

1

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PREPARATION OF BLOOD FRACTION FOR USE IN Rh TESTING PROCEDURES

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This invention relates to a process for the fractionation of mammalian serum and relates particularly to a process for preparing a serum fraction containing albumin in a major amount and globulin in a minor amount, said serum fraction being suitable for use in demonstrating the presence in blood of an Rh antibody known variously as incomplete antibody, blocking antibody, albumin agglutinin, and agglutuinoid.

In 1939, Levine and Stetson, J. A. M. A., 113:126, 1939 reported a transfusion reaction in a woman who delivered a macerated fetus and whose serum contained agglutinins. In 1940, Landsteiner and Wiener, Proc. Exper. Biol. and Med. 43:223, 1940, reported the preparation of an anti-serum against the red cells of the rhesus monkey and found that this anti-rhesus serum, when properly absorbed, agglutinated the blood of approximately 85% of the members of the white race that were tested. They labelled the antigen in the human bloods that reacted with the anti-rhesus serum the Rh factor. Human bloods containing the Rh factor were termed Rh-positive. It was shown that Levine and Stetson, who reported the earlier transfusion reaction, were dealing with a case of Rh immunization and that the unnamed property was the property later named the Rh factor by Landsteiner and Wiener. Levine, Katzin, and Burnham, J. A. M. A., 116:825, 1941, reported that intragroup incompatibility could be produced by immunization of an Rh-negative mother by an Rh-positive fetus. These investigators demonstrated at the same time that Rh immunization explained the majority of cases of erythroblastosis fetalis.

It was at first thought that the Rh types could be explained on the basis of the presence or absence of the Rh factor, a single positive factor. Later it was discovered that the problem was much more complex and at the present time at least eight Rh subtypes are demonstrable. The anti-Rh agglutinins are not found normally in the blood of any individual. Antibodies that agglutinate Rh-positive cells may develop in the serum of some Rh-negative individuals under certain conditions. Anti-Rh agglutinins, which appear in the blood of certain Rh-negative individuals as a result of immunization by transfusion of Rh-positive blood or by bearing Rh-positive children, are demonstrable in the serum or plasma of the immunized individual and are generally divided into two types; the saline agglutinins (complete antibodies) and albumin agglutinins (incomplete antibodies). Anti-Rh agglutinins in general are similar to other isohemagglutinins except that they are more active at 37° C. than at lower temperatures. The anti-Rh agglutinins act very well when the serum is tested against a 1% or 2% suspension of Rh-positive cells in 0.85% sodium chloride. It was later discovered that there were antibodies other than the saline-active, anti-Rh agglutinins responsible for immunization. It was noted that a large proportion of the Rh-negative women who bore erythroblastic children did not show anti-Rh agglutinins in their serum when the serum was tested against a 1% or 2% suspension of Rh-positive cells in 0.85% sodium chloride. It was demon-

2

strated that these women were also immunized against the Rh factor even though Rh antibodies were not demonstrated when the serum was tested against Rh-positive cells suspended in salt solution.

It was later demonstrated that the serum of those women had the capacity to prevent the action of known anti-Rh agglutinins against Rh-positive cells suspended in salt solution, and it was assumed that their serum possessed antibodies capable of nullifying the effect of ordinary anti-Rh agglutinins on Rh-positive cells suspended in saline. This antibody has been called incomplete antibody, blocking antibody, albumin agglutinin, and agglutuinoid.

