CONTROLLED PROTEIN FRACTIONATION

A controlled protein fractionation method in which a cold protein-containing solution is circulated in contact with one side of a membrane and the temperature is controlled by circulating a protein precipitant solution in contact with the opposite side of the membrane. The flow rate is increased and back pressure is exerted on the precipitant solution to cause it to penetrate the membrane, mix with the protein solution and precipitate protein in fine particles along an inside membrane wall. The precipitated protein may be concentrated and separated by centrifugation or diafiltration. The membrane is preferably in the form of a plurality of hollow fibers. Control of process variables increases yield and purity, decreases processing time and reduces degree of denaturation. The method is especially applicable to carrying out the Cohn cold alcohol fractionation process.
FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT Austria
AU Australia
BR Brazil
CF Central African Republic
CG Congo
CH Switzerland
CM Cameroon
DE Germany, Federal Republic of
DK Denmark
FR France
GA Gabon
GB United Kingdom
HU Hungary
JP Japan
KP Democratic People's Republic of Korea
LI Liechtenstein
LU Luxembourg
MC Monaco
MG Madagascar
MW Malawi
NL Netherlands
NO Norway
RO Romania
SE Sweden
SN Senegal
SU Soviet Union
TD Chad
TG Togo
US United States of America
CONTROLLED PROTEIN FRACTIONATION

Technical Field
This invention relates generally to a protein fractionation process. More specifically, the invention is directed to novel combinations of technology that allow for the simultaneous addition of potentially harmful denaturing solvents to protein solutions at controlled rates, the simultaneous efficient exchange of heat generated by the addition of these solvents to liquid, the control of concentration of protein, solvent, pH and ionic strength in complex mixtures of proteins such as blood plasma. Also this methodology can be utilized to remove organic solvents from protein solutions circumventing the harsh denaturing effect of solvent removed by freeze drying, flash evaporation or lyophilization and eliminating need for centrifugation in certain phases of the Cohn cold ethanol process, specifically Cohn methods 6 and 9. The method is ideally adapted to a continuous cold ethanol process for protein or plasma fractionation. Finally, addition of the organic solvent in the manner described in this invention allows for the most efficient formation of small particles during precipitation. The temperature control, pH control, ionic strength control, the formation of small particles during precipitation and the agglomerating effect of the use of hollow fibers are synergistic in effect to reduce the total process time to prepare Cohn Fraction V (albumin) from 5 to 7 days to less than 3 days with high yield, high purity, and minimal denaturation.

Background of the Invention
The fractionation of blood proteins is generally practiced according to the alcohol precipitant method introduced by E. J. Cohn (U. S. Patents No. 2,390,074 and No. 2,770,616). While fractionation of proteins by this process has met with success, a significant body of evi-
vidence has accumulated which indicates that proteins isolated principally through the use of alcohol and other protein precipitants, are not natural products. Both human albumin and particularly immune gamma globulin invariably contain aggregates when isolated with ethanol. These aggregates are present in amounts which indicate that denaturation has occurred. Many commercially available products prepared by the ethanol fractionation and use of other protein precipitants contain up to 25 per cent of aggregates (see U. S. Patent No. 4,136,094 to Condie).

The method proposed by Cohn depends upon balancing the precipitating action of the organic solvent with the solvent actions of the electrolytes present. In such a method, five independent variables have been controlled only to varying degrees: electrolyte concentration, alcohol concentration, hydrogen ion concentration, temperature, and protein concentration. To the extent that these variables are controlled depends the purity the yields, and the extent of denaturation of each of the plasma proteins isolated.

The Cohn process is currently practiced as a batch process. Cooled plasma is treated with reagents in very large vessels. That is, after pH adjustment of the plasma or subfraction thereof, the protein solution is cooled to a specific temperature at or below 0°C to decrease solvent denaturing of the protein and to achieve conditions for correct precipitation. Precipitation is achieved by adding the precipitant (usually ethanol) with stirring, the quantity being predetermined to achieve a final concentration appropriate to the separation of the desired protein fraction. The art as practiced today can neither control localized temperature variations, localized protein concentration variations, nor localized variation in alcohol concentration. It is not unusual to see localized temperature variation as great as ±15°C. Stirring of the mixture cannot be carried out efficiently without excessive foaming which is undesirable. The
process is essentially a slow one with the result that the concentration of precipitant fluctuates continuously up to the point at which all the precipitant has been added. Consequently, precipitation of fractions takes place progressively and a long period of aging is required in order to approach final equilibrium. It is in practice very rare to achieve the equilibrium condition required and the final product is almost invariably contaminated and denatured.

Such bulk systems also have the disadvantage that large volumes of plasma are continually at risk due to plant failure and staff errors. Incorporation under appropriate conditions to obtain complete fractionation cycle with the Cohn method requires 160 hours or approximately 7 days.

Cohn in U. S. Patent No. 2,469,193 taught that when a readily denatured protein such as gamma globulin is to be precipitated, considerable care should be exercised in the addition of the precipitant to the plasma or sub-fractionation thereof. Thus, it is recommended that after a suitable pH and temperature adjustment of the plasma or subfraction thereof, the precipitant be added by way of a semi-permeable membrane to avoid denaturation. This has not been possible in large scale systems. However, addition of alcohol by diffusion through semi-permeable membranes does not control wide fluctuations in protein concentration, alcohol concentration or temperature fluctuation.

It is naturally to be expected that the process is essentially slow and the concentration of the precipitant varies continuously up to the point which all the precipitant has been added. Long periods of aging are required in order to approach final equilibrium. The inordinate time requirements are documented in U. S. Patent No. 3,826,740 to Jewitt, a method and apparatus for treating multi-phase systems, said U. S. Patent No. 3,769,009 of John G. Watt, directed to an improved system of blood plasma fractionation.
Use of the Cohn method currently requires a large installation, the yields of certain fractions are low, and it requires prolonged exposure of proteins to high alcohol content which has a denaturing effect on some of these proteins. Up to the present time, control of the five variables has only been partially achieved and always under less than optimal conditions. The fact that the Cohn cold ethanol fractionated gamma globulin contains molecular aggregates makes it unsuitable for intravenous administration. This fact is documented and mentioned in Chanbrom U. S. Patent No. 3,763,135, and Falksvenden U. S. Patent No. 3,869,436. Even following closely the recommended Cohn method, it has been shown that proteins isolated with alcohol as a precipitant cause albumin and immune serum globulin aggregates.

