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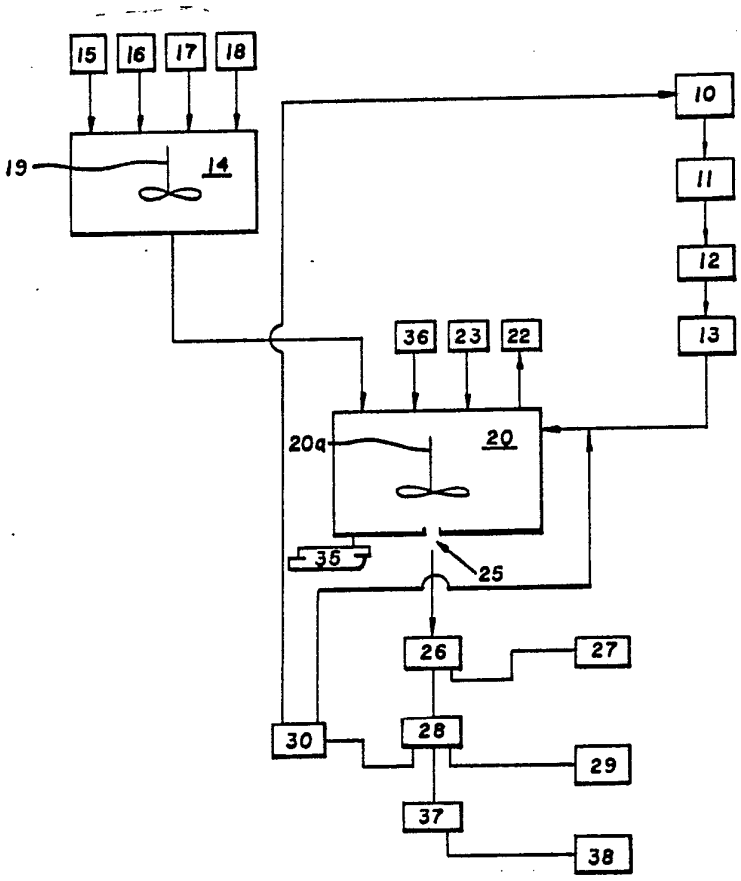
(54) Title: PREPARATION OF SYNTHETIC ERYTHROCYTES

(57) Abstract

The preparation of synthetic erythrocytes by forming a mixture of phospholipid and stroma-free hemoglobin and passing this mixture under pressure of from 500 to 10,000 pounds per square inch through an orifice to thereby form synthetic erythrocytes which contain stroma-free hemoglobin within membranes of phospholipid material. Also, subjecting the synthetic erythrocytes so formed to lyophilization to convert the liquid crystal structure of the erythrocytes to anon-crystal liquid which is capable of reconstitution into its liquid crystal form. Also, the recirculation of stroma-free hemoglobin not encapsulated within phospholipid membranes to be introduced again along with other stroma-free hemoglobin into mixture with lipids.



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PREPARATION OF SYNTHETIC ERYTHROCYTES

BACKGROUND OF THE INVENTION

Erythrocytes are the red blood cells of blood which serve the biological function of carrying oxygen to body tissues. In nature, the walls of the cells are membranes which contain many different kinds of proteins. Oxygen passes through these erythrocyte walls and is exchanged for carbon dioxide which the erythrocytes carry away from the tissues.

It has long been common in the practice of medicine to take blood from a donor and transfuse this into the blood circulatory system of a patient being treated. There are, however, difficulties in the preparation of blood for transfusion.

One difficulty is that the natural erythrocytes in the blood of animals and humans deteriorate relatively soon after the blood is drawn, and present regulations require that the blood must be used for transfusion within 21 days after it is drawn. Another serious inconvenience is that the blood of the donor must be typed and transfusions made into subjects whose blood is of the same type as that of the donor. Both of these disadvantages are due to the presence of the proteins which are contained within the membranes of the natural erythrocytes.

U.S. Patent No. 4,133,874 discloses a process in which a lipid in an organic solvent is spun to form a film on the interior walls of a container and this film allowed to dry. Stroma-free hemoglobin is added and by the use of ultra sound, hemoglobin is encapsulated within lipid membranes to form synthetic erythrocytes.

