyzer computer system (9, 10) are shown in Table 1. All samples were read 10 times during rotation, and the results either displayed alpha numerically on a cathode ray tube (CRT) or automatically printed out. The absorbance due to the dyes used in commercial anti-A and anti-B serum was found to be only 0.016 for the area of wick used. In these experiments drop volumes were not calibrated, not all of the red cells migrated onto the wick because of well geometry, the size of the tip removed for elution could not be accurately measured, and part of the dried cell mass at the tip often broke off and was lost before the tips were cut off. The results obtained, however, amply confirm the visual observations. The high and low values shown in Table 1 indicate the range of values seen and show that there was no overlap between positive and negative results for the 99 wicks eluted. One sample was judged to be borderline for type C by both manual and wick test and was intermediate between the 18 C-positive and 1 C-negative bloods examined in this series. The feasibility of preparing a more reproducible sample was next examined using a small punch to cut $\frac{1}{8}$ inch diameter discs $\frac{3}{4}$ inch from the wick tip, close to the liquid front. These were eluted with 2 ml of 0.1 *N* NaOH and read as described for the tips. The results are shown

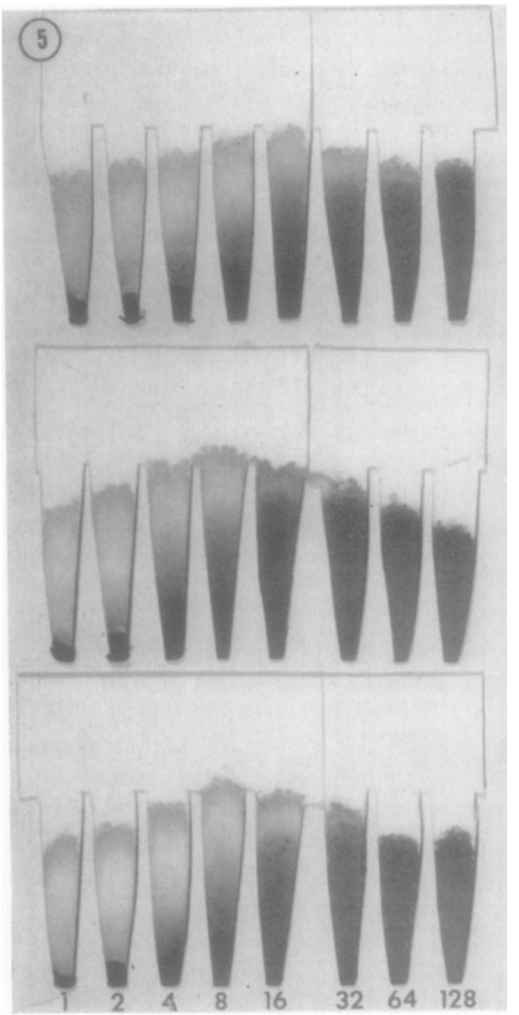

FIG. 5. Titration of anti-A typing serum with type A blood using three different blood samples. Using the highest dilution causing observable retardation of the cells, the titers were: upper, 1:64; center, 1:32; and lower, 1:32.

in Table 2. Note that the results are the reverse of those obtained when the tips were eluted. Thus A-negative blood gave a higher value than A-positive blood using A antiserum with discs cut high up the wick, while the opposite is true when tips are used.

With the discs, positive and negative bloods differed by a factor of

TABLE 1

Absorbance of Hemoglobin Eluted from Wick Tips

(Hemoglobin extracted overnight from tips (3/16 inch) into 10 ml of 0.1 *N* NaOH and read at 395 nm in 1 cm path GeSAEC G-IIC rotor)

Antiserum:	A		B		C[a]		D		E	
Cell type:	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.
Average:	0.414	1.506	0.444	1.237	0.100	1.185	0.080	1.277	0.215	1.207
High value:	0.540	1.760	0.739	1.457	0.100	1.627	0.097	1.517	0.314	1.598
Low value:	0.200	1.225	0.227	1.018	0.100	0.798	0.063	0.834	0.142	1.029
Number tested:	11	9	18	2	1	18	2	17	15	5

[a] One sample was judged borderline by both manual assay and the wick test and gave an absorbance of 0.725, almost midway between the negative and positive values.