Watt (U. S. Patent No. 3,764,009) has attempted to control temperature and alcohol addition by introducing a method for the fractionation of protein solutions, particularly plasma, in which spatially projected convergent jets are combined with the protein precipitant to form a mixture in which plasma protein is instantaneously precipitated and from which the protein is subsequently separated. In practice Watt claims that the spatially projected jets are sufficiently fine, that upon combination with the plasma the mixing is substantially instantaneous. The exothermic reaction of adding the alcohol to plasma and the dissipation of the generated heat is accomplished, Watt claims, if the jets merge smoothly to form a composite stream, impinging thereafter on a cool surface so that undue rise in temperature, which is liable to cause protein denaturation, is prevented. Turbulence which is another problem in the addition of the alcohol is minimized or reduced to an insignificant level.

The degree that the five variables are controlled determines the yield, purity, and extent of denaturation of the resultant protein fractions. Denaturation of protein can occur from a combination of any or all of the following - turbulence and foaming, high alcohol
concentrations, and heat generated by the alcohol addition or failure to keep temperature of the solution constant or, in the last step, when alcohol is removed from the protein. With the exception of the concentration of protein which is only initially controlled at the start of the process, these variables are all essentially uncontrolled until near the final stages of each step. During the critical phases which take place where localized differences in alcohol concentration occur, there is high heat, high alcohol concentration, and low concentration of proteins. These wide fluctuations resulting from the inability to control critical factors result in denaturation of proteins, low purity, and low yields. In order to minimize two critical variables, namely the alcohol concentration and temperature, the alcohol is added very slowly to the protein solution and with a minimum amount of agitation to avoid foam denaturation. This then requires approximately 7 days for the completion of the total cycle. In addition to the extensive time requirements, the protein concentrations have never been adequately controlled. Finally, because of the profound denaturing action and exothermic release of heat when added to aqueous solutions at high concentrations, ethyl alcohol must be added slowly and at fairly low concentrations (55 per cent) resulting in requirements for combined volume four times that of the starting liquid or plasma.

Summary of the Invention

The present invention incorporates a number of principles for the precise control at any time of the six major variables of the Cohn method including (1) protein concentration, (2) rate of alcohol addition, (3) temperature control by a unique heat exchange system, (4) control of pH and (5) conductivity, and (6) controlled agglomeration of the precipitating protein. This allows for the first time control of the significant variables of the Cohn method. Time required for completion of the complete fractionation cycle is reduced from 7 to 3 days, yields and purity are increased, and degree of denaturation is significantly reduced.
Broadly stated, the invention is a controlled rapid protein fractionation method which comprises the following steps. A cold protein-containing solution, such as plasma, is circulated through a confined space defined by one side of a porous membrane, such as a hollow fiber. The temperature of the protein solution is controlled by circulating a liquid protein precipitant solution in contact with the opposite side of the membrane. By increasing the flow rate and adjusting the back pressure, the precipitant solution is caused to penetrate the membrane and cause the precipitation of fine particles of plasma protein along the first side of the membrane. Then the precipitated protein particles are separated. The system may be backflushed with buffers to adjust pH levels, Na+ concentration, and ionic strength.

Brief Description of the Drawings

The invention is illustrated by the accompanying drawings in which corresponding parts are identified by the same numerals and in which:

FIGURE 1 is a schematic representation of one form of system incorporating the present invention;

FIGURES 2a and 2b are schematic representations of one form of fractionation cell showing one mode of operation in FIGURE 2a and another mode of operation in FIGURE 2b;

FIGURE 3 shows graphically the rate and concentration of alcohol in plasma, the temperature of ingoing and outgoing plasma and the temperature of ingoing and outgoing alcohol, as measured in the practice of Example 1;

FIGURE 4 shows graphically the rates of alcohol addition and concentration in plasma, temperature of ingoing and outgoing plasma before and after alcohol addition and the temperature of the ingoing and outgoing heat exchanging alcohol measured in the practice of Example 2; and

FIGURE 5 shows graphically the conditions measured in the practice of Example 3.
Detailed Description of the Preferred Embodiment

Referring to the drawings, and particularly to FIGURE 1, there is shown one exemplary system for carrying out the fractionation method of this invention. The system includes an insulated plasma vessel 10, an insulated alcohol vessel 11, and a buffer vessel 12, each equipped with a stirrer 13, heat exchange means 14 and temperature indicating means 15. In addition, tank 12 is provided with conductivity sensor 16 and pH sensor 17. A pair of alcohol and plasma mixing and heat exchange fractionation vessels 18 are provided, arranged in parallel.

As best seen schematically in FIGURE 2, each vessel or cell 18 includes a plurality of porous membrane fibers 19, all communicating at one end with an entry chamber 20 and communicating at the other end with a discharge chamber 21. Each vessel is provided with an inlet port 22 and discharge port 23, each provided with a temperature sensor 15. All of the fibers 19 extend through and are surrounded by a central chamber 24 having an inlet port 25 and discharge port 26, each also having a temperature sensor 15. FIGURE 2a shows the cell in one mode of operation with ethanol (indicated by shading) passing through fibers 19 and plasma passing through the space surrounding the fibers. FIGURE 2b shows the opposite mode 2 in which the plasma passes through the fibers and the alcohol, again indicated by shading, passes through the surrounding space.