Hill, Reid, and Haberman, Texas State Journal of Medicine, 45:477-481, 1949, concluded that the antibody-antigen reactions are reversible and that there is competition between the different varieties of antibodies which explains various antibody patterns observed clinically. These investigators employed the term "agglutuinoid" to designate the Rh antibody found in the euglobulin associated with the albumin fraction which saturates the antigen and blocks the action of the saline agglutinin to distinguish it from the antibodies which these investigators have designated cryptagglutinoids which do not block, do not agglutinate saline suspensions, and show chemical differences to these two antibodies. These investigators postulate that the reaction of antibodies with antigen is reversible and that union of antigen and antibody occurs much more readily than disassociation. The three antibodies were found to vary in the affinity for the antigen in the probable order agglutuinoid (albumin agglutinins), agglutinin (saline agglutinins), and cryptagglutuinoid. Reactions between antibodies and antigens follow the laws of mass action and therefore, are greatly influenced by the concentrations of any of the three orders of antibody that might be present in a given serum. Blocking effects as well as apparent masking of agglutinins are well explained by this concept of heterogeneous antibodies acting in reversible reactions with the antigen and following the laws of mass action. Fractionation studies of these investigators furnished further evidence for these three orders of Rh antibodies and it has also been demonstrated that there are significant chemical differences between the agglutinins (saline agglutinins), and agglutinoids (albumin agglutinins), and cryptagglutinoids.

A test for demonstrating the presence of the incomplete antibody was developed by Diamond and Abelson, J. Lab. and Clin. Med., 30:204, 1945, which demonstrated this antibody produced agglutination of Rh-positive cells. In the test, one drop of the serum containing the incomplete antibody was mixed with two drops of whole oxalated or citrated Rh-positive blood and spread thinly on a warm slide, and this resulted in clumping of the Rh-positive cells. The same results were obtained and could be demonstrated by testing the serum containing the incomplete antibody against a 50% suspension of Rh-positive cells suspended in serum. It was originally thought that the heavy cell suspension was a factor which permitted such a serum to clump Rh-positive cells, but it was later concluded that the serum or plasma in which the cells were suspended was the determining factor. It was still later shown that the same effect is possible when incomplete antibody is tested against a 2% suspension of Rh-positive cells suspended in a 20% to 30% solution of human or bovine albumin, acacia, globin and many other macromolecular substances. The test to produce agglutination by incomplete antibodies was later modified and in this modification, the serum and Rh-positive cells in saline are incubated for one hour at 37° C. and centrifuged. The supernatant saline is removed and serum or other macromolecular substance added followed by resuspension of the cells. The mixture is again centrifuged and observed for clumping. In this test, the Rh-positive cells

in saline absorb the incomplete antibody but do not clump until the macromolecular solution is added.

E. A. McCulloch, *Nature* 165:276-277, 1950, reported on the requirements for bovine albumin used in demonstrating incomplete Rh antibodies by suspending the reacting cells in a concentrated albumin solution according to the test described by Diamond, *J. Clin. Invest.*, 24:793, 1945. Prior to the report of McCulloch, variation in the efficiency of different preparations of albumin suggested that the activating protein was not albumin but some contaminating protein associated with it. Kekwick and MacKay, *Proc. First International Congress of Biochemistry*, 1949, reported that solutions of beta- and gamma-globulin prepared by the ether fractionation of human plasma were inactive but that an active fraction could be prepared from the supernatant left after their precipitation which was soluble in 0.9% saline at pH 5.0. This soluble fraction consisted of albumin 38%; alpha-globulin, 33.4%; beta-globulin, 19.7%; and gamma-globulin, 8.9%. Albumin, separated electrophoretically from this preparation and concentrated by freeze-drying, showed no activity, but at a comparable protein concentration, the globulins were highly active. McCulloch concluded from these reported results that since the albumin was consistently negative, and no activity could be found in beta- and gamma-globulin; the activity appeared to be associated with alpha-globulin. This author suggested that alpha-globulin is responsible for the demonstration of incomplete Rh antibodies in the Diamond test.

