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2,847,348

PLASMA FRACTIONATION AND PRODUCT THEREFROM

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No Drawing. Application May 27, 1954
Serial No. 432,895

4 Claims. (Cl. 167—74)

This invention relates to a method for the preparation and isolation of a substance from horse, bovine, human or rabbit plasma capable of increasing the activity of thromboplastic material. More specifically, the invention relates to the fractionation of horse or rabbit plasma whereby there is obtained a substance having the ability to increase the activity of a thromboplastic material which has a prothrombin time, as determined by the Shapiro-Weiner method, longer than nine seconds.

Thromboplastin has accepted value for use in the determination of prothrombin time, which is a measure of the amount of prothrombin present in a tested blood sample. The determination of prothrombin time is useful clinically, for the fact that it varies with a variety of clinical situations has been well established. It is known that vitamin deficient diets may result in prolonged prothrombin time. Biliary diseases frequently result in prolonged prothrombin time and are considered to be related to impaired vitamin K absorption. Impaired liver function results in prolongation of prothrombin time. A variety of drugs such as the salicylates and especially Dicumarol affect the prothrombin time to a degree considered sufficient to be of clinical significance.

The two-stage theory of Morawitz for the mechanism of blood coagulation postulates, as a first stage, the interaction of prothrombin, calcium ion, and thromboplastin which results in the formation of thrombin and, as a second stage, the reaction of thrombin with fibrinogen to form fibrin. Fibrin fibers are largely responsible for the characteristic properties of clotted blood. It has been shown that the addition to blood of small amounts of thromboplastin can accelerate clotting time, generally referred to as prothrombin time, from the usual several minutes down to a few seconds. Thromboplastin, otherwise known as the platelet-tissue factor, is essential to the blood clotting mechanism but has not been definitely identified chemically. The mechanism of the activity and function of thromboplastin in blood clotting is not settled but most workers believe it to be enzymatic, and that thromboplastin acts to catalyze the conversion of prothrombin to thrombin probably through an intermediate prothrombin - thromboplastin - calcium complex. Since thrombin is a protein essential to the formation of fibrin and thromboplastin is necessary for the conversion of prothrombin to thrombin, the measurement of prothrombin time, wherein a standardized preparation of thromboplastin is used, has come to be considered as yielding information of great clinical value.

It is an object of this invention to provide a process for the fractionation of horse, bovine, human, or rabbit plasma whereby a substance is isolated which is capable of increasing the activity of thromboplastic material which has a prothrombin time of longer than nine seconds.

It is another and further object of this invention to provide a substance from horse, bovine, human, or rabbit plasma which upon addition to thromboplastic material obtained from rabbit brain or lung tissue, or a mixture thereof, results in a decrease in the prothrombin time of

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plasma as determined by our modification of the Shapiro-Weiner method.

Other objects and more particular advantages of the invention will appear from the following description as well as the appended claims.

The objects of this invention are accomplished by a process in which de-prothrombinized horse, bovine, human, or rabbit plasma is substantially freed of albumin and alpha-globulins by extraction with a first buffered aqueous alcohol solution and extracted with a second buffered aqueous alcohol solution to obtain a substance which, upon addition to thromboplastic material having a prothrombin time of longer than nine seconds, increases the activity thereof.

