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Research Article

CHEMICAL, CLINICAL, AND IMMUNOLOGICAL STUDIES ON THE PRODUCTS OF HUMAN PLASMA FRACTIONATION

XXIX. SERUM ALBUMIN AS A DILUENT FOR Rh TYPING REAGENTS [1, 2]

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During the last two years methods for the separation and concentration of the anti-Rh isoagglutinins have been developed as a part of the study of the fractionation of human plasma (1). The solubility characteristics on which the separation has been based resemble those of the anti-A and anti-B isoagglutinins, and have been summarized in a previous communication (2). The final products have been prepared as protein powders, stable and readily soluble, which may be packaged in convenient quantities and reconstituted to yield potent reagents useful for Rh typing or for A or B grouping, respectively.

In contrast to the anti-A and anti-B isoagglutinins, which may be obtained readily from grouped plasma pools from normal donors (3), the available supply of anti-Rh material is limited. The incidence of anti-Rh agglutinins in the general population is very low, and bleedings suitable as a source of original plasma for processing must be obtained from sensitized Rh negative donors, specially selected (2). Moreover, the phenomenon, termed "blocking," [3] whereby the antibodies in certain anti-Rh serums fail to agglutinate homologous Rh positive cells in sodium chloride solutions, but appear to combine with them and to inhibit the action of Rh agglutinins (4, 7, 8), has made these serums and the anti-Rh globulin fractions obtained from them unsuitable for use as typing reagents by ordinary methods.

Anti-Rh agglutination with serums containing "blocking" antibodies may be demonstrated by the slide method [4] if whole blood is used (9), or by the test tube method [4] when compatible plasma or serum, instead of isotonic sodium chloride, is the reaction medium (10, 11). Plasma, or serum, is similarly effective with anti-Rh globulin fractions high in "blocking" antibodies (12). These globulin fractions, which in isotonic sodium chloride systems either on the slide or in the test tube, agglutinate specific Rh positive red cells poorly or not at all, cause strong agglutination in whole plasma. Plasma also intensifies, to a lesser degree, the reactions of "agglutinating" antiserums, *i.e.*, those in which no "blocking" antibodies are demonstrable.

Influence of whole plasma on anti-Rh avidity

Reaction times of cells in plasma and in sodium chloride, when measured against each of these kinds of anti-serum, are reported in Table I. For these tests, packed Rh_1 cells, twice washed in isotonic sodium chloride, were resuspended in their own plasma and in the salt solution. A measured volume, 0.03 ml. of cell suspension, was mixed on a slide with 0.01 ml. of antiserum, and the reaction was timed as the slide was rocked over a viewing box. The times for first agglutination visible to the unaided eye, and for an advanced stage, which has been called "complete" agglutination, were recorded. The maximum size of clumps attained

[1] This work has been carried out under contracts, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

[2] This paper is Number 40 in the series "Studies on Plasma Proteins" from the Harvard Medical School, Boston, Massachusetts, on products developed by the Department of Physical Chemistry. The blood was collected by the Blood Grouping Laboratory, Children's Hospital, Boston.

[3] The terminology of Wiener (4, 5) has been used throughout the paper. The terms "late" and "early" as alternatives to "blocking" and 'agglutinating," have been proposed elsewhere (6).

[4] The slide method consists essentially of mixing, on an open glass slide, suitable proportions of cells and antibody solution, and then gently rocking the slide while observing the mixture for macroscopic agglutination. In the test tube method, cells and antibody are incubated together in a small test tube, usually at 37° C., for about 1 hour. The tube is then centrifuged briefly, and the contents examined for agglutination.

TABLE I

The avidities on the slide of an "agglutinating," and a "blocking" Rh antiserum, against cells in plasma and in isotonic sodium chloride

Type of antiserum	Rh_1 cell concentration	Slide agglutination times				Size of final clumps *	
		First visible		Complete			
		In plasma	In sodium chloride	In plasma	In sodium chloride	In plasma	In sodium chloride
	per cent	*seconds*					
"Agglutinating"	50	15	20	60	100	lg	<lg
	25	15	25	70	210	lg	>sm
"Blocking"	50	20	25	110	>300	lg	sm
	25	20	40	120	>300	lg	<sm

* Symbols: "lg" means "large"; "sm" means "small."