Reid and Jones, *Amer. J. Clin. Pathology*, 19:10-15, 1949, described a process for the production of therapeutic fractions of human blood serum by the removal of salt with ion exchange resins. In the process of these investigators, cation and anion exchange resins were employed alternately for removing salts from blood serum or plasma, while keeping the pH between 6 and 8. As the salt concentration progressively decreased, the globulins precipitated out until the solution was essentially salt-free and at this time only euglobulins, albumin, hemoglobin, and other impurities were left in the solution. Precipitated globulins were removed and the pH was brought down to 5 which resulted in precipitation of the euglobulins. The euglobulin fraction contains the alpha-globulin components suggested by McCulloch to be necessary for demonstration of incomplete Rh antibodies in the Diamond test, as well as beta-globulins. The precipitated euglobulins were removed and a solution of albumin containing the hemoglobin which was in the original plasma remained. Sodium caprylate was added to 0.05 molarity of the albumin solution and the pH was adjusted to 7.4. The caprylate stabilized the albumin so that the solution could be pasteurized. The pH of the solution was adjusted to 5.0 and this resulted in denaturation of the hemoglobin which was then quantitatively removed by centrifugation. The solution was pasteurized and the caprylate and pyrogenic contaminants were removed by passage of the solution through successive columns of a stable cation exchange resin. The albumin solution was dessicated under vacuum from the frozen state and by this means could be safely carried to any concentration desired. The process of these investigators produced an undenatured albumin suitable for clinical use and free from globulin fractions.

It is an object of this invention to prepare a serum fraction suitable for use in demonstrating incomplete Rh antibodies in a method described by Diamond.

It is another object of this invention to prepare a serum fraction suitable for use in demonstrating incomplete Rh antibodies by suspending the reacting cells in a concentration of the serum fraction.

It is still another object of this invention to prepare a serum fraction, suitable for use in the method of demonstrating incomplete Rh antibodies of Diamond in which the reacting cells are suspended in concentrated albumin

solution, which fraction contains albumin and alpha-globulins.

It has now been discovered that a serum fraction containing albumin and alpha-globulins which is suitable for use in the Diamond test may be produced from mammalian blood serum by a process in which salts are removed from the serum with ion exchange resins to precipitate salt-soluble euglobulins and, after removing the precipitate, globulins in the serum are heat denatured in the presence of a salt of an organic acid without denaturation of albumin and alpha-globulins, and subsequently precipitated by an acid adjustment of the pH of the solution and removed, following which the alpha-globulins are precipitated by pH adjustment, the albumin is precipitated by methanol addition and albumin and alpha-globulins are removed together by appropriate means such as centrifugation.

In the process of this invention, serum is passed through a resin column containing both cation and anion exchange resins in a proportion such that a solution having a pH within the range of from 6 to 8, and preferably neutral, results on passage through the column of an acidic, basic, or neutral solution. Cation exchange resins having active phenolic, carboxyl or sulfonic groups which are saturated with hydrogen ions such as phenol methylene sulfonic acid resin, nuclear sulfonic acid resin, and polymerized acrylic acid resin and anion exchange resins having active amine groups such as a polymer resulting from nitration and subsequent reduction of a styrene-divinylbenzene copolymer are suitable for use in the process of the invention. As the resins remove the salts from the serum, the salt-soluble globulins, also referred to as euglobulins, are precipitated and subsequently removed by appropriate means such as centrifugation. An alkali metal salt of a fatty acid having 4 to 10 carbon atoms is then added to the solution to bring its molarity with respect thereto at least about 0.15. An alkali metal salt of a fatty acid such as butyric, caproic and hexanoic acid and of acetyl tryptophane has been found particularly suitable for addition to the solution. Below about 0.15 molar, heat subsequently applied to the solution destroys active alpha-globulin components necessary for the test; when the molarity is greater than about 0.3, alpha-globulins are precipitated. The pH is then adjusted to between 6.8 and 7.4, but it is preferred that the pH be at 7.0. The solution is brought to a temperature of approximately 75° C. and maintained at this temperature for about 25 to 35 minutes. The alkali metal salt prevents denaturation by heating of the alpha-globulins and albumin present in the solution. Heating to this temperature substantially denatures globulins present in the solution other than the active portions of the alpha-globulins. The solution is then cooled to a temperature not greater than about 25° C. and adjusted to a pH of 5.5 to 5.8, the preferred pH being 5.6. Adjustment of the pH of the solution to 5.5 to 5.8 after heating results in precipitation of substantially all denatured globulins leaving undenatured alpha-globulins in solution. If the pH is adjusted to a more acid pH than 5.5, alpha-globulins are precipitated and if the pH is adjusted to a more alkaline reaction there is insufficient precipitation of denatured globulins. The precipitated denatured globulins are removed by appropriate means such as centrifugation and the pH of the supernatant is adjusted to within the range of from 3.0 to 5.5. Upon adjustment of the pH of the supernatant to a point within this range, alpha-globulins are precipitated. It is preferred that the pH be adjusted to a point within the range of 4.4 to 4.8 because this is the point of minimum solubility of albumin in water and is known as the isoelectric range. If the pH of the supernatant is adjusted to a point below 3.0, albumin is destroyed and if the pH is adjusted to a point above 5.5, alpha-globulins are not precipitated. After adjustment of the pH, the supernatant is cooled to a temperature within the range of from 2° to -1° C. and methanol in an amount to bring