More particularly, in the process of this invention, horse, bovine, human, or rabbit plasma to which a heparin sodium solution has been added to prevent coagulation, is centrifuged in order to obtain the plasma. The plasma is slurried with two to five percent, and preferably five percent, of barium sulfate, the barium sulfate being expressed in grams, and the plasma in cubic centimeters. At least two percent of barium sulfate is required to remove all the prothrombin, and the presence of more than five percent results in a mixture of such a consistency that physical handling thereof is extremely difficult. The slurry is stirred for one hour in order that the maximum amount of prothrombin be completely adsorbed, and is then centrifuged. It is preferred that the adsorption be repeated to insure complete removal of prothrombin, preferably with the same amount of barium sulfate, and upon centrifugation after the second adsorption, the plasma is prothrombin free. Albumin and alpha-globulins are extracted from the prothrombin-free plasma by the addition of a first buffered aqueous alcohol solution and centrifugation. The alcohol may be ethanol, methanol or a mixture of methanol and ethanol and may be present in an amount of from about 15 to 30 percent by volume, but it is preferred that the amount be about 25 percent. If the amount is less than about 15 percent, a significant amount of active substances remain in solution, and if the amount is greater than about 30 percent a substantial amount of inactive material is present in the precipitate. The first aqueous alcohol solution is buffered within the pH range of from 4 to 5, the preferred pH being 4.2 to 4.6. Buffering below a pH of 4 results in incomplete solution of albumin in the extracting solution and buffering above a pH of 5 results in a significant amount of the desired plasma fraction being lost in the extracting solution. In general, any buffer system capable of maintaining the pH of the mixture of the first aqueous alcohol solution and prothrombin-free plasma within the range of 4 to 5 may be used. The preferred buffer system is sodium acetate-acetic acid; however, buffer systems such as sodium succinate-succinic acid, and sodium acid phthalate-phthalic acid, have been found suitable.

The volume of first aqueous alcohol solution used may vary widely, but the most efficient removal of albumin and alpha-globulin from the prothrombin-free plasma is accomplished when the volume is three to five times the volume of the plasma. It is preferred that the volume of the first aqueous alcohol solution be about four times the volume of the plasma.

It is necessary that the extraction and removal by centrifugation of albumin and alpha-globulins in solution in the first aqueous alcohol solution be accomplished at a low temperature in order that they be efficiently removed from the plasma. Extraction and removal at a higher temperature results in denaturation of albumin and alpha-globulins by the alcohol and incomplete removal thereof in the extracting solution. The prothrombin-free horse, bovine, human, or rabbit plasma is cooled to 5 to 0° C., and preferably to 0° C., and

the first aqueous alcohol solution, which has been cooled to -5 to -10°C ., is slowly added with stirring and during the course of the addition, the temperature of the mixture is maintained between 0 and -5°C ., and preferably at about -5°C . After addition is completed the mixture is stirred for about 30 minutes and during this time the temperature of the mixture is maintained at 0 to -5°C ., and preferably at -5°C . Immediately after stirring is discontinued, the mixture is centrifuged and during the centrifugation the temperature is maintained at 0 to -5°C ., and preferably -5°C . The supernatant, which consists mainly of albumin and alpha-globulins is discarded and the residue is extracted with a second buffered aqueous alcohol solution to obtain the desired plasma fraction.

The alcohol in the second aqueous alcohol solution may be ethanol, methanol, or a mixture of ethanol and methanol and the alcohol, or mixture of alcohols, is present in an amount from 5 to 20 percent by volume and preferably in an amount of about 16 percent by volume. The second aqueous alcohol solution is buffered at a pH within the range of from 6 to 8, and preferably 6.8 to 7.2. A solution buffered outside this pH range extracts significantly less of the desired plasma fraction from the residue of the first extraction. The second aqueous alcohol solution contains alkali metal salt of an amino acid having not more than six carbon atoms, such as alanine, glycine, proline, or serine. In general, other amino acids are not sufficiently soluble in the second extracting solution. The alkali metal salt acts as a stabilizing agent for the material to be extracted from the plasma, as a solubilizer for beta- and gamma-globulins, and as an active part of the buffer system. The amino acid is present in an amount within the range of 4 to 6 percent by weight, and preferably 5.4 to 5.8 percent by weight. At a concentration less than 4 percent by weight, the solubilizing effect of the amino acid is significantly decreased; on the other hand, the solubilizing effect is not increased when the concentration is greater than 6 percent. Phosphates such as mono- or di-sodium phosphate are also present in the second aqueous alcohol solution as part of the buffer system and the pH of the solution is adjusted to the desired value with an alkali metal hydroxide. Any buffer system may be used which is effective within a pH range of 6 to 8.

