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A Low Ionic Strength, Hemagglutinating, AutoAnalyzer® for Rhesus Typing of Dried Bloodstains

The use of absorption-elution processes has resulted in a rapid advance in the grouping of dried bloodstains [*1*]. An absorption-elution method was successfully applied to the detection of the rhesus antigens using tube techniques by Bargagna and Pereira [*2*] and Lincoln and Dodd [*3*].

AutoAnalyzers® designed for antibody screening are capable of detecting very low antibody concentrations, and Douglas and Staveley [*4*] described the use of an AutoAnalyzer® for detecting antibodies in eluates from bloodstains by using the apparatus and reagent system of Rosenfield and Haber [*5*]. This technique and other methods [*6, 7*] used a normal saline medium, and the reference cells were treated with an enzyme. The present paper describes the use of a single channel, low ionic strength method for rhesus typing of dried bloodstains based on the system described by Douglas and Staveley [*8*].

Materials

1. Control bloodstains were prepared from fingerprick samples dried onto cotton cloth at room temperature. The stains were stored at room temperature without special precautions until testing was completed.

2. Ortho® and Dade® antisera for slide or tube testing were used. Anti-Rh_0(D) and anti-hr′(c) were diluted 1:10 with saline, and anti-rh′(C), anti-rh″(E), and anti-hr″(e) were diluted 1:3 with saline for incubating the bloodstains.

3. Donor blood of rhesus phenotype Rh_1Rh_2 (CcDEe) in acid citrate dextrose packs was supplied by the Wellington Blood Transfusion Service. Each pack of blood supplied had been routinely checked for atypical antibodies. Rhesus grouping of bloodstains was not affected by the ABO group of the cells, and although most of the cells supplied were group O, two packs of group A cells and one pack of group B cells were also used without any problems. Each pack of cells was used for approximately 4 to 6 weeks, and changes in sensitivity were negligible during this time.

The blood was centrifuged, and after removal of the plasma (which was retained for

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use), the cells were washed at least three times with saline. A 40% (v/v) suspension of the packed cells was prepared in saline containing 0.5% Ficoll.[2] This solution was filtered through a silk disk to remove any small clots of fibrin. During use the container of cells was immersed in an ice bath and the solution was stirred to maintain an evenly suspended cell concentration.

4. Protamine sulfate (Calbiochem®), 0.1 g, was dissolved in 30 ml of isotonic sugar solution and the mixture was filtered before use.

5. Isotonic sugar solution was prepared by dissolving 9.25% (w/v) sucrose or 5.0% (w/v) glucose in distilled water.

6. Trisodium citrate (E. Merck, Darmstadt) was used as a 3.5% (w/v) solution of the dihydrate salt in water.

7. Triton-X-100® was used as a 0.5% solution in water. The solution was prepared by mixing the Triton-X-100® with an equal volume of ethanol and adding the mixture to distilled water.

Methods

The bloodstains, cut into five portions, one for each Rh factor, were placed in labelled, 2-ml sample cups essentially as described by Culliford [6]. The size of each portion was determined by the concentration of blood in the stain and the age of the stain. Larger portions were required to detect the rh′, rh″, and hr″ factors than the hr′ and Rh_o factors.

The sample cups were placed in racks holding 40 cups in rows of 5, and three drops of the appropriate antiserum diluted with saline were added to each cup. The trays were sealed in plastic wet boxes and incubated overnight at 37°C. After incubation, unabsorbed antiserum was removed by washing the stained material at least five times with saline at room temperature; then the absorbed antiserum was eluted into 0.2 ml of saline at 55°C. The antibodies present in the eluates were detected using a Technicon AutoAnalyzer® II with the flow diagram and flow rates shown in Fig. 1.