Plasma vessel 10 is connected to the mixing cells 18 by means of pipe or tube forming line 27 extending from vessel 10 to the entry ports 22 of the mixing cells. Line 27 includes a controlled metering pump 28 to circulate the plasma. In addition, a temperature sensor 15, pH sensor 17, alcohol concentration sensor 29, protein sensor 30, flow gauge 31, pressure control 33, and pressure gauge 34 are all provided to detect and control conditions of liquid flowing through the line. Three-way valves 32 in the line enable the flow to be directed as desired. The discharge ports 22 of the mixing cells 18
are connected back to plasma vessel 10 through pipes or tubes forming line 35, these lines having temperature sensors 15, pH sensors 17, alcohol sensors 29, flow gauges 31, pressure valves 36, and pressure gauge 34 to permit detection and control of flow conditions. Alcohol vessel 11 is connected through line 37 and pump 28 to the entry port 25 to the mixing cell 18 and through line 38 to the discharge port 26 of that cell. Line 37 includes flow gauge 31, pressure valve 36, and pressure control 33. Buffer vessel 12 is similarly connected through lines 39 and 40 to the other of the mixing cells.

The process involves preferably the use of hollow fibers of either Teflon, divinyl acrylonitrile, polysulfone or cellulose acetate constitution. Alternate systems can include the utilization of spiral wound tubular membranes, plate and frame systems utilizing membranes of similar composition and pore size.

The preferred hollow fiber membranes are manufactured as single, coherent structures that can tolerate pressures on either side of the active membrane surface without rupture or damage to a support structure. Hollow fibers are made by "spinning" a polymeric solution which upon setting or solidifying forms the anisotropic membrane in a tubular configuration. These hollow fibers consist of a very thin smooth membrane surface on the inside of lumen of the fiber with a controlled pore density and molecular rejection coefficient, and a rough spongy membrane structure on the outside of the fiber with a looser or more open pore configuration.

This membrane structure lends itself to pressurizing both the inside and the outside of the hollow fiber. Operating the hollow fiber module in an ultrafiltration mode is accomplished by pressurizing the inside of the hollow fiber. Operating the hollow fiber module in a "backflush" mode is accomplished by pressurizing the outside or shell side of the hollow fiber. The capability of backflushing hollow fibers is important to the use of hollow fibers for the addition of cold ethanol in the Cohn process.
Although ethanol is a preferred protein precipitant, a number of alternative solvents and solutions may be used. These include methanol, butanol, acetone, members of the glycol series, polyethylene glycol, dioxane, etc., neutral salts such as phosphate, sulfates, etc., or a mixture of reactants such as alcohol and salts, and finally block copolymer of ethylene oxide and polyisopropylene. Although described in terms of fractionation of blood plasma, the invention is applicable to the fractionation of protein solutions from whatever source, animal (human, bovine, porcine, etc.), bacterial, or plant.

Plasma at between 4°C and -7°C is pumped through the lumen of the hollow fibers. This temperature can be sensitively and accurately controlled by pumping 50 per cent to 80 per cent ethanol at temperatures between -20°C to -10°C on the outside of the hollow fibers. The cold ethanol is added to the recirculating plasma inside the fibers simply by increasing the flow rate and adjusting the back pressure on the outside of the hollow fibers sufficient to cause the alcohol to penetrate the pores of the hollow fiber. By contacting the cold ethanol with the plasma in this manner, local heat buildup is minimal due to the velocity (2-10 ft/sec) of the plasma through the hollow fiber and the thin (monomolecular) layer of alcohol continuously contacting the plasma at the membrane surface. This high surface area contact created by the hollow fiber membranes causes very small particles to precipitate promoting sharp fractions, high yield, and minimal contact time for complete precipitation.

This critical temperature control of plasma at the alcohol plasma interface is achieved by adjustments of the following easily controlled factors. The temperature and flow rate of the alcohol act in concert to effect efficient and instantaneous heat exchange and act synergistically with the flow rate and temperature of the plasma. Therefore, heat is effectively dissipated, localized concentrations of alcohol are minimized, and the plasma protein concentration is kept at concentrations...
favoring optimal formation of optimally sized protein precipitates.

The addition of buffers can be carried out in similar "backflushing" manner allowing for rapid and smooth equilibration to various pH levels, Na+ concentration, and ionic strength.

The addition of cold alcohol and buffer solutions can be accurately controlled by the pressure differential between the inside (lumen) of the hollow fibers and outside (shell) of the hollow fibers. The high velocity of the plasma flowing through the lumen of the fibers promotes the most efficient contact of alcohol with plasma and acts as an agglomerator to create fine particles of precipitating plasma while minimizing any heat of reaction caused by the mixing of plasma and cold alcohol.

The formation of optimal particle size during precipitation, temperature control, pH control, and agglomerating effect of the hollow fibers are synergistic in effect to reduce the total process time to make Cohn Fraction V (human albumin) from 5 to 7 days to less than 3 days.

Another advantage of the proposed use of hollow fibers to add protein precipitants such as cold ethanol to plasma is the elimination of the need for open batch tanks, thereby reducing the risk of pyrogenic lots of plasma proteins. This process can be carried out in closed tanks since the pH, temperature, and alcohol addition are controlled within the hollow fiber module. Special mixing devices and alcohol addition devices in the tanks are also eliminated since mixing and alcohol addition is carried out in the hollow fiber. Since the temperature, particle size, and pH can be finely controlled by the use of hollow fibers, the need for very expensive low shear pumps is eliminated and more moderately priced low shear modified centrifugal pumps can be employed.

It has been suggested by some experts that shear caused by pumping and flow through narrow channel (hollow fibers) tubes causes considerable denaturation. (Charm,
S.E.: Shear Inactivation in Processing Biologic Material, DHEW Publication No. NIH 78-1422, pg. 27). However, Romicon hollow fibers have been used to process enzymes on a commercial scale with minimal loss of enzyme activity. These commercial systems also employ centrifugal type pumps.

Other experts (Dunhill, P.: The Action of Shear on Enzymic Proteins, DHEW Publication No. NIH 781422, pg. 40) have proposed that shear itself is not detrimental (does not cause denaturation) to enzymes and labile proteins, but that exposure to oxygen (air) and/or elevated temperatures are the denaturing conditions causing loss of enzyme activity and protein integrity.