within 5 minutes was also noted and was classified in a series from "small" to "large." [5] It may be seen that against the "agglutinating" antiserum, a 50 per cent cell suspension in plasma reacted somewhat faster, and showed larger clumps than did the same cell concentration in sodium chloride. With a 25 per cent suspension the plasma medium gave a markedly better reaction than the sodium chloride medium. The contrast between plasma and sodium chloride was much more striking when "blocking" antiserum was used. Agglutination was excellent in plasma and very poor in the salt solution.[6]

Influence of plasma fractions on anti-Rh avidity

In an effort to discover which of the components of plasma contribute to the beneficial effects observed, a preliminary avidity study of some of the separated fractions of human plasma (1) was made. Approximately 10 per cent protein solutions were prepared at isotonicity and neutral pH. To detect any non-specific effects, 2 per cent suspensions of cells (type Rh_1) were first made in these solutions without the addition of antibody and were studied microscopically.

Fractions I (fibrinogen) and II (γ-globulin) caused non-specific clumping,[7, 8] and therefore are not included in the study which follows. Subfractions of III and IV (described later) caused at most only a trace of clumping. No non-specific clumping occurred with fraction V (albumin) or with a similar solution of crystallized bovine albumin.[9] Cell suspensions in whole plasma showed considerable *rouleau* formation, as is often the case.

The effects of the media upon speed of agglutination on the slide were next compared. Twenty-five per cent suspensions of Rh_1 cells were prepared in the various protein solutions, in whole plasma, and in sodium chloride alone. This concentration of cells, rather than a 50 per cent concentration, was chosen in order to observe more accurately the progress of cell aggregation. An anti-Rh globulin composed largely of "agglutinating" antibodies [10] and dissolved in buffered sodium

[5] We have considered these slide reactions to be satisfactory if agglutination is first visible within 30 seconds and complete within 120 seconds and if the clumps are "medium" or "large."

[6] These results are discussed further in connection with Table VIII.

[7] Gelatin solutions in sodium chloride have a similar effect which is inhibited by glycine (13). Glycine likewise prevents the clumping of red cells by γ-globulin (14).

[8] While the non-specific effects of both fractions decreased upon dilution with isotonic sodium chloride, an interesting difference in their influence upon avidity was noted. Fraction II, when used in 2 per cent concentrations as a cell suspension medium, caused no non-specific clumping, nor did it improve specific agglutination on the slide. Fraction I, on the other hand, showed no macroscopic non-specific effects at 0.8 per cent concentration, yet was still effective in improving avidity. Further studies are indicated.

[9] See footnote number 12.

[10] The antibody molecules themselves probably comprise less than 1 per cent of the protein in these preparations.

TABLE II

The effects of human plasma fractions on the avidity of anti-Rh globulin

Cell suspension medium *			Slide agglutination times	
Designation	Principal protein component	Final concentration on the slide	First visible	Complete
		per cent	*seconds*	
Isotonic sodium chloride	— — —	—	110	>300
Whole plasma	— — —	"5"†	40	120
Fraction III–0	β Lipoprotein	8	45	165
Fraction III–1, 2	γ Euglobulin, β Globulin	8	40	140
Fraction IV–1	α Lipoprotein	8	40	210
Fraction IV–3, 4	α Globulin	7	30	150
Fraction V (human)	Albumin	8	80	250
Fraction V (bovine)‡	Albumin‡	8	80	260

* 25 per cent suspensions of Rh_1 cells were used.

† "5 per cent" plasma means that 3 parts of cell suspension in whole plasma were added to 1 part of antibody reagent in sodium chloride.

‡ The material used here was a crystallized product, prepared from Fraction V by the Armour Laboratories, Chicago, Illinois. See also footnote 12 in text.

chloride was used; the tests were performed as described earlier in the paper. The results are presented in Table II.

Cells in sodium chloride, as expected, reacted very poorly, while those in plasma showed rapid agglutination. Several fractions of human plasma comprising lipoproteins and globulins, and known as Fractions III–0, III–1, 2, IV–1, and IV–3, 4, approached unfractioned plasma in their effects at the concentrations tested. The albumin fractions, both human and bovine, which had earlier been observed to increase speed of agglutination on the slide, were less effective than these fractions but much better than sodium chloride. Certain mixtures of albumin with the other proteins were more effective than albumin alone. Further study of the subfractions of III and IV is clearly indicated. Even though it was realized that these fractions might be useful, bovine serum albumin was chosen, because of its high stability, its low viscosity, and its availability in quantity, for additional experiments which are the subject of this paper.