The amount of the second aqueous alcohol solution used in the extraction of the albumin and alpha-globulin-free residue may vary widely but an amount of solution three to five times, and preferably four times, the volume of the original plasma results in the most efficient recovery of the desired plasma fraction. The second aqueous alcohol solution is preferably cooled to 0° to -5°C ., and added at that temperature to the residue. The mixture of residue and second aqueous alcohol solution is stirred at a temperature of 0 to -5°C ., and preferably at -5°C ., for about 30 minutes, and then centrifuged and the residue from the centrifugation which consists mainly of beta-globulins and fibrinogen is discarded. During centrifugation the temperature is preferably kept at -1° to -5°C . The supernatant, which contains the desired plasma fraction, is filtered, dialyzed against distilled water at a temperature of 1 to 5°C ., and the dialysate is lyophilized. (The term "dialysate" as used in this specification designates the material which has failed to pass through the dialysis membrane.) Denaturation of the desired plasma fraction is at a minimum when the temperature during dialysis is within the range of 1 to 5°C . The solid material obtained upon addition to thromboplastic material enhances its thromboplastic activity.

In order that those skilled in the art may better understand how the present invention may be carried into effect, the following examples are given by way of illustration but not by way of limitation.

Fifty ml. of heparin sodium solution were added to 20 liters of horse blood and the mixture was centrifuged for 30 minutes at 25°C . Nine liters of the supernatant plasma were slurried with 450 grams of barium sulfate at 25°C ., stirred for one hour and centrifuged. The supernatant was slurried with 450 grams of barium sulfate, stirred for one hour at 25°C . and centrifuged. The prothrombin-free plasma obtained, which had a volume of eight and one-half liters, was cooled to 0°C . Thirty-four liters of an aqueous alcohol solution buffered at a pH of 4.6 was cooled to a temperature of 0°C . and added to the plasma at such a rate that the temperature of the mixture throughout the addition was below 0°C . The mixture was stirred for 30 minutes at -5°C . after addition was complete and then centrifuged and the temperature of the mixture was maintained at a temperature of -5° during the centrifugation. The supernatant, which contained substantially all the albumin and alpha-globulins of the plasma, was discarded. The aqueous alcohol solution contained per liter, $12\frac{1}{2}$ ml. of methanol, $237\frac{1}{2}$ ml. of 95 percent ethanol, and 0.6 ml. of an acetate buffer solution prepared by diluting a mixture of 20 ml. of 4 molar sodium acetate and 40 ml. of 10 molar acetic acid to 100 ml. with water. The residue from the centrifugation was finely dispersed with a spatula in 34 liters of the aqueous alcohol extracting solution buffered at a pH of 7. The temperature of the extracting solution and residue during the dispersion was maintained at -5°C . and after dispersion was complete the mixture was stirred for 30 minutes at a temperature of -5°C . and centrifuged. The temperature of the mixture during the centrifugation was maintained at -5°C . The supernatant was filtered and dialyzed against distilled water. The temperature of the supernatant during filtration and dialysis was maintained at 5°C . The dialysate was lyophilized and 350 grams of solid were obtained. The solid had no demonstrable thromboplastic activity. The aqueous alcohol extracting solution contained, per liter, 8 ml. of methanol, 152 ml. of 95 percent ethanol, 56 grams of glycine, 3.12 ml. of a solution of sodium glycinate, prepared by dissolving 4.5 grams of glycine and 2.0 grams of sodium hydroxide in 100 ml. of water, 4 ml. of 0.5 molar disodium hydrogen phosphate, and 2.76 ml. of 0.5 molar monosodium dihydrogen phosphate.