This hypothesis appears to be supported by the work done with Romicon hollow fibers processing both enzymes and plasma proteins. It is possible that what was interpreted as shear denaturing of enzymes and proteins was actually denaturation caused by elevated temperatures and exposure to air, particularly foaming.

This ability to control temperature with the cold ethanol on either the outsides or insides of the hollow fibers similar to a tubular heat exchanger over an area of 15 ft$^2$ or 26.5 ft$^2$ per module almost eliminates localized heating, while the elimination of open tanks and mixers in the batch tanks prevents foaming and excessive exposure to air. This enables the use of centrifugal pumps in conjunction with hollow fibers for plasma pumping while controlling the cold alcohol addition by use of a refrigerated pressurized tank or a refrigerated tank with centrifugal pump. Denaturation of protein aggregation will be minimized under these conditions as well as minimizing the risk of pyrogen contamination.

Alternatively the alcohol may be added to the plasma by reversing the compartments, i.e., alcohol flowing through the inside of the hollow fiber with plasma circulating on the outside. See FIGURE 2. In mode 1, FIGURE 2a, the alcohol is circulated inside the hollow fiber. In mode 2, FIGURE 2b, the alcohol is circulated outside the hollow fiber.
Finally, the capacity to control protein concentration eliminates the need for large storage vessels used in current practice to accommodate up to 4-fold increase in volume following alcohol addition.

The invention is further illustrated by the following:

Example I

Processing Data - Cohn Run #12 - Mode 1, FIGURE 2a
-Cryo-Precipitated Plasma

Separation of Fraction I

The pilot plant used for these examples is illustrated in FIGURE 1. The alcohol and plasma compartments in cells 18 are shown in FIGURES 2a and 2b. The mixing of alcohol and plasma and the heat exchange occurs also in this interface. Starting cryo-precipitated plasma (1.6 liters, 12.2 mS at 24°C, containing 93 grams total protein: 54.4 grams albumin and 13.5 grams IgG) is cooled to +1°C without permitting ice to form. Three milliliters of sodium acetate buffer (4.0 M HAc + 0.2 M sodium acetate, pH 4.0) is added to adjust the pH of the plasma from 7.86 to 7.1±0.1 (all pH determinations are 1:5 dilutions of sample to 0.9 per cent saline). Alcohol (80 per cent) for addition is stirred in a closed tank at a temperature of -11°C. The plasma is stirred in a closed tank and temperature adjusted to comply with desired conditions. The protein is recirculated through the outside of the hollow fiber at a rate of 2500 ml/min. At the same time, the cold ethanol is recirculated through the inside of the fibers at a rate of 300 ml/min. To obtain a final ethanol concentration in the plasma of 8 per cent, 178 ml of 80 percent ethanol were added to the plasma by increasing the rate of the ethanol flow until 20 psi of back pressure is observed. Under these conditions the addition of alcohol was completed in 70 minutes. During this time the plasma ethanol concentration went from 0 per cent to 8 per cent, the plasma temperature dropped from 0°C to -2.5°C and the ethanol temperature climbed from -11°C to -8.5°C. The rate and concentra-
-13-

tion of alcohol in plasma, the temperature of ingoing plasma (prior to mixing with alcohol), the temperature of outgoing plasma (after alcohol addition), and the temperature of ingoing and outgoing alcohol are illustrated in FIGURE 3.

Precipitate I was removed by centrifugation for 15 minutes, 27000 x G. Precipitate I was resuspended with ice to 420 ml. This precipitate is mainly fibrinogen. It contains 1.9 grams total protein (0.6 gram albumin and 0.4 gram IgG). Supernatant I contains 87 grams total protein (51 grams albumin, 12.1 grams IgG) in a volume of 1550 ml. The conductivity of Supernatant I is 8.7 mS at 24°C. The protein cooling tank and the hollow fiber outside are cleaned with distilled water and rinsed with 20 per cent ethanol.

Separation of Fraction II + III

Supernatant I is placed into the plasma cooling tank and maintained at -2.5 to -3°C. The pH is adjusted to 6.8±0.05 by the addition of 3.1 ml of sodium acetate buffer. The supernatant is brought from 8 per cent ethanol to 20 percent ethanol by the addition of 310 ml of 80 per cent ethanol using the same flow rate and pressure conditions as outlined in the "Separation of Fraction I". FIGURE 3 shows that during this 30 minute addition, the supernatant I temperature is lowered to -6°C as the ethanol temperature climbs from -13°C to -6°C.

Precipitate II + III is removed by centrifugation for 15 minutes, -6°C, 27000 x G. Precipitate II + III is resuspended with ice to a volume of 1.16 liters. It contains 19.7 grams of total protein (1.9 grams albumin and 10.5 grams IgG). Supernatant II + III contains 60.5 grams total protein (43.7 grams albumin and 1.4 grams IgG) in a volume of 1.7 liters. Its conductivity is 4.8 mS at 24°C. The protein cooling tank and hollow fiber outside are rinsed with distilled water and 40 per cent ethanol. The 80 per cent ethanol is allowed to drop to -15°C in preparation for the separation of Fraction IV.1.
Separation of Fraction IV\textsubscript{1}.

Supernatant II + III is placed back into the plasma cooling tank and maintained at \(-6^\circ C\). The pH is adjusted to 5.4±0.1 with the addition of 13 ml of sodium acetate buffer. The supernatant is diluted to 18 per cent ethanol with the addition of 190 grams of distilled crushed ice which is allowed to mix until the ice is dissolved. This step takes 25 minutes.

Precipitate IV\textsubscript{1} is removed by centrifugation for 15 minutes, \(-6^\circ C\), 27000 x G. It is resuspended with ice to a final volume of 830 ml. Precipitate IV\textsubscript{1} contains 6.6 grams of total protein (1.6 grams albumin and 0.9 gram IgG). Supernatant IV\textsubscript{1} contains 50.4 grams of total protein (39.4 grams albumin and 0.2 gram IgG) in a volume of 1.8 liters. Its conductivity is 5.3 mS at 24\(^\circ\) C.