Influence of serum albumin on anti-Rh agglutination by the test tube method

Several pools of anti-Rh plasma have recently been fractionated. The original material had been segregated into 2 categories, 1 containing mostly "agglutinating" antibodies, the other high in "blocking" antibodies. The corresponding fractionation products have likewise been found to be low and high, respectively, in "blocking" antibodies.[11] The behavior of both kinds of material and the effects of albumin upon their agglutinating properties *in vitro* and upon their stability is considered in this communication. A related paper (6) deals with the use of albumin in the detection of anti-Rh agglutinins in whole serums. After tests had shown that human and bovine albumins are very similar in their effects, we adopted crystallized bovine albumin[12] for these experiments. A number of bovine albumin preparations, both of the crystallized protein and of Fraction V which is over 95 per cent albumin and the starting material for crystallization, have proved satisfactory.[13] The reactions have always been noted in systems at neutral pH and made isotonic with sodium chloride or some other suitable electrolyte.

Albumin, like plasma, when used as part of the reaction medium, enables anti-Rh globulin high in

[11] The two varieties of antibody have not as yet been separated by chemical fractionation.

[12] We are indebted to the Armour Laboratories, Chicago, Illinois, for this material, which was prepared under a contract recommended by the Committee on Medical Research of the Office of Scientific Research and Development.

[13] Certain very old preparations of crude Fraction V have given equivocal results.

TABLE III

*Titrations of anti-Rh globulin fractions high in "blocking" antibodies in sodium chloride systems with and without albumin**

Globulin preparation number	Final reaction medium	Type of cells †	Anti-Rh globulin dilution series ‡													
			1	2	4	8	16	32	64	128	256	512	1024	2048	4096	8192
14	Isotonic sodium chloride	Rh_1	0	0	0	0	0	0								
		rh	0													
	Albumin (20 per cent)	Rh_1	++++	++++	++++	++++	++++	+++	+++	+++	++	+	tr	0	0	
		rh	0													
17	Isotonic sodium chloride	Rh_1	tr	0	0	0	0									
		rh	0													
	Albumin (20 per cent)	Rh_1	++++	++++	++++	++++	++++	++++	+++	+++	++	++	+	tr	tr	0
		rh	0													

* Geometric dilutions of the anti-Rh globulin solution were made in small test tubes so that each tube contained 0.05 ml. One-tenth of a ml. of a 2 per cent suspension of red cells was added to each tube. After incubation for 1 hour at 38° C., followed by brief centrifugation, the packed cells in each tube were gently shaken up and the degree of agglutination recorded. Weak reactions were checked microscopically. The symbol "tr" means "trace agglutination."

† "rh" means "Rh negative."

‡ Column headings in this and similar tables are reciprocals of the dilutions of the antibody reagent.

"blocking" antibodies, to agglutinate homologous Rh positive red cells. This is shown in Table III, which presents the titrations of 2 anti-Rh preparations assayed in sodium chloride systems with and without albumin. Preparation 14, which showed no titer in the salt solution alone, showed agglutination in albumin to a dilution of 1:1024. Preparation 17, with only a trace reaction at its highest concentration in sodium chloride, had a titer of 1:4096 in albumin. The reactions were specific, as was indicated by the Rh negative control tests.[14]

Further experiments have been performed with anti-Rh globulins containing "blocking" antibodies in sodium chloride systems with and without the addition of serum and albumin. Representative results are reported in Table IV. For each of these tests, 1 part of anti-Rh globulin solution in sodium chloride or albumin was mixed in a small test tube with 2 parts of a 2 per cent suspension of Rh_2 cells in sodium chloride, serum, or albumin. The final systems thus contained varying percentages of protein. No agglutination occurred with any of the anti-Rh preparations in the sodium chloride medium alone. Preparation 8 gave strong agglutination in all the other systems. Agglutination with Preparation 17 was weak in 5 per cent albumin, stronger in "5 per cent" serum, and still stronger in the higher protein concentrations. Agglutination with preparation

[14] The soluble A and B factors of Witebsky have usually been added to neutralize the anti-A and anti-B isoagglutinins. Complete elimination of agglutination due to these antibodies requires a large excess of the group-specific substances, particularly if the typing reagent is to be used in an albumin medium.