the quantity thereof to at least 38% by volume is added to the supernatant at such rate that the temperature of the solution does not rise above 2° C. The addition may be facilitated if the methanol is chilled before addition to the supernatant. It is essential that alpha-globulins be precipitated before methanol is added to the supernatant and that methanol not be in contact with the alpha-globulins and albumin at a temperature above 2° C. because methanol will denature any alpha-globulins in solution and denaturation of both alpha-globulins and albumin will occur at a higher temperature. The methanol precipitates the albumin fraction from the solution and precipitated albumin and alpha-globulins are removed by centrifugation. Residual alcohol present in the precipitate is removed by evaporation, preferably under vacuum at a temperature lower than 2° C. The residual solid material may be redissolved in an appropriate amount of a solution of 0.9% aqueous sodium chloride and adjusted to a pH of from 7.0 to 7.4 to give a final solution with a concentration of from 26 to 30% protein. This is the concentration and pH range of the protein fraction used in the Diamond test. The invention is further illustrated by the following example, but it should be understood that the invention is not limited to the specific details set forth in the examples.

50 liters of bovine serum were passed through a resin column consisting of a mixture of a strong-base anion exchange resin, Amberlite IRA-410 having a total capacity of 2.5 milliequivalents per gram, the resin being a reaction product of trimethyl amine and an insoluble, cross-linked copolymer of styrene and divinyl benzene, which copolymer contains chloroalkyl groups having the formula $C_nH_{2n}Cl$, in which C_nH_{2n} is an alkylene group and in which n is an integer from 1 to 4, and a phenol methylene sulfonic cation exchange resin, Amberlite IR-105G. The proportion of the cation exchange resin and anion exchange resin in the column was such that a neutral solution resulted on passage through the column of an acidic, basic, or neutral solution. The effluent from the column contained precipitated globulin which was removed from the solution by centrifugation. The total solution of the effluent was 60 liters and after precipitated globulins had been removed therefrom, 1467 grams of sodium caprylate were added to the supernatant. The pH was adjusted to 7.0 and the solution was heated at 75° C. for thirty minutes, cooled to room temperature and the pH was then adjusted to 5.6 at which time a precipitation of denatured globulins resulted which was removed by centrifugation. The supernatant having a volume of 40 liters was adjusted to a pH of 4.5 and cooled to 0° C. 26.6 liters of chilled methanol were added slowly and at such a rate that the temperature of the solution was maintained approximately at 0° C. throughout the addition. The precipitate resulting from the addition of methanol was removed by centrifugation and residual alcohol present in the removed precipitate was evaporated under vacuum at -5° C. The precipitate was dissolved in 3.5 liters of 0.9% aqueous sodium chloride and the pH was adjusted to 7.2. 4.5 liters of 28.25% protein fraction containing bovine albumin and alpha-globulins were obtained.

The following example illustrates the use of the solution of protein fraction obtained by the above procedure in the test procedure of Diamond, the said solution of protein fraction being substituted for the solution of human or bovine albumin, acacia, globin or other macromolecular substance used in the Diamond procedure.

A blood sample suspected of containing the "blocking antibody" was allowed to clot and the clotted sample was centrifuged. Two drops of the serum obtained by centrifugation were placed in a test tube and to this was added one drop of a physiological salt solution containing in solution 22% by weight of the blood fraction prepared according to the example above, and 2% by weight of Rh positive blood cells suspended therein. The mixture was incubated for one hour in a water bath maintained at

37° C., removed from the water bath; centrifuged at 1000 revolutions per minute for one minute, removed from the centrifuge, agitated gently and examined microscopically. The red blood cells exhibited the phenomenon known as "clumping," thus demonstrating the presence in the test serum of the "blocking antibody."