The protein cooling tank is cleaned with distilled water. The 40 per cent ethanol is evacuated from the outside of the hollow fiber.

Separation of Fraction IV\textsubscript{4}.

Supernatant IV\textsubscript{4} is placed into the cooling tank and maintained at \(-6^\circ C\). The pH is adjusted to 5.8±0.05 by the addition of 20 ml of 1.0 M NaHCO\textsubscript{3}. The supernatant is brought from 18 per cent ethanol to 40 per cent ethanol by the addition of 998 ml of 80 per cent ethanol. The same initial flow rates are used as in separation of Fraction I, but during the addition the ethanol flow is increased until 25 psi of back pressure is observed. FIGURE 3 shows that during this 80 minute addition, the plasma temperature is maintained between \(-6^\circ C\) and \(-9^\circ\) C as the ethanol temperature is kept below \(-7^\circ\) C.

Precipitate IV\textsubscript{4} is removed by centrifugation for 15 minutes, \(-6^\circ C\), 27000 x G and is resuspended in ice to obtain a final volume of 730 ml. It contains 6.6 grams of total protein (1.6 grams albumin and 0.9 gram IgG). Supernatant IV\textsubscript{4} contains 40.2 grams of total protein (35.8 grams albumin and 0.0 gram IgG) in a volume of 2.68 liters. Its conductivity is 2.5 mS at 24\(^\circ\) C.
The protein cooling tank is cleaned with distilled water and is separated from the hollow fiber. The hollow fiber and ethanol cooling tank are cleaned at this time.

Separation of Fraction V

Supernatant IV₄ is placed into the cooling tank and maintained between -7° C and -11° C. The pH is adjusted to 4.8±0.05 by the addition of 38 ml of sodium acetate buffer. The plasma becomes milky white. This step takes 60-70 minutes and the plasma temperature is below -7° C throughout.

Precipitate V is removed by centrifugation for 15 minutes, at -6° C, 27000 x G and is resuspended to a volume of 3.0 liters with ice. This precipitate is mainly albumin. It contains 37.5 grams of total protein by Biuret determination (37.7 grams albumin by immunochemical assay and 0.0 gram IgG). Supernatant V contains 2.6 grams total protein (0.1 gram albumin and 0.0 gram IgG) in a volume of 2.18 liters. Its conductivity is 3.0 mS at 24° C.

The essential features such as total protein yields of IgG and albumin are summarized in Table I.

Example 2

Processing Data - Cohn Run #14 - Mode 1 and Mode 2 -Stabilized Human Plasma

Stabilized human plasma was prepared by the method outlined in U.S. Patents No. 3,998,946 and No. 4,136,094 (Condie). Stabilization of plasma results in the removal of fibrinogen, the clotting factors, the complement system, the kininogens, the lipoproteins, and the proteolytic enzyme plasmin plasminogen. Since it is free of fibrinogen, separation of Fraction I is not required. For the example given, the protein is recirculated through the outside of the hollow fiber and is referred to as Mode #1 (FIGURE 2a). When the addition of alcohol is accomplished with the protein on the inside of the hollow fiber, it is referred to as Mode #2 (FIGURE
2b). Rates of alcohol addition and concentration in plasma, temperature of ingoing and outgoing plasma before and after alcohol addition, and the temperature of the heat exchanging alcohol (ingoing and outgoing) are presented graphically in FIGURE 4.

Separation of Fraction II + III

Starting stabilized plasma (1.8 liters, 11.4 mS at 24° C, containing 120.1 grams total protein: 98.1 grams albumin and 11.4 grams IgG) was cooled to +1° C without permitting ice to form. Four milliliters of sodium acetate buffer (4.0 M HAc + 0.2 M sodium acetate, pH 4.0) was added to adjust the pH from 7.53 to 7.1 ±0.1 (all pH determinations are 1:5 dilutions of sample to 0.9 per cent saline). Alcohol (80 per cent) for addition is stirred in a closed tank at a temperature of -11.5° C. The plasma is stirred in a closed tank and temperature adjusted to desired conditions. The protein is recirculated through the outside of the hollow fiber (Mode #1) at a rate of 2500 ml/min. At the same time, the cold ethanol is recirculated through the inside of the fibers at a rate of 300 ml/min.

To obtain a final ethanol concentration in the plasma of 8 per cent, 178 ml of 80 per cent ethanol was added to the plasma by increasing the rate of the ethanol flow until 25 psi of back pressure is observed. Under these conditions the addition of alcohol was completed in 25 minutes. During this time the plasma ethanol concentration went from 0 per cent to 20 per cent, the plasma temperature dropped from 0° C to -5° C and the ethanol temperature climbed from -11.5° C to -5.5° C (see FIGURE 4-II + III).

When Mode #2 is used, the protein is recirculated through the inside of the hollow fiber at a rate of 400 ml/min. As the ethanol is recirculated through the outside of the hollow fiber, the back pressure due only to the plasma is 12 psi. A screw clamp is applied to the ethanol out line until the total back pressure reaches 20 psi. This addition takes 70 minutes. The ethanol tem-
-17-

temperature is kept below -5°C and the protein temperature is kept below -3°C throughout the addition.

Precipitate II + III was removed by centrifugation for 15 minutes, at -5°C, 27000 x G. Precipitate II + III was resuspended with ice to 1270 ml. This precipitate is mainly IgG. It contains 132. grams total protein (3.4 grams albumin and 8.4 grams IgG). Supernatant II + III contains 89.1 grams total protein (75.9 grams albumin, 2.5 grams IgG) in a volume of 2160 ml. The conductivity of Supernatant II + III is 5.0 mS at 24°C. The protein cooling tank and the hollow fiber outside are cleaned with distilled water and rinsed with 40 per cent ethanol.

Separation of Fraction IV₁

Supernatant II + III is placed back into the cooling tank and maintained at -3.5°C. The pH is adjusted to 5.4±0.1 with the addition of 12 ml of sodium acetate buffer. The supernatant is diluted to 18 per cent ethanol with the addition of 240 grams of distilled crushed ice which is allowed to mix until the ice is dissolved. This step takes 25 minutes. The addition of ice causes the plasma temperature to drop to -8°C (see FIGURE 4-IV₁).