Non-specific reactions with Group 0, Rh negative cells have been observed in systems where relatively high concentrations of anti-Rh globulin had been superimposed upon 20 per cent albumin. Satisfactory typing reagents must of course be free from such effects. It is important, in the fractionation of anti-Rh serums, to remove as completely as possible γ-globulin and fibrinogen, which have been shown to be especially conducive to such reactions.

TABLE IV

*Agglutination by anti-Rh globulin fractions high in "blocking" antibodies, in sodium chloride systems with and without serum and albumin**

Final reaction medium	Globulin preparation number		
	8	17	2
0.9 per cent sodium chloride	0	0	0
5 per cent albumin	Solid†	+	0
"5 per cent" serum‡	++++	+++	0
10 per cent albumin	++++	++++	0
"5 per cent" serum and 5 per cent albumin	Solid	Solid	+++**
15 per cent albumin	Solid	++++	++

* The readings were made as in Table III, after the usual incubation and centrifugation. See text for further description.

† The term "solid" means "one large clump."

‡ Group AB, Rh positive. "5 per cent" serum means that whole serum was diluted 2 : 3 in the final system.

** Includes rouleaux.

TABLE V

*Titrations of an anti-Rh globulin fraction low in "blocking" antibodies, in sodium chloride systems with and without albumin**

Type of cells	Final reaction medium	Anti-Rh globulin dilution series											
		1	2	4	8	16	32	64	128	256	512	1024	2048
Rh_2	Isotonic sodium chloride	tr	+++	++++	++++	++++	++++	+++	++	+	tr	0	
	Albumin (20 per cent)	Solid	Solid	Solid	Solid	Solid	Solid	++++	++++	+++	+++	++	++
Rh_1	Isotonic sodium chloride	++++	+++	+++	+++	+++	++++	++	++	+	tr	0	
	Albumin (20 per cent)	Solid	Solid	Solid	++++	++++	++++	++++	+++	+++	++	+	0
Rh′	Isotonic sodium chloride	++++	++++	++++	++++	+++	++	+	+	tr	0		
	Albumin (20 per cent)	Solid	++++	++++	++++	+++	+++	++	++	+	tr	0	

* The method used is described in a footnote to Table III. The term "solid" means "one large clump." The symbol "tr" means "trace agglutination."

2, which was very high in "blocking" antibodies by earlier tests, occurred only in the highest albumin concentration (15 per cent) and in the albumin-serum mixture.[15] Albumin and serum enabled the antibodies to react to a degree dependent upon the nature and concentration of the proteins in these media.[16] The total strength of antibody present and the relative amount of "blocking" antibodies no doubt also affected the final result with any particular preparation.

The reactions of an anti-Rh globulin very low in "blocking" antibodies will next be considered. Preparation 16 was made from serums containing for the most part "agglutinating" antibodies. Its titers against Rh_1, Rh_2, and Rh′ cells in sodium chloride systems with and without albumin were measured, and in addition "blocking" tests were performed.[17] The results are presented in Tables V and VI. It may be seen that agglutination with each of the 3 cell types was intensified in the albumin medium as compared with the sodium chloride medium. A definite prozone appeared in the lower dilutions of the series in sodium chloride against Rh_2 cells. This we have attributed to the presence of a small amount of "blocking" antibody, which decreased the agglutination in these dilutions. This explanation appears to be confirmed by the related "blocking test" against the same Rh_2 cells in Table VI, since the standard anti-Rh_0 "agglutinating" serum was unable to produce its maximum effect at the lowest dilution of globulin. The series in sodium chloride against Rh_1 cells also showed a prozone, partly masked by agglutination due to anti-Rh′ antibodies. This slight prozone was not reflected in the "blocking" test (Table VI). The Rh′ series showed no "blocking" antibody by either test, yet its reactions were strengthened in albumin. Thus, when "blocking" antibodies were absent or present only in low concentration, albumin has also proven beneficial.[18]

TABLE VI

"Blocking tests" using the cells and anti-Rh globulin of Table V

Type of cells	Standard agglutinating serum	Anti-Rh globulin dilution series			
		1	2	4	8
Rh_2	Anti-Rh_0	+++*	++++	++++	++++
Rh_1	Anti-Rh_0	++++	++++	++++	++++
Rh′	Anti-Rh′	++++	++++	++++	++++

* Definitely weaker than succeeding dilutions.