Example II

Three ml. of heparin sodium solution were added to 1200 ml. of rabbit blood and the mixture was centrifuged for 30 minutes at 25°C . Six hundred-twenty ml. of the supernatant plasma were slurried with 31 grams of barium sulfate at 25°C ., stirred for one hour and centrifuged. The supernatant was slurried with 31 grams of barium sulfate, stirred for one hour at 25°C ., and centrifuged. The prothrombin-free plasma obtained, which had a volume of 656 ml. was cooled to 0°C . Two thousand two hundred-sixty ml. of an aqueous alcohol solution buffered at a pH of 4.6 was cooled to a temperature of 0°C . and added to the plasma at such a rate that the temperature of the mixture throughout the addition was below 0°C . The mixture was stirred for 30 minutes at -5°C . after addition was complete and then centrifuged and the temperature of the mixture was maintained at a temperature of -5° during the centrifugation. The supernatant, which contained substantially all the albumin and alpha-globulins of the plasma, was discarded. The aqueous alcohol solution contained per liter, $12\frac{1}{2}$ ml. of methanol, $237\frac{1}{2}$ ml. of 95 percent ethanol and 0.6 ml. of an acetate buffer solution prepared by diluting a mixture of 20 ml. of 4 molar sodium acetate and 40 ml. of 10 molar acetic acid to 100 ml. with water. The residue from the centrifugation was finely dispersed with a spatula in 2260 ml. of the aqueous

alcohol extracting solution buffered at a pH of 7. The temperature of the extracting solution and residue during the dispersion was maintained at -5°C . and after dispersion was complete the mixture was stirred for 30 minutes at a temperature of -5°C . and centrifuged. The temperature of the mixture during the centrifugation was maintained at -5°C . The supernatant was filtered and dialyzed against distilled water. The temperature of the supernatant during filtration and dialysis was maintained at 5°C . The dialysate was lyophilized and eight grams of solid were obtained. The solid had no demonstrable thromboplastic activity. The aqueous alcohol extracting solution contained, per liter, 8 ml. of methanol, 152 ml. of 95 percent ethanol, 56 grams of glycine, 3.12 ml. of a solution of sodium glycinate, prepared by dissolving 4.5 grams of glycine and 2.0 grams of sodium hydroxide in 100 ml. of water, 4 ml. of 0.5 molar disodium hydrogen phosphate, and 2.76 ml. of 0.5 molar monosodium dihydrogen phosphate.

A plasma fraction prepared as above has the ability to increase the activity of a thromboplastic material which has a prothrombin time, as determined by our modification of the Shapiro-Weiner method, longer than nine seconds. A suitable thromboplastic material was prepared as follows:

Seventy-six grams of frozen rabbit brain and 1440 grams of frozen rabbit lung were homogenized at 5°C . for one minute in the presence of 7600 ml. of an aqueous solution containing, per liter, 150 ml. of an alcoholic solution prepared by adding 7.5 ml. of methanol, 15 grams of glycine, 4.8 ml. of one molar aqueous sodium acetate solution, and 2.6 ml. of one molar aqueous acetic acid solution to 142.5 ml. of 95 percent ethanol. The homogenate was stirred for two hours at 5°C . and centrifuged at -5°C . for thirty minutes. The supernatant liquid was filtered, dialyzed against distilled water at 5°C . and lyophilized. Forty-four and forty-six hundredths grams of thromboplastic solid material were obtained.