Precipitate IV₁ is removed by centrifugation for 15 minutes, -6°C, 27000 x G. It is resuspended with ice to a final volume of 960 ml. Precipitate VI₁ contains 5.1 grams of total protein (2.0 grams albumin and 1.4 grams IgG). Supernatant IV₁ contains 86.3 grams of total protein (77.1 grams albumin and 1.0 gram IgG) in a volume of 2.34 liters. Its conductivity is 5.4 mS at 24°C.

The protein cooling tank is cleaned with distilled water. The 40 per cent ethanol is evacuated from the outside of the hollow fiber.

Separation of Fraction IV₄

Supernatant IV₁ is placed into the cooling tank and maintained at -6°C. The pH is adjusted to 5.8±0.05 by the addition of 17 ml of 1.0 M NaHCO₃. The supernatant is brought from 18 per cent ethanol to 40 per cent ethanol by the addition of 1294 ml of 80 per cent ethanol. The
same flow rates and back pressure are used as in separation of Fraction II + III. During this 55 minute addition, the plasma temperature is maintained between -6° C and -8.5° C as the ethanol temperature is kept below -9° C (see FIGURE 4-IV4).

When Mode #2 is used, the conditions are the same as in Fraction II + III Mode #1 except that the back pressure is allowed to reach 25 psi. The ethanol temperature is kept below -8° C and the protein temperature is kept below -5° C throughout the addition. The addition was adjusted to completion in 250 minutes.

Precipitate IV4 is removed by centrifugation for 15 minutes, -6° C, 27000 x G and is resuspended in ice to obtain a final volume of 1100 ml. It contains 19.9 grams of total protein (13.0 grams albumin and 0.9 grams IgG). Supernatant IV4 contains 64.6 grams of total protein (56.4 grams albumin and 0.0 gram IgG) in a volume of 3.40 liters. Its conductivity is 2.4 mS at 24° C.

The protein cooling tank is cleaned with distilled water and is separated from the hollow fiber. The hollow fiber and ethanol cooling tank can be cleaned at this time.

Separation of Fraction V

Supernatant IV4 is placed into the cooling tank and maintained between -6.5° C and -7.5° C. The pH is adjusted to 4.8±0105 by the addition of 33 ml of sodium acetate buffer. The plasma becomes milky white. This step takes 55 minutes and the plasma temperature is below -6° C throughout (see FIGURE 4-V).

Precipitate V is removed by centrifugation for 15 minutes, at -6° C, 27000 x G and is resuspended to a volume of 2.8 liters with ice. This precipitate is mainly albumin. It contains 57.4 grams of total protein according to Biuret assay (58.7 grams albumin by immunochromical assay and 0.0 grams IgG). Supernatant V contains 3.8 grams total protein (0.8 gram albumin and 0.0 gram IgG) in a volume of 2.56 liters. Its conductivity is 2.8 mS at 24° C.
The essential features concerning IgG and albumin yield for this run are presented in Table II.

Table III summarizes the IgG and albumin yields of five runs of cryopoor plasma and four runs of stabilized human plasma. The albumin yields averaged 69.5±3.1 per cent for the stabilized human plasma runs and 69.8±1.8 per cent for cryo-precipitated human plasma runs. The albumin isolated during these runs contained less than 2 per cent contamination by other plasma proteins.

The IgG Precipitate II + III contained appreciable concentrations of albumin with the yields of IgG being 76.6±7.5 per cent for stabilized human plasma and 73.9±4.3 per cent for cryo-precipitated human plasma. Reprocessing Precipitate II + III in this system following Cohn method 6 gave an essentially pure IgG with an overall yield based on the IgG present in starting plasma of 64.7 per cent.

---

Plasma Temperature Control During Ethanol Addition

There are major points to be made regarding the precise nature of temperature control that is routinely achieved in this system when adding and mixing concentrated ethanol to plasma. First, alcohol may be added through hollow fibers at controlled rates which can be very slow or quite rapid. FIGURES 3 and 4 illustrate that this addition can be controlled so that ethanol addition is at a constant, steady rate. Second, that regardless of the rate of addition the temperature of the plasma during ethanol addition can be rigorously controlled so that there is on the average 0.4° C differential between the temperature of plasma immediately before ethanol addition and immediately after mixing in the hollow fiber. This fine control of temperature is achieved and controlled by: (a) the alcohol temperature and alcohol flow rate through the hollow fiber, and (b) plasma temperature and plasma flow rate through the hollow fiber. As is clearly demonstrated in FIGURES 3 and 4, the alcohol serves as the major source of heat exchange and this control is enhanced.
by the design and construction of the hollow fiber system
when utilized according to this invention.

Time Required to Fractionate Plasma
According to Cohn Method 9

Another significant feature of the process is the reduction in time required to fractionate human plasma completely to albumin. As can be seen in FIGURE 3, the time required for each step is short with a total elapsed time being 10-1/2 hours, albumin yield averages 70 per cent with less than 2 per cent non-albumin contaminants.

When stabilized plasma is used (Example 2 and FIGURE 4), the total time is reduced to 8 hours and results in an essentially pure albumin with 70 per cent yields.

Example 3
Diafiltration

Diafiltration may be used to replace centrifugation and solvent removal. The following is an example of its use to process albumin eliminating centrifugation and solvent removal by other denaturing methods. After the addition of ethanol to 40 per cent, diafiltration may be used to concentrate the protein and to remove the ethanol. The process is identical to Examples 1 or 2 until after the addition of ethanol to a plasma concentration of 40 per cent.