No significant difference has been found in the titer endpoint of unfractionated, "agglutinating" serums whether or not albumin was present (6). The anti-Rh globulin in Table V likewise showed only a slight difference in this respect when measured against Rh′ cells for which no "blocking" antibodies were demonstrable. Against Rh_2 cells, where anti-Rh_0 "blocking" antibodies could act,

[15] *Rouleaux* occurred in this mixture, but not in the albumin media alone.

[16] It is noteworthy that the 10 per cent albumin-serum medium, but not 10 per cent albumin alone, enabled agglutination to occur with one of the preparations. Here, as in the reactions reported in Table II, the influence of other serum proteins is indicated.

[17] These tests were done according to the method of Wiener, modified by Diamond and Abelson (9).

[18] With certain preparations very high in "blocking" antibodies, 20 per cent albumin has enabled strong agglutination to occur in most dilutions of the globulin, but has not completely eliminated the prozone phenomenon.

the titer in albumin was significantly higher than in sodium chloride alone.

Stability

High stability is important to the usefulness of blood typing and grouping reagents. Low moisture content (less than 1 per cent) and evacuation have been of aid in maintaining the potency both of anti-Rh and of anti-A and anti-B isoagglutinins. Nevertheless, additional means of assuring stability are desirable. Serum albumin has proved highly satisfactory for this purpose when used with anti-Rh globulin.

Experiments have been performed in which anti-Rh globulin samples were dried from the frozen state in isotonic sodium chloride with and without albumin. These samples were then heated as dry products at 50° C. for varying periods of time, after which they were redissolved and assayed together with unheated controls. Table VII presents the results of 1 such experiment in which an anti-Rh globulin low in "blocking" antibodies was used. In the titrations shown, the serial dilutions and the cell suspensions were in sodium chloride. Therefore, the only albumin present was that added before drying. The titration of an anti-Rh sample, dried with or without albumin and then heated, may be directly compared with its unheated control. The anti-Rh globulin, dried with albumin and heated for 8 days, showed little or no loss in titer against any of the 3 Rh positive cell types used. In contrast, titers of samples dried with sodium chloride alone, dropped so sharply after heating as to render the materials untrustworthy as typing reagents. Their avidities as observed on the slide were also greatly reduced, and the solutions themselves were more cloudy than before heating. The samples dried with albumin, on the other hand, showed no loss in avidity and yielded clear solutions. An anti-Rh globulin high in "blocking" antibodies has shown equal stability by appropriate tests, *i.e.*, its agglutinating properties as measured in an albumin system were maintained. A 42-day heat stability trial at 50° C. was made with an anti-Rh preparation dried with albumin as above. After this heating period, there was still no loss in titer or avidity against Rh_1 test cells. Other samples in albumin, with 0.1 gram per cent of carboxylmethoxylamine hemihydrochloride added as a preservative,[19] were allowed to stand in solution at room temperature for periods as long as 30 days. At the end of this time, the samples showed some loss in

[19] We are indebted to Dr. C. B. Favour of the Peter Bent Brigham Hospital for experiments which show that this antibiotic prevents the multiplication of common bacterial contaminants, unless these are present in very large numbers. These experiments were carried out under his contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

TABLE VII

*The stability at 50° C. of a dried anti-Rh globulin fraction, with and without albumin**

Diluent added before drying	Days heated dry at 50° C.	Type of cells	Dilution series of redissolved anti-Rh globulin									
			1	2	4	8	16	32	64	128	256	512
Isotonic sodium chloride	0	Rh₁	++++	++++	++++	+++	+++	tr	0	0		
	8		+++	+++	++	tr	0	0				
Albumin (15 per cent)	0		Solid	Solid	Solid	Solid	++++	+++	++	+	tr	0
	8		Solid	Solid	Solid	++++	++++	+++	++	+	tr	0
Isotonic sodium chloride	0	Rh₂	Solid	++++	++++	+++	++	+	tr	0	0	
	8		++	+++	++	++	tr	tr	0	0		
Albumin (15 per cent)	0		Solid	Solid	Solid	Solid	++++	+++	++	+	0	
	8		Solid	Solid	++++	++++	+++	+++	++	tr	0	
Isotonic sodium chloride	0	Rh′	+++	+++	+	tr	0	0				
	8		++	0	0	0						
Albumin (15 per cent)	0		Solid	++++	++++	++++	+++	++	tr	0		
	8		Solid	Solid	++++	+++	++	tr	0	0		

* The titration method is described in a footnote to Table III. The term "solid" means "one large clump"; the symbol "tr" means "trace agglutination."

both titer and avidity but were still useful typing reagents as measured with Rh_1 cells.