Since certain changes may be made in the above process and different embodiments of the invention could be made without departing from its scope, it is intended that all matter contained in the above description should be interpreted as illustrative and not in a limiting sense.

This application is a continuation in part of our application Serial No. 273,781, filed February 27, 1952.

What is claimed is:

1. The process of preparing blood serum proteins suitable for use in demonstrating incomplete Rh antibodies by suspending reacting blood cells in a solution of the blood serum proteins which comprises passing mammalian blood serum through a column containing both cation and anion exchange resins in a proportion such that a solution having a pH within the range of from 6 to 8 results on passage through the column of a solution selected from the class consisting of acidic, basic, and neutral solutions, whereby a precipitate of euglobulins is formed in the solution; removing the precipitate, adding to the solution an alkali metal salt of an organic acid selected from the group consisting of fatty acids having from 4 to 10 carbon atoms and acetyltryptophane in an amount sufficient to bring the molarity of the solution with respect thereto to at least about 0.15; adjusting the pH of the solution to 6.8-7.4; heating the solution to approximately 75° C. and maintaining the temperature of the solution at approximately this temperature for a period of from 25 to 35 minutes; cooling the solution to a temperature not greater than about 25° C.; adjusting the pH of the solution to 5.5-5.8; whereby a precipitate of denatured globulins is formed; removing the precipitated denatured globulins from the solution; adjusting the pH of the solution to 3.0-5.5, whereby alpha-globulins are precipitated, cooling the solution to a temperature of 2° to -1° C. and adding methanol to the solution in an amount sufficient to bring the amount with respect thereto to at least 38% by volume and at such a rate that the temperature of the solution does not rise above 2° C. during the course of the addition, whereby albumin is precipitated; and removing precipitated alpha-globulins and albumin from the solution.

2. A process according to claim 1 in which the alkali metal salt of an organic acid is sodium caprylate.

3. A process according to claim 1 in which the cation exchange resin has an active group selected from the class consisting of phenolic, carboxyl, and sulfonic groups and in which the anion exchange resin has an active amine group.

4. A process according to claim 1 in which the cation exchange resin is a phenol methylene sulfonic acid resin and in which the anion exchange resin is a polymer resulting from nitration and subsequent reduction of a styrene-divinylbenzene copolymer.

5. The process of preparing bovine blood serum proteins suitable for use in demonstrating incomplete Rh antibodies by suspending reacting blood cells in a solution of the blood serum proteins which comprises passing bovine blood serum through a column containing both cation and anion exchange resins in a proportion such that a solution having a pH within the range of from 6 to 8 results on passage through the column of a solution selected from the class consisting of acidic, basic, and neutral solutions, whereby a precipitate of euglobulins is formed in the solution; removing the precipitate, adding to the solution an alkali metal salt of an organic acid selected from the group consisting of fatty acids having from 4 to 10 carbon atoms and acetyltryptophane in an amount sufficient to bring the molarity of the solution with respect thereto to at least about 0.15; adjusting the pH of the solution to 6.8-7.4; heating the solution to ap-

7

proximately 75° C. and maintaining the temperature of the solution at approximately this temperature for a period of from 25 to 35 minutes; cooling the solution to a temperature not greater than about 25° C.; adjusting the pH of the solution to 5.5-5.8; whereby a precipitate of denatured globulins is formed; removing the precipitated denatured globulins from the solution; adjusting the pH of the solution to 3.0-5.5, whereby alpha-globulins are precipitated, cooling the solution to a temperature of 2° to -1° C. and adding methanol to the solution in an amount sufficient to bring the amount with respect thereto to at least 38% by volume and at such a rate that the temperature of the solution does not rise above 2° C. during the course of the addition, whereby albumin is precipitated; and removing precipitated alpha-globulins and albumin from the solution.

6. A process according to claim 5 in which the alkali metal salt of an organic acid is sodium caprylate.

8

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