The essential features of plasma temperature, plasma volume, protein concentration, and plasma ethanol concentration are presented in FIGURE 5. Prior to centrifugation, the plasma containing 40 per cent ethanol is concentrated from 3.45 liters to 1.88 liters. This process was adjusted for completion in 250 minutes. The protein temperature is kept below -3° C. The protein concentration of the plasma is increased from 26 mg/ml to 48 mg/ml. The plasma is pumped through the inside of the hollow fiber at a rate of 3000 ml/min which gives a back pressure reading of 25 psi. Filtrate removal proceeds at an average rate of 390 ml/hr at the temperature described.
The concentrated plasma is now centrifuged for 15 minutes, at -6° C, 27000 x G. The composition of Precipitate IV₄ and Supernatant IV₄ is the same as described for Examples 1 and 2. The volume of Supernatant IV₄, however, has been reduced to 1.70 liters at a protein concentration of 42.5 mg/ml.

Supernatant IV₄ is placed into the cooling tank and kept at -6° C. Instead of reducing the pH to 4.8 followed by centrifugation, the plasma is simply brought to 0 per cent ethanol by diafiltration with ice and cold water.

Begin recirculating the plasma through the inside of the hollow fiber until 25 psi of back pressure is observed. The flow rate of plasma will be 3000 ml/min. Add 1200 grams of distilled ice to the plasma to reduce the ethanol concentration from 40 per cent to 23 per cent. This addition increases the plasma volume to 2.95 liters. As the ice dissolves, concentration proceeds until the plasma volume is reduced to 1.95 liters. An additional 120 grams of distilled ice are added to the plasma to reduce the ethanol concentration from 23 per cent to 10 per cent. This addition increases the plasma volume to 2.95 liters. As the ice dissolves, concentration proceeds until the plasma volume is reduced to about 2.50 liters. The filtrate flow rate averages 900 ml/hr during the ice addition which is accomplished in 120 minutes.

Since the ethanol concentration in the plasma is reduced to 10 per cent, it is advantageous to allow the plasma temperature to increase above 0° C. The concentration rate of the plasma is highly temperature dependent and the rate of filtrate out increases from 900 ml/hr to 5800 ml/hr as the plasma temperature increases to +14° C. The reduction of the ethanol concentration from 10 per cent to 0 per cent is accomplished by adding 3.5 liters of cold distilled water to the plasma over a two hour period. Since the rate of concentration is greater than the rate of water addition, the plasma volume decreases to a final volume of 1.6 liters and a final protein concentration of 45.5 mg/ml. The resulting albumin is free of alcohol and
IgG, is in a concentrated state, and was never allowed to precipitate. Although the separation of albumin by diafiltration is illustrated, the same method may be used to remove and separate alcohol and other solvents from protein fractions from any step of the overall system.

It is apparent that many modifications and variations of this invention as hereinbefore set forth may be made without departing from the spirit and scope thereof. The specific embodiments described are given by way of example only and the invention is limited only by the terms of the appended claims.
The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A controlled rapid protein fractionation method which comprises:
   A) circulating a cold protein solution through a confined space defined by one side of a porous membrane,
   B) controlling temperature by circulating a liquid protein precipitant solution in contact with the opposite side of said membrane,
   C) Increasing the flow rate and pressure of the precipitant solution on the opposite side of said membrane to cause the protein precipitant solution to penetrate the membrane, mix with the protein from the solution along the first side of the membrane wall, and
   D) separating the precipitated protein particles.

2. A method according to Claim 1 wherein the temperature of the protein solution is controlled by circulating a cold protein precipitant solution.

3. A method according to Claim 2 wherein:
   A) the resulting supernatant is recooled,
   B) the pH, Na+ concentration and ionic strength of the supernatant are adjusted and the supernatant is recirculated through a confined space defined by one side of a porous membrane,
   C) the temperature is controlled by pumping a cold liquid protein precipitant solution in contact with the opposite side of said membrane,
D) the flow rate and pressure of the precipitant solution on the opposite side of the membrane is increased to cause the protein precipitant solution to penetrate the membrane, mix with the supernatant and precipitate further fine particles of protein along the first side of the membrane wall, and

E) the further precipitated protein particles are separated.

4. A method according to Claim 3 wherein:

A) the pH, Na+ concentration and ionic strength of successive supernatants are further adjusted and the supernatant is circulated in contact with one side of the membrane,

B) further cold protein precipitant solution is circulated in contact with the opposite side of the membrane to control the temperature of the supernatant,

C) the flow rate and pressure of the precipitant solution is increased to cause that solution to penetrate the membrane wall and precipitate further find protein particles along the first side of the membrane wall, and

D) the precipitated protein particles are separated successively until all desired fractions have been recovered.

5. A method according to Claim 3 wherein the protein containing solution is acidified by:

A) circulating an acidic buffer in contact with the opposite side of the membrane, and

B) increasing the flow rate and pressure of the buffer to cause the buffer to penetrate the membrane.
6. A method according to Claim 5 wherein:
A) the membrane is in the form of a plurality of small diameter hollow fibers,
B) one of said solutions is circulated through the fibers, and
C) the other of said solutions is circulated around the outside of the fibers.

7. A method according to Claim 1 wherein the precipitated protein particles are separated by centrifugation.

8. A method according to Claim 1 wherein the precipitated protein particles are concentrated and solvent is removed by diafiltration.

9. A method according to Claim 8 wherein:
A) a solution containing precipitated protein particles is circulated through a confined space defined by one side of a porous membrane,
B) another liquid is circulated on the opposite side of the membrane wall, and
C) the flow rate and pressure are increased on said solution to cause solvent therein to penetrate the membrane wall and concentrate the protein particles.

10. A method according to Claim 1 wherein:
A) said protein solution is blood plasma at an initial temperature between about 4°C and -7°C, and
B) said protein precipitant is ethanol in initial concentration between about 50 per cent to 80 per cent at an initial temperature between about -20°C to -10°C.
11. A method for the concentration of protein by the removal of organic solvents from protein solutions, which method comprises:

A) while maintaining a back pressure, adding ice to a cold protein solution, thereby increasing the volume of the solution reducing the concentration of protein and organic solvent, said ice functioning as a heat sink,

B) circulating the cold protein solution including organic solvent at a temperature at or near the freezing point of the solution through a confined space defined by one side of a porous membrane,

C) maintaining the flow rate of the protein solution to produce the back pressure on the membrane to decrease the volume of the solution to increase the concentration of protein by forcing dializable materials through the membrane,

D) gradually increasing the temperature of the protein solution to remove liquid including the organic solvent through the membrane to reduce the concentration of organic solvent in the solution, the passage of organic solvent through the membrane increasing as the temperature increases, and

(E) continuing until the organic solvent is removed.

