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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 agglutinoïd.

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 per cent 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 erythroblastotic children did not show anti-Rh agglutinins in their serum when the serum was tested against a 1% or 2% suspen-

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sion of Rh-positive cells in 0.85% sodium chloride. It was demonstrated 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 agglutinoïd.

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 reports 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, report 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.

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 selective precipitation process in which, by a first precipitation step, globulins other than alpha-globulin are denatured, and precipitated from serum containing an alkali metal salt of an organic acid dissolved therein while alpha-globulin and albumin remain in solution in an undenatured condition; and a second precipitation step, in which alpha-globulin and albumin are precipitated by addition of a lower aliphatic alcohol to the solution after removal of previously precipitated denatured globulins other than alpha-globulins.

In the process of this invention, an alkali metal salt of a fatty acid having from four to ten carbon atoms, or an alkali metal salt of acetyltryptophane is added to a serum in an amount such that the molarity with respect to the added salt is 0.13 to 0.17. At a molarity below this range, the application of heat to the solution destroys alpha-globulin components necessary for the Diamond test and at a molarity substantially above this range, alpha-globulins are precipitated. Salts of acids such as butyric, caproic, and hexanoic have been found particularly suitable. The pH of the solution is adjusted to approximately 6.8 to 8.2; the preferred pH range is 7.0 to 8.0. The temperature of the solution is raised and maintained substantially above room temperature for a short period of time, preferably 25 to 35 minutes; the preferred temperature to which the solution is brought is 70° to 75°. Following the heating period, the solution is cooled to a temperature approximating room temperature. The presence of the alkali metal salt of the acid in the serum prevents heat inactivation of alpha-globulins and albumin and the region of greatest stability of these two substances in the presence of the alkali metal salt of the acid is within the pH ranges specified above. Globulins other than alpha-globulins are denatured by the heat treatment. The pH of the cooled solution is adjusted to 5.55 to 5.60. At a pH within this range denatured beta-globulins precipitate and are removed by any suitable means such as centrifugation. If the pH is adjusted to lower than 5.55, some alpha-globulins are precipitated and if the pH is adjusted to higher than 5.60, the denatured globulins are not completely precipitated. The pH of the solution after removal of the beta-globulins is adjusted to 3.0 to 5.5; the preferred pH range is 3.3 to 4.8. Alpha-globulins are precipitated at a pH within this range and the isoelectric point of the albumin is also within this range. If the pH is below 3.0, albumin is destroyed and if the pH is above 5.5, alpha-globulins are not precipitated. Following adjustment of the pH to 3.0 to 5.5, the solution is cooled to a temperature not substantially greater than 5° C. A low molecular weight aliphatic alcohol in an amount sufficient to bring the concentration of alcohol in the solution to at least 10 mols per liter is added to the cooled solution at such a rate that the temperature does not rise above 5° C. Addition is facilitated if the methanol is chilled before addition to the solution. Methanol, ethanol, and isopropanol have been found particularly suitable. Albumin is precipitated upon the addition of alcohol and the combined precipitate of alpha-globulins and albumin is removed from solution by appropriate means such as centrifugation. It is essential that

alpha-globulins be precipitated before alcohol is added to the solution and that alpha-globulins in solution not come in contact with alcohol or they will be denatured. The temperature of a solution containing albumin must not be substantially above 5° C. when alcohol is added in order that there be no denaturation of the albumin. Upon adjustment of the pH to 6.5 to 7.5, an aqueous solution of the precipitate containing alpha-globulins and albumin is suitable for use in the Diamond test. If desired, the precipitate may be dissolved in distilled water and dialyzed to remove salts and alcohol. The invention is further illustrated by the following example but it should be understood that the invention is not to be limited to the specific details set forth in the example.

249 grams of sodium capryllate were added to ten liters of bovine serum and this made the solution 0.15 molar with respect to sodium capryllate. The pH of the solution was adjusted to 7.0 by the addition of five normal hydrochloric acid. The solution was heated for thirty minutes at a temperature of 75° C. while being continually stirred mechanically and then cooled to room temperature and adjusted to a pH of 5.6 with hydrochloric acid. The precipitate of denatured beta-globulins formed at this point were removed by filtration and the clear liquid was adjusted to a pH of 4.5 with hydrochloric acid. After cooling the solution to 0.5° C., 2334 cc. of methanol, previously cooled to -5° C. were added at such a rate that the temperature remained between 0° and 5° C. Precipitated alpha-globulins and albumin were removed from the solution by centrifugation and during the time of removal the temperature of the solution was maintained at -5° C. The alcohol associated with the precipitate removed by centrifugation was removed under vacuum. The precipitate was dissolved in 125 cc. of 0.1% saline and adjusted with sodium hydroxide to a pH of 7.2.

The following example illustrates the use of the saline solution of alpha-globulins and albumin prepared according to the above procedure in the test procedure of Diamond, the said saline solution 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 the physiological salt solution obtained by the above procedure which contained in solution 22% by weight of the blood fraction comprising alpha-globulins and albumin; two per cent by weight of Rh positive blood cells were suspended in the mixture. 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 Number 283,974, filed April 23, 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: adding to mammalian blood serum 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 serum

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with respect thereto to within the range of 0.13–0.17; adjusting the pH of the solution to 6.8–8.2; heating the solution; cooling the solution; adjusting the pH of the solution to 5.55–5.60, 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 not substantially above 5° C.; adding a low molecular weight aliphatic alcohol to the solution in an amount sufficient to bring the concentration of alcohol in the solution to at least 10 mols per liter and at such a rate that the temperature of the solution does not rise above 5° C. during the course of the addition, whereby albumin is precipitated; and removing precipitated alpha-globulins and albumin from the solution.

2. The process for 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: adding to mammalian blood serum 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 serum with respect thereto to within the range of 0.13–0.17; adjusting the pH of the solution to 7.0–8.0; heating the solution to a temperature within the range of 70°–75° C. and maintaining the solution at this temperature for 25–35 minutes; cooling the solution to approximately room temperature; adjusting the pH of the solution to 5.55–

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5.60; whereby a precipitate of denatured globulins is formed; removing the precipitated denatured globulins from the solution; adjusting the pH of the solution to 3.3–4.8 whereby alpha-globulins are precipitated; cooling the solution to a temperature not substantially greater than 5° C.; adding methanol to the solution in an amount sufficient to bring the concentration thereof in the solution to at least 10 mols per liter and at such a rate that the temperature of the solution does not rise above 5° C. during the course of the addition, whereby albumin is precipitated; and removing precipitated alpha-globulins and albumin from the solution.

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

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

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