Variables affecting avidity on the slide

Among the factors which influence anti-Rh agglutination reactions *in vitro,* cell concentration, antibody concentration, and the nature of the reaction medium are of particular importance. In order to devise a slide typing test of maximum usefulness, the optimum relationships of these 3 variables were determined. The avidity studies with plasma fractions, discussed earlier in this paper, were concerned with the reaction medium. A further series of experiments has been performed to determine how avidity on the slide varies with varying cell, antibody, and albumin concentrations. Twice-washed Rh_2 cells and anti-Rh globulin were prepared separately in solutions of bovine albumin, isotonic with sodium chloride. A series of cell and globulin concentrations was used. An anti-Rh preparation high in "blocking" antibodies was chosen in order that any complications due to such material might be taken into account in arriving at a practical slide test method.

TABLE VIII

The effects of cell, antibody, and albumin concentrations on anti-Rh avidity on the slide

	Antibody solution		Rh₂ cell suspension		Slide agglutination times		Size of final clumps*
	Anti-Rh globulin concentration	Albumin diluent concentration	Cell concentration	Albumin concentration	First visible	Complete	
	per cent				*seconds*		
Part I	6	20	50	20	13	50	>lg
			25		18	75	<lg
			10		40	240	sm
			2		>300	—	—
	3	20	50	20	13	55	lg
			25		24	130	<md
			10		60	>300	<sm
	1.5	20	50	20	20	110	<lg
			25		40	180	>sm
			10		90	>300	<sm
	1.5	20	50 in plasma		20	60	>lg
Part II	3	20	50	20	20	100	lg
		10		10	35	180	<lg
		5		5	45	210	>md
		0		0	50	300	>sm

*Symbols: "lg" = large; "md" = medium; "sm" = small.

The tests were performed as described earlier in this paper. The results are reported in Table VIII.

The speeds of agglutination and the sizes of clumps in Part I of this table may be directly compared with one another, since the tests were all performed within a period of a few hours. The times shown in Part II are relatively slower, having been observed with cells 24 hours old, but are likewise comparable among themselves. It may be seen that lowering the concentration of any one of the 3 variables decreased both speed of agglutination and size of clumps. The system was particularly sensitive to cell concentration; that is, 25 per cent cell suspensions gave much poorer results than 50 per cent, and 10 per cent suspensions were extremely poor, even though a high concentration of albumin was present. Reducing the concentration of the anti-Rh globulin was less critical. A system with 50 per cent cells and 20 per cent albumin showed very good agglutination (20", 110", < lg) even when the anti-Rh globulin was as low as 1.5 per cent, which is only one-fourth of the maximum concentration tested.[20] For a comparison of reaction mediums, a 50 per cent cell suspension in whole plasma was tested. The reaction of this suspension was faster and showed larger clumps than the comparable 50 per cent suspension in albumin when the same antibody concentration was used with both. The effects of reducing the albumin concentration, using a constant 50 per cent cell suspension, are shown in Part II of Table VIII. Each reduction resulted in a poorer reaction upon the slide.

The high sensitivity of "blocking" antibodies to cell concentration on the slide is in agreement with other findings (9). In those experiments, however, diluted cell suspensions were made by adding isotonic sodium chloride to a plasma medium. In Table I of the present paper comparisons of slide agglutination with 50 per cent and 25 per cent cell concentrations, each in an undiluted plasma medium, were presented. In this medium, against both "blocking" and "agglutinating" antiserums, there was no significant difference in the behavior of the 2 cell concentrations.

It appears, therefore, that under proper conditions, any one of the 3 variables here discussed

[20] The cell and anti-Rh globulin concentrations referred to are those before the reagents were combined on the slide.

may be a limiting factor, particularly in the presence of "blocking" antibodies. With whole plasma as the reaction medium, high cell concentration is not as essential as it is with an albumin medium. On the other hand, high antibody concentration can at least partly offset the effects of lower cell or albumin levels. By specifying a 40 to 50 per cent cell suspension in plasma or in 20 per cent albumin, definite economy may be achieved as regards the concentration of anti-Rh globulin necessary to provide a fast, accurate reaction on the slide.