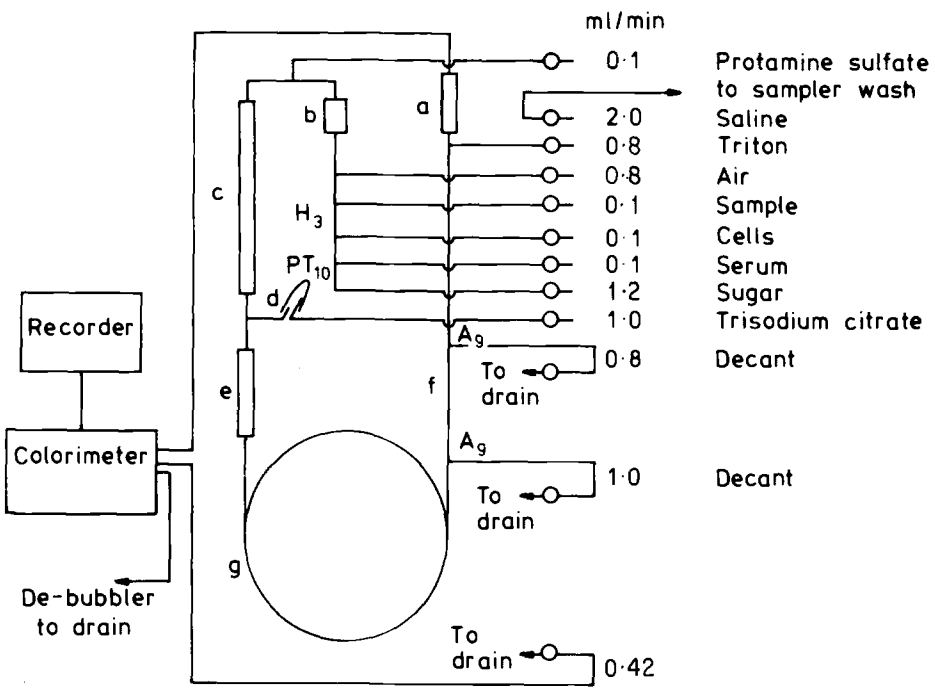
The sample cups containing the eluates, and sample cups containing saline wash solution, were placed in alternate positions around the sampler tray. After each series of five antisera, two cups containing anti-Rh_o diluted 1:2000 with saline were place in consecutive holes on the sampler tray. These adjacent cups produced a distinctive double peak which simplified sample identification. The AutoAnalyzer® was operated at 90 samples per hour with a sample to wash ratio of 2:1. Results obtained from an R_2R_2 bloodstain and an unstained portion of cloth are shown in Fig. 2.

Results and Discussion

Elution of Antigens

The absorbed antigens were eluted from the stained cloth into saline at 55°C. The strongest eluate was obtained after 2 h, and heating for periods up to 4 h did not affect the titer. The Weiner elution technique used by Douglas and Staveley [4] was not necessary, and when this method was used the amount of anti-rh′ and anti-rh″ detected was much lower. The bloodstained samples were not removed after elution, and there was no obvious reabsorption of the antibody. Attempts to reuse the same portion of bloodstain for further rhesus antigens were unsuccessful, and it is probable that the rhesus binding sites were destroyed by prolonged heating to 55°C.

[2] Polysucrose, molecular weight 400 000, manufactured by Pharmacia of Sweden.



a 4 turn 1mm internal diameter mixing coil
b 4 turn 1mm internal diameter mixing coil
c 28 turn 2·4mm internal diameter mixing coil, jacketed at 32°C
d Pulse suppressor
e 14 turn 2·4mm internal diameter mixing coil
f Straight settling tube 2·4mm internal diameter 20cm long
g $2\frac{1}{2}$ turn settling coil

FIG. 1—*Flow diagram of the Technicon AutoAnalyzer® used for detecting antigens eluted from bloodstains.*

Use of Plasma

The original system described by Douglas and Staveley [*8*] required AB serum to prevent the formation of gross rouleaux during incubation. This reagent was not readily available, and the same result was obtained with the plasma removed from the blood cells in use. This was diluted with an equal volume of isotonic sugar solution and the mixture was acidified by adding 0.05*M* hydrochloric acid (5% v/v). A portion of the plasma was kept undiluted to allow some change to be made in the concentration if necessary. (When there is too little plasma or serum present, the rouleaux tend to adhere to the mixing coils.)

Titer of Antisera

A series of dilutions of antisera were prepared in saline. Ortho anti-Rh_o was readily detected at 1:16 000, anti-hr′ at 1:8000, and anti-rh′, anti-rh″, and anti-hr″ could all be detected at 1:1000 dilutions in saline. The bloodstains tested each day always in-