A principal object of the present invention is to discover improved methods by which synthetic erythrocytes may be produced effectively and efficiently and in quantities sufficient to supply medical needs. A further object is to discover processes for the encapsulation of stroma-free hemoglobin within

phospholipid membranes where the interaction of the lipids and hemoglobin to form synthetic cells is not limited to the surface of the lipid film. Another object is to discover processes which avoid waste of hemoglobin and produce a maximum quantity of synthetic erythrocytes from a given quantity of natural red blood cells. Yet another object is to discover processes for treating or modifying the synthetic erythrocytes to preserve them in a viable state for extended periods of time.

SUMMARY

We have discovered that it is possible to prepare stromafree hemoglobin from mammalian blood, combine this with a phospholipid composition, and pass this combination under pressure through an orifice to thereby encapsulate stroma-free hemoglobin within membranes of the phospholipid compositions so as to produce synthetic erythrocytes which, being free of the proteins contained in natural erythrocytes, remain useful over a much longer period than do the natural erythrocytes. Further, the interaction between the hemoglobin and the lipid is not limited to the surface of a lipid film on the interior of a container as was the case in the process described in Patent No. 4,133,874. Also, we have discovered ways in which waste of excess hemoglobin may be avoided by recirculating the unused hemoglobin; and though our synthetic erythrocytes when formed have long shelf life, we have discovered that, by changing the structure of the improved erythrocytes to a non-crystalline structure by lyophilization, we are able to extend still further the usable life of the erythrocytes. Details of these discoveries will be set forth as follows:

DETAILED DISCLOSURE OF INVENTION

One embodiment of our invention is illustrated in the accompanying drawing in which --

Fig. 1 is a schematic flow diagram of our process;

Fig. 2 is a view in cross section of one type of orifice which may be used to produce encapsulation of the stroma-free hemoglobin within phospholipid membranes;

Fig. 3 is a view in cross section of another type of orifice which may be used and

Fig. 4 is a sectional view of yet another type of orifice which may be used in the practice of the invention.

Referring first to Fig. 1, squares or blocks are used to represent steps or units of equipment. Beginning at the top of Fig. 1, the character 10 designates a container for a saline solution. Saline solution is passed through a centrifuge 11 used to separate plasma from whole blood. The blood may have been drawn from humans or from other mammals. The plasma is separated off, and the fraction containing the red blood cells, or erythrocytes, is passed to step 12 where the natural erythrocytes are subjected to alternate freezing and thawing to rupture the membranes of any remaining cells, after which the membranes and any tissue is removed by filtration, leaving stroma-free hemoglobin. The stroma-free hemoglobin may be held in the receptable 13.

Turning our attention now to the left-hand side of Fig. 1, there is a mixing vessel 14 into which a quantity of a phospholipid is fed from a container 15. This may be any kind of phospholipid, preferably lecithin. There is also added a sterol such as cholesterol from container 16, and dicetyl phosphate may be added from container 17 to give the mixture the desired electrical charge. An organic solvent may be added from the container 18. The phospholipid and other added ingredients are mixed, using mixer 19, and the

mixture fed into the pressure vessel 20. Associated with the pressure vessel 20 is a mixing device 20a, a vacuum pump 22 by the which the solvent may be evaporated and drawn off from this vessel, and a high pressure pump 23 capable of developing a pressure of at least 500 pounds per square inch and preferable at least 1500 pounds per square inch. We prefer that the pump be capable of producing pressures as high as can practicably be reached up to pressures of the order of 10,000 pounds per square inch.

After the mixture from the container 14 has been discharged into vessel 20, the vacuum pump 22 may be activated to evaporate and draw off the organic solvent. After the solvent has been withdrawn, the stroma-free hemoglobin from receptable 13 may be introduced into the vessel 20 and the mixer 20a operated to bring the ingredients within this vessel into a homogeneous mass.

The vessel 20 has means 35 for heating vessel 20 to maintain a temperature of about 35-40°C.

At the lower portion of vessel 20 is an orifice 25 which may be of the type illustrated in either of Fig. 1, Fig. 2, or Fig. 3. The type illustrated in Fig. 1 is the ordinary orifice in the wall of a vessel. The type shown in Fig. 2 is like that shown in Fig. 1 but with rounded corners at the edge of the orifice which causes the orifice to resemble that which is found in a common nozzle. The type shown in Fig. 3 resembles the orifice in a common valve where the valve may be opened slightly to produce an orifice in the form of a narrow crack.