12. A method according to claim 11 wherein an aqueous liquid is circulated in contact with the opposite side of the membrane.

13. A method according to claim 11 wherein the protein is a blood protein fraction and the solvent is alcohol.
14. A method according to claim 11 wherein the protein is a paste.

15. A method according to claim 11 wherein the protein is a powder.

16. A method according to claim 11 wherein the protein is a suspension.

17. A method according to claim 11 wherein the protein is albumin.

18. A method according to claim 13 wherein the alcohol is ethanol.

19. A method for the concentration of protein by the removal of salt solution from protein solutions, which method comprises:

   A) circulating a cold protein solution including a solution of an inorganic neutral salt through a confined space defined by one side of a porous membrane,

   B) maintaining the flow rate of the protein solution to produce a back pressure on the membrane to decrease the volume of the solution to increase the concentration of protein by forcing dializable materials through the membrane,

   C) while maintaining the back pressure adding water to the protein solution increasing the volume of the protein solution reducing the concentration of protein and salt,

   D) gradually increasing the temperature of the protein solution to remove liquid containing the salt through the membrane to reduce the concentration of salt in the solution, the passage of salt through the membrane increasing as the temperature increases, and
E) continuing until the salt is removed or is the osmolarity of blood.

20. A method according to claim 19 wherein water is circulated in contact with the opposite side of the membrane.

21. A method according to claim 19 wherein the protein is a blood fraction protein.

22. A method according to claim 19 wherein the protein is a paste.

23. A method according to claim 19 wherein the protein is a powder.

24. A method according to claim 19 wherein the protein is a suspension.

25. A method according to claim 21 wherein the protein is albumin.

26. A method according to claim 19 wherein the salt is selected from the group consisting of phosphates and sulfates.
EXAMPLE 2

FIG. 4
EXAMPLE 3

Concentration and Diafiltration

Temperature °C Plasma

-12
-8
-4
0
4
8
12
16

IN
OUT

SUPT IV → SUPT V

Diafiltration vs. ICE
Diafiltration vs. H₂O

(2400 g ICE)  (3350 ml H₂O)

1200 g ICE

Plasma Volume (ml)

3500
3000
2500
2000
1500

Protein Concentration (mg/ml)

48
44
40
36
32
28
24

% Plasma ETOH

40
30
20
10
0

Time (minutes)

0 100 200 300 400 500

FIG. 5
### I. CLASSIFICATION OF SUBJECT MATTER

According to International Patent Classification (IPC) or to both National Classification and IPC:

- **INT. CL.** B01D 13/00; C07G 7/00; C09H 7/00
- **U.S. CL.** 210/22R; 260/112R; 210/DIGEST 23

### II. FIELDS SEARCHED

<table>
<thead>
<tr>
<th>Classification System</th>
<th>Classification Symbols</th>
<th>Minimum Documentation Searched</th>
</tr>
</thead>
<tbody>
<tr>
<td>U.S.</td>
<td>210/DIGEST 23, 22R; 260/112B,R; 424/101,177</td>
<td></td>
</tr>
</tbody>
</table>

**Documentation Searched other than Minimum Documentation**

*to the extent that such Documents are Included in the Fields Searched*

### III. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of Document, <em>A</em> with indication, where appropriate, of the relevant passages <strong>B</strong></th>
<th>Relevant to Claim <strong>C</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>U.S.A, 4,136,094, PUBLISHED 23 JANUARY 1979, CONDIE.</td>
<td>1-26</td>
</tr>
<tr>
<td>A</td>
<td>U.S.A, 2,390,074, PUBLISHED 04 DECEMBER 1945, COHN.</td>
<td>1-26</td>
</tr>
<tr>
<td>A</td>
<td>U.S.A, 2,469,193, PUBLISHED 03 MAY 1949, COHN.</td>
<td>1-26</td>
</tr>
<tr>
<td>A</td>
<td>U.S.A, 2,770,616, PUBLISHED 13 NOVEMBER 1956, COHN ET AL.</td>
<td>1-26</td>
</tr>
<tr>
<td>A</td>
<td>U.S.A, 3,763,135, PUBLISHED 02 OCTOBER 1973, SHANBRON ET AL.</td>
<td>1-26</td>
</tr>
<tr>
<td>A</td>
<td>U.S.A, 3,764,009, PUBLISHED 09 OCTOBER 1973, WATT.</td>
<td>1-26</td>
</tr>
<tr>
<td>A</td>
<td>U.S.A, 3,869,436, PUBLISHED 04 MARCH 1975, FALKSVEDEN.</td>
<td>1-26</td>
</tr>
<tr>
<td>A</td>
<td>U.S.A, 3,998,946, PUBLISHED 21 DECEMBER 1976, CONDIE ET AL.</td>
<td>1-26</td>
</tr>
</tbody>
</table>

**X** "Shear Inactivation in Processing Biological Material" CHARM ET AL, DHEW PUB. NO. NIH 78-1422, PAGE 27.

---

*Special categories of cited documents:

**A** document defining the general state of the art
**E** earlier document but published on or after the international filing date
**L** document cited for special reason other than those referred to in the other categories
**O** document referring to an oral disclosure, use, exhibition or other means

**P** document published prior to the international filing date but on or after the priority date claimed
**T** later document published on or after the international filing date or priority date and not in conflict with the application, but cited to understand the principle or theory underlying the invention

**X** document of particular relevance

---

### IV. CERTIFICATION

**Date of the Actual Completion of the International Search**

29 AUGUST 1980

**Date of Mailing of this International Search Report**

1 SEP 1980

**International Searching Authority**

ISA/US

**Signature of Authorized Officer**

ETHEL R. CROS