The thromboplastic activity, as measured by the prothrombin time, of the thromboplastic material prepared as above was determined as follows:

A calcium-thromboplastin suspension was prepared in a test tube by adding 100 milligrams of lyophilized solid to 20 ml. of 0.85 percent aqueous sodium chloride solution, admixing by inverting the tube three or four times until a uniform suspension was obtained, keeping the suspension in a water bath at $46-50^{\circ}\text{C}$. for twenty minutes, centrifuging, cooling the supernatant to room temperature, adding 0.1 ml. of 0.25 molar calcium chloride solution to 4 ml. of the suspension, mixing as above, and centrifuging again. Two-tenths ml. of the slightly turbid supernatant liquid was added to 0.1 ml. of fresh, oxalated human plasma which had been prepared by the addition of 0.1 molar aqueous sodium oxalate solution to fresh human blood in the proportion of one part sodium oxalate solution to nine parts of blood and centrifugation of the oxalated blood. The mixture of supernatant solution and oxalated human plasma was agitated at 37°C . by tilting the test tube back and forth and timing the first appearance of a fibrin clot. Clot formation was detected in twenty-five seconds after addition of the supernatant liquid to human plasma.

The calcium-thromboplastin suspension, in the form of the slightly turbid supernatant liquid prepared above, was mixed with a solution of the plasma fraction, prepared according to Example II and containing five mg. of plasma fraction per ml. of 0.85 percent aqueous sodium chloride solution, so that a series of mixed solutions containing varying proportions of the calcium-thromboplastin suspension and the saline solution of the plasma fraction resulted. The first and second columns in the table below give the amounts in milliliters of calcium-thromboplastin suspension and saline solution of the plasma fraction, respectively, in the series of mixed so-

lutions. The thromboplastic activity, as measured by prothrombin time, of each of the solutions of the series was determined by adding 0.2 ml. of the solution to 0.1 ml. of fresh, oxalated human plasma which had been prepared by the addition of 0.1 molar aqueous sodium oxalate solution to fresh human blood in the proportion of one part sodium oxalate solution to nine parts of blood and centrifugation of the oxalated blood. The mixture of solution and oxalated human plasma was agitated at 37°C . by tilting the test tube back and forth and timing the first appearance of a fibrin clot. The values in the third column of the table below represent the time in seconds for clot formation to occur following addition of the solution to the human plasma.

0.9	0.1	14
1.9	0.1	17
2.9	0.1	20
3.9	0.1	21
4.9	0.1	22
1.0	0	25

All determinations of thromboplastic activity, as measured by prothrombin time, were determined by our modification of the Shapiro-Weiner method for determining prothrombin time of blood, which is described in a book entitled: "Coagulation, Thrombosis and Dicumarol," by Shapiro and Weiner, published in 1949 by the Brooklyn Medical Press, Brooklyn, New York.

It will be obvious to those skilled in the art that various changes may be made without departing from the spirit of the invention and therefore it is to be understood that the invention is not limited to what is described in the specification and examples but only as indicated in the appended claims.

What is claimed is:

1. A process for the preparation of a substance capable of increasing the activity of thromboplastic material, conducted at a low temperature throughout, comprising the steps: adding to a unit volume of prothrombin-free plasma selected from the class consisting of horse, bovine, human, and rabbit prothrombin-free plasma, three to five volumes of a first aqueous alcohol solution buffered at a pH of 4-5 and containing about 15-30 percent by volume of an alcohol selected from the class consisting of methanol and ethanol and mixtures thereof, stirring and centrifuging the mixture, separating the supernatant from the residue; and adding to the residue three to five volumes based on plasma volume of a second aqueous alcohol solution buffered at a pH of 6-8 and containing 5-20 percent by volume of an alcohol selected from the class consisting of methanol and ethanol and mixtures thereof and 4-6 percent by weight of an alkali metal salt of an amino acid having not more than six carbon atoms, stirring and centrifuging the mixture, filtering the supernatant, dialyzing the filtered supernatant against distilled water, and lyophilizing the dialyzed supernatant.