The area of the orifice may vary, as a practical matter from as small as 0.001 square inches to 1 square inch. The smaller size orifice within this range will require a pressure applied to the mixture of phospholipids and hemoglobin to force it through the

orifice of at least 500 pounds per square inch; and with the use of larger orifices, pressures of from 1500 to 3000 pounds per square inch are appropriate; and with the use of orifices having an area greater than 0.5 square inch, even greater pressures are appropriate up to the limit of pumping equipment or near 10,000 pounds per square inch.

Quite unexpectedly, the mixture which passes from vessel 20 through the orifice 25 emerges in the form of hemoglobin encapsulated in membranes of the phospholipid composition. These are synthetic erythrocytes which are suspended in hemoglobin solution. They may be retained in receptable 26 and used as a product, or may be subjected to lyophilization at step 27.

The material from receptable 26 may also be washed by addition of saline solution, and the washed synthetic erythrocytes centrifuged and filtered at step 28. These may be used as a product, or may be subjected to lyophilization at 29.

The washed and filtered synthetic erythrocytes obtained at step 28 may be resuspended in the saline solution to which human albumin is added to form a plasma-like solution at step 37. This may be used as a product, or may be subjected to lyophilization at step 38.

The synthetic erythrocytes, when formed as a result of our processes as above described, are liquid crystals, but when they are subjected to lyophilization (freeze-drying), their moisture content is diminished. As the moisture content is reduced below 50%, based on the total weight of the synthetic erythrocytes, their structure changes to non-crystalline; and in this state, they are quite stable and have a very slow rate of deterioration. It was not previously known whether reconstitution to the liquid crystal form would be

possible; but we have found that when moisture is added and the moisture content comes to be in excess of 50%, they do revert to their original liquid crystal structure and function.

The diluted hemoglobin solution, obtained as a by-product of washing the synthetic erythrocytes at step 28, may also be passed to a concentrator 30. The concentrated stroma-free hemoglobin fraction of the solution may be reintroduced along with other stroma-free hemoglobin into vessel 20. The pure saline fraction of the solution may be recirculated back to 10 from which it is recycled again with whole blood.

The synthetic erythrocytes formed as a result of our processes typically have a size of from 0.01 to 10 microns at their largest dimension.

In a modified process, we may use a vessel 20 which is associated with a source of inert gas such as nitrogen. In this modified process, the nitrogen, or other inert gas, is pumped from source 36 into the vessel 20 and much of the gas is absorbed into the mixture of phospholipids and hemoglobin in vessel 20.

However, when the erythrocytes containing the absorbed gas emerge rapidly from the orifice 25 with a sudden drop in the applied pressure, they explode; and as a result, the cells previously formed are destroyed, but as a secondary result of the sudden drop in pressure, there is a violent foaming which reforms the synthetic erythrocyte cells containing the stroma-free hemoglobin within membranes of phospholipid material.

Following are specific examples which describe ways of carrying out our invention.

EXAMPLE 1

In the first part of the process, 40.7 grams of egg lecithin, 20.7 grams of cholesterol, and 6.9 grams of dicetyl phosphate were dissolved in 200 ml of organic solvent consisting of 9 volumes of chloroform mixed with

1 volume of methanol. This solution was poured into a high pressure reactor vessel provided with a stirrer. Organic solvent was evaporated by heating the solution at 40°C and by evacuation by means of a vacuum pump, until all solvent was removed, leaving the phospholipid material in the vessel.

In the second part of the process, stroma-free hemoglobin solution was made by freezing and thawing of 300 ml of washed and packed human erythrocytes, and separating stroma-free hemoglobin from membranous material.

In the third part of the process, 150 ml of stroma-free hemoglobin solution was added to the high pressure reactor vessel containing the phospholipid material made in the first part of the process. The phospholipid material and stroma-free hemoglobin solution was then thoroughly mixed in the vessel. The reactor vessel was then pressurized with nitrogen gas, at 2500 psi, for 15 minutes. The mixture was then expelled, under 2500 psi pressure, through a valve attached to the bottom of the reactor vessel into a receiving vessel. During the passage of the mixture through the valve, pressure was suddenly reduced from 2500 psi to atmospheric; nitrogen was released from the mixture; and very small spheroid particles were formed, from 0.5 to 1.0 microns in diameter, densely suspended in the stromafree hemoglobin solution. These particles represented the synthetic erythrocytes.