TABLE 2
Absorbance of Hemoglobin Eluted from Wicks
(Hemoglobin eluted overnight from 1/8 inch diameter circles cut 3/4 inch from wick tips into 2 ml 0.1*N* NaOH and read at 395 nm in 1 cm path GeMSAEC G-IIC rotor)

Antiserum:	A		B		C		D		E	
Cell type:	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.
Average:	0.337	0.009	0.325	0.014	0.576	0.030	0.456	0.029	0.472	0.042
High value:	0.596	0.012	0.536	0.019	0.821	0.081	0.545	0.038	0.624	0.124
Low value:	0.209	0.004	0.199	0.009	0.356	0.015	0.372	0.017	0.345	0.026
Number tested:	23	13	30	6	11	25	6	30	23	13

approximately 20, and no overlap between positive and negative values occurred. For quantitation based on elution the use of discs cut ¾ inch (approximately ¾ of the distance from tip to the advancing front) is therefore preferable.

Application to Other Problems

Hemagglutination is widely used for virus and antivirus serum titration. Exploratory studies employing chicken erythrocytes and influenza virus[9] showed that agglutination could be assayed by the wick system. A wide variety of assays have been developed using tanned erythrocytes and antigens or antibodies fixed to the cell surface with agglutination being the end measurement. The wick technique appears to be readily adaptable to many of these methods. In addition, tissue and other hemolysins may be measured by using specific antisera to agglutinate cells and ghosts. The amount of hemoglobin in the wick then indicates the degree of hemolysis. The feasibility of this technique has been demonstrated using synthetic detergents in low concentration to produce partial hemolysis.

Permanence of Records

The wicks rapidly change color after drying as the oxyhemoglobin is denatured, yielding a brown color. The results are still clearly evident, however, especially by transmitted light. Since tissue samples several thousand years old have recently been successfully blood typed (11), it appears that the dried wicks will form a record sufficiently permanent for most present purposes. The possibility of keeping a blood sample, untreated with antiserum, as part of the record for future reference also deserves consideration.

DISCUSSION

Cellulose wicks have been used to separate by capillary filtration agglutinated cells from unagglutinated ones. Blood samples may be reproducibly typed for ABO and C (rh′), D (Rh_0), and E (rh″) and cross-matched for presence of antibodies in donor serum against A or B cells. The dried wicks provide a permanent record of the results which may form part of the donors record or be affixed to the blood bag. Results in this form may prove useful for detecting increases in antibodies occurring as a result of pregnancy or transfusion mismatch.

In these studies untreated filter paper has been used. Preliminary studies with thin-layer chromatographic media showed that unaggregated

[9] Kindly supplied by Dr. Irving Johnson of Eli Lilly & Co., Indianapolis, Indiana.

red cells can move through much finer porous supports, suggesting that better resolution may be obtained using filter paper filled with glass or other fine particulate material, and experimental studies with such materials are indicated.

One of the objectives of this work is the development of small blood-typing devices which may be attached to the blood bag and in which typing may be accomplished while the blood is being drawn. These results provide the first steps toward attaining this goal. *Such experimental systems, and the system described here, require extensive evaluation under operating laboratory conditions in parallel with accepted methods before being put into general use.* It should be noted, however, in instances in which subjective decisions are involved, little evidence of borderline tests may find its way into records while, with permanent records, such as are described here, these problems are observed to persist. Subjective evaluations may therefore have a superficial orderliness that objective measurements may lack.

A clear distinction should be made between the use of filter paper as described here and as employed in mechanized blood-typing machines. In the latter, agglutinated cells are allowed to sediment away from unagglutinated ones before being sieved out on filter paper. In the studies reported here, agglutinated cells at the tip of the wick entrap and agglutinate additional cells carried up the wicks by capillarity. Additional opportunity for agglutination occurs as the blood continues to ascend the wick. The results are judged on the basis of the mass of cells at the tip, on the gradient of cells along the wick, and on the mass of cells close to the advancing edge. With some batches of A and B typing sera a small retardation of cells is seen with nonreacting cells (O cells in anti-A or anti-B, A cells in anti-B, B cells in anti-A). This effect has not been seen in Rh-typing antisera, and Rh-negative cells always concentrate at the advancing liquid front.

It is evident that aggregation of noncolored particles including white cells, tissue culture cells, and subcellular particles may also be studied with wicks using protein or other stains to indicate the position of the particles. Results of further photometric studies on wicks, proposed methods of scoring, and comparison with titrations obtained with classical methods will be described elsewhere.

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