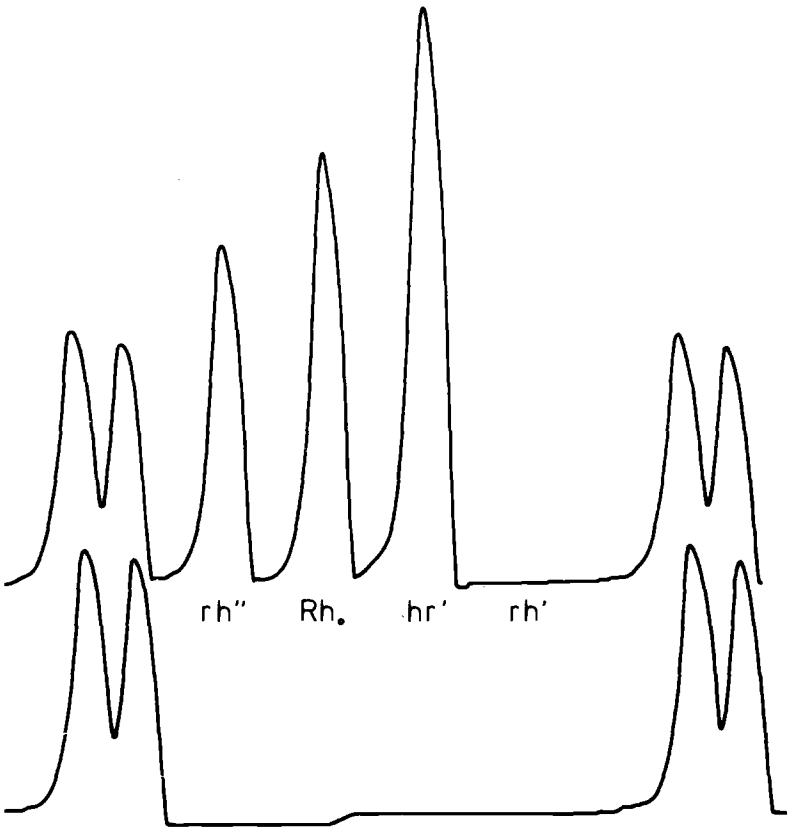


FIG. 2—(Top line) *Results obtained with an R_2R_2 bloodstain incubated with anti-rh', anti-hr', anti-Rh_o, and anti-rh".* (Bottom line) *Portion of the unstained material incubated with the same antisera as the bloodstain.*

cluded samples known to be Group R_1R_1, R_1R_2, R_2R_2, and rr. This series demonstrated whether there were any false positive or negative reactions.

Bloodstains on Different Materials

Bloodstains on cotton or wool gave no problems, and good results were also obtained with bloodstained paper. Some vinyl fabrics, denim, and other tightly woven materials were more difficult to wash and occasionally gave small false positive Rh_o or hr' peaks. Bloodstains on nonabsorbent materials and some synthetics were often lost during the washing stage, and it was better to absorb these bloodstains onto damp cotton cloth before incubation.

Age of Bloodstains

Stains less than 30 days old gave good results with all five antisera. With stains between 30 days and 60 days old, the reaction with anti-rh', anti-rh", and anti-hr" decreased, and these antigens were rarely detectable in bloodstains more than 3 months old. The Rh_o and hr' factors could be detected in stains which were at least 1 year old.

Bloodstains with the rh^w antigen gave small peaks with anti-rh'. Good results were obtained for rh^w using specific antisera and indicator cells containing the rh^w factor.

In this case, rh^w bloodstains gave peaks comparable to the results obtained with rh′ stains using the normal R_1R_2 indicator cells. The rh′ bloodstains gave no peaks when anti-rh^w sera and rh^w indicator cells were used.

The low ionic strength method described by Douglas and Staveley [*8*] used glycine wash solutions, and the antenatal sera were also diluted with glycine solution. For rhesus grouping of dried bloodstains the antibodies bound to the bloodstain are eluted into saline. When glycine wash solutions were used in combination with the saline eluates a number of false positive peaks were recorded with the samples which followed immediately after a strong peak. This problem was eliminated by completely replacing the glycine solutions with saline.

Douglas and Staveley [*8*] found the low ionic strength method was at least as sensitive as the bromelin-polyvinylpyrrolidone (PVP) method, with the advantages that there was no need to prepare enzyme solutions of standardized activity and that the low ionic strength method was free of the technical difficulties involved in pumping the viscous PVP solution.

Some antigen sites on blood cells may be destroyed by enzyme treatment, and it is possible the use of the low ionic strength method may permit automation of a number of other blood grouping systems, including Ss and Duffy, which have only recently been detected in bloodstains by a microelution technique using antihuman globulin [*1*].

Acknowledgments

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