2. A process for the preparation of a substance capable of increasing the activity of thromboplastic material comprising the steps: adding at a temperature of -5° to -10°C . to a unit volume of prothrombin-free plasma selected from the class consisting of horse, bovine, human, and rabbit prothrombin-free plasma at a temperature of 5 to 0°C ., about four volumes of a first aqueous alcohol solution buffered at a pH of 4.2-4.6 and containing about 25 percent by volume of an alcohol selected from the class consisting of methanol and ethanol and mixtures thereof, stirring and centrifuging the mixture, and separating the supernatant from the residue while the temperature is maintained at 0° to 5°C . and adding while the temperature of 0° to -5°C . to the residue at a temperature is 0° to -5°C ., three to five volumes based on plasma volume of a second aqueous alcohol solution, at a temperature of 0° to -5°C ., buffered at a pH of 6.8 to 7.2 and containing about 16 percent by

volume of an alcohol selected from the class consisting of methanol and ethanol and mixtures thereof and 5.4-5.8 percent by weight of an alkali metal salt of an amino acid having not more than six carbon atoms, stirring the mixture while the temperature is at 0° to -5° C., centrifuging the mixture, filtering the supernatant, and dialyzing the filtered supernatant against distilled water while the temperature is at 1° to 5° C., and lyophilizing the dialyzed supernatant.

3. A substance capable of increasing the activity of thromboplastic material prepared at a low temperature from prothrombin-free plasma, selected from the class consisting of horse, bovine, human, and rabbit prothrombin-free plasma, by adding to a unit volume of the plasma 3-5 volumes of a first aqueous alcohol solution buffered at a pH of 4-5 and containing about 15-30 percent by volume of an alcohol selected from the class consisting of methanol and ethanol and mixtures thereof, stirring and subsequently centrifuging the mixtures, separating the supernatant from the residue; and adding to the residue 3-5 volumes based on plasma volume of a second aqueous alcohol solution buffered at a pH of 6-8 and containing 5-20 percent by volume of an alcohol, selected from the class consisting of methanol and ethanol and mixtures thereof and 4-6 percent by weight of an alkali metal salt of an amino acid having not more than six carbon atoms, stirring and centrifuging the mixture, filtering the supernatant and dialyzing the filtered supernatant against distilled water, and lyophilizing the dialyzed supernatant.

4. A substance capable of increasing the activity of thromboplastic material prepared from prothrombin-free plasma, selected from the class consisting of horse, bovine, human, and rabbit prothrombin-free plasma, by adding, at a temperature of 0 to -5° C., to a unit volume of the plasma, maintained at a temperature of -5° to -10° C.

about four volumes of a first aqueous alcohol solution buffered at a pH of 4.2-4.6 and containing about 25 percent by volume of an alcohol selected from the class consisting of methanol and ethanol and mixtures thereof, stirring and subsequently centrifuging the mixture while the temperature thereof is maintained at 0° to -5° C., separating the supernatant from the residue; and adding to the residue while the temperature is 0° to -5° C., 3-5 volumes based on plasma volume of a second aqueous alcohol solution, at a temperature of 0° to -5° C., buffered at a pH of 6.8-7.2 and containing about 16 percent by volume of an alcohol selected from the class consisting of methanol and ethanol and mixtures thereof, and 5.4-5.8 percent by weight of an alkali metal salt of an amino acid having not more than six carbon atoms, stirring and subsequently centrifuging while the temperature is at 0° to -5° C., filtering and dialyzing the filtered supernatant against the distilled water while the temperature is at 1°-5° C., and lyophilizing the dialyzed supernatant.

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UNITED STATES PATENT OFFICE
CERTIFICATE OF CORRECTION

Patent No. 2,847,348

August 12, 1958

Heron O. Singher et al.

It is hereby certified that error appears in the printed specification of the above numbered patent requiring correction and that the said Letters Patent should read as corrected below.

Column 4, line 58, for "656 ml." read -- 565 ml. --; column 6, line 71, strike out "while the" and insert instead -- at a --; lines 71 and 72, strike out "at a" and insert instead -- while the --.

Signed and sealed this 28th day of October 1958.

(SEAL)
Attest:

KARL H. AXLINE
Attesting Officer

ROBERT C. WATSON
Commissioner of Patents