DISCUSSION

The usefulness of many anti-Rh serums as typing reagents has been sharply limited by interfering phenomena which have been attributed to "blocking" antibodies. Agglutination often fails to occur with such materials when the usual isotonic sodium chloride medium is used for testing. The fractionated and concentrated anti-Rh typing globulins often behave in a similar manner when they contain "blocking" antibodies.

Cell agglutination takes place *in vitro,* however, in the presence of "blocking" antibodies if the medium of reaction is plasma. Plasma is particularly useful as a suspension medium when the unknown cells are to be typed by the slide method. Since high cell concentrations are desirable, the samples may be taken and used as whole blood; or cells from clotted blood samples may be recovered, in concentrated suspension, in their own serum. As a routine diluent for Rh typing by the test tube method, which requires dilute cell suspensions and may include titrations, plasma is less satisfactory. Except when obtained from bloods of group AB, any stock plasma to be used with a variety of cell samples must be completely neutralized for the anti-A and anti-B isoagglutinins. Plasma is not highly stable when stored as a liquid, and often causes *rouleau* formation which confuses microscopic readings.

The value of serum albumin in making "blocking" antibody products useful as typing reagents, and in stabilizing anti-Rh globulin fractions, has been demonstrated by the experiments reported. Much plasma, or serum, formerly considered useless because of the presence of "blocking" antibodies, may now be fractionated and the products used in conjunction with albumin. Since a high concentration of albumin is required with such preparations, it is useful to supply this material both as a constituent of the dried anti-Rh reagent and as an auxilliary diluent. Typing tests by the slide and the test tube methods can then usually be performed with the same anti-Rh globulin solution.

The properties which enable plasma and its components to bring about agglutination in the presence of "blocking" antibodies are of interest.[21] Since a much higher concentration of serum albumin than of whole plasma is necessary to obtain comparable results, it must be concluded that factors other than albumin are effective in whole plasma. Several plasma fractions improved the avidity of anti-Rh globulin to a greater extent than did albumin, even when the antibodies present were largely of the "agglutinating" variety. Other preliminary experiments have indicated that these fractions are also effective as reaction media with "blocking" antibodies. Since the principal protein components of the various fractions differ widely from one another, no single specific protein would seem to be required.

The relationship between specific and non-specific effects of proteins upon red cell agglutination may be significant. Plasma fractions which caused non-specific clumping at high concentrations were usually among those which most improved specific agglutination at lower concentrations. An investigation of the effects of "blocking" antibodies and of various proteins on the electrical potentials of Rh positive and Rh negative red cells would be of interest. It has been reported (15) that changes in potential accompany isoagglutination.

Note added in proof: In a paper just published (Nature, 1945, **156**, p. 233) Coombs and Race have shown that changes in the mobility of red cells in an electric field occur after treatment with either "blocking" or "agglutinating" antibodies.

Fractionation has not been found to alter the properties of "blocking" antibodies. Plasma pools high in these substances yield anti-Rh globulin fractions of the same nature. This may be interpreted as evidence that the "blocking" antibodies are real entities and not merely the effects

[21] An explanation for the effect has recently been suggested (10).

of the original plasma environment. Since, under certain conditions, strong agglutination occurs in the presence of these antibodies, it is important to determine whether this reaction involves the "blocking" antibodies or is caused by "agglutinating" antibodies, present in the mixture but not always manifest.

SUMMARY

1. The presence of "blocking" antibodies heretofore has seriously limited the usefulness of many anti-Rh serums and globulin fractions as typing reagents for the detection of the Rh factor in red cells.

2. Plasma as a reaction medium is known to enable agglutination to occur *in vitro* with these materials.

3. Several different fractions of plasma have been found to improve the avidity of anti-Rh globulin preparations.

4. Serum albumin in high concentrations enables agglutination to occur with anti-Rh globulin fractions high in "blocking" antibodies and stabilizes anti-Rh globulin in general. Albumin as a reaction medium possesses certain technical advantages over plasma.

5. The influence of erythrocyte, antibody, and albumin concentrations on anti-Rh avidity on the slide has been studied.

6. Practical applications of the experiments are discussed, and the significance of certain observations is considered.

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