EXAMPLE 2

Synthetic erythrocytes, prepared as described in Example 1, were filtered through a filter with 1 micron pores. The resulting filtrate was diluted with the physiologic saline solution in 1:1 ratio by volume, then centrifuged at 4°C and 40,000 g for 30 minutes. The supernatant was discarded. The sediment was again diluted with the physiologic saline solution and

centrifugation was repeated. The sediment represented washed synthetic erythrocytes. The washed synthetic erythrocytes were then suspended, in 1:1 volume ratio, in a solution containing 5 gram percent human albumin dissolved in physiologic saline solution. This was synthetic erythrocyte suspension in albumin solution.

EXAMPLE 3

200 Grams of synthetic erythrocytes, obtained as in Example 1, were poured into a 1 liter flask. A vacuum pump was attached to the flask and vacuum pumped from the space above the mixture, at 4°C, until a sufficient amount of water was evaporated so that the remaining mass could not flow. This was the partially dried, unwashed synthetic erythrocyte suspension.

EXAMPLE 4

50 Grams of the partially dried, unwashed synthetic erythrocyte suspension, obtained in Example 3, was subjected to additional drying, at 4°C using a vacuum pump, until the mass was dried so that the remaining mass flaked off the sides of the flask. This was the completely dried, unwashed synthetic erythrocyte suspension.

EXAMPLE 5

200 Grams of synthetic erythrocytes suspension in albumin solution, obtained in Example 2, was poured into a 1 liter flask. A vacuum pump was attached to the flask and vacuum pumped from the space above the mixture, at 4°C, until a sufficient amount of water was evaporated so that the remaining mass could not flow. This was the partially dried, washed synthetic erythrocyte suspension.

EXAMPLE 6

50 Grams of the partially dried, washed synthetic erythrocyte suspension, obtained in Example 5, was subjected to additional drying, at 4°C using a vacuum pump, until the mass was dried so that the

remaining mass flaked off the sides of the flask. This was the completely dried, washed synthetic erythrocyte suspension.

EXAMPLE 7

In the first part of the process, 40.7 grams of egg lecithin, 20.7 grams of cholesterol, and 6.9 grams of dicetyl phosphate were dissolved in 200 ml of organic solvent consisting of 9 volumes of chloroform mixed with 1 volume of methanol. This solution was poured into a high pressure reactor vessel provided with a stirrer and a piston to produce high pressures. Organic solvent was evaporated by heating the solution at 40°C, and by evacuation by means of a vacuum pump, until all solvent was removed, leaving the phospholipid material in the vessel.

In the second part of the process, stroma-free hemoglobin solution was made by freezing and thawing of 300 ml of washed and packed human erythrocytes, and separating stroma-free hemoglobin from membranous material.

In the third part of the process, 150 ml of stroma-free hemoglobin solution was added to the high pressure reactor vessel containing the phospholipid material made in the first part of the process. The phospholipid material and stromafree hemoglobin solution were then thoroughly mixed in the vessel. The air was removed from the vessel with a vacuum pump. The reactor vessel was then pressurized with the piston to 2500 psi pressure. The mixture was expelled, under 2500 psi pressure, through a valve attached to the bottom of the reactor vessel into a receiving vessel. During the passage through the valve, the mixture was extruded producing spheroid particles, of approximately 0.5 microns in diameter, densely suspended in the stroma-free hemoglobin solution. These particles represented synthetic erythrocytes.

EXAMPLE 8

Synthetic erythrocytes, prepared as described in Example 7, were filtered through a filter with 1 micron pores. The resulting filtrate was diluted with the physiologic saline solution in 1:1 ratio by volume, then centrifuged at 4°C and 40,000 g for 30 minutes. The supernatant was discarded. The sediment was again diluted with the physiologic saline solution and centrifugation was repeated. The sediment represented washed synthetic erythrocytes. The washed synthetic erythrocytes were then suspended, in 1:1 volume ratio, in a solution containing 5 gram percent human albumin dissolved in physiologic saline solution. This was synthetic erythrocyte suspension in albumin solution.

EXAMPLE 9

200 Grams of synthetic erythrocytes, obtained in Example 7, were poured into a 1 liter flask. A vacuum pump was attached to the flask and vacuum pumped from the space above the mixture, at 4°C, until a sufficient amount of water was evaporated so that the remaining mass could not flow. This was the partially dried, unwashed synthetic erythrocyte suspension.

EXAMPLE 10

50 Grams of the partially dried, unwashed synthetic erythrocyte suspension, obtained in Example 9, was subjected to additional drying, at 4°C using a vacuum pump, until the mass was dried so that the remaining mass flaked off the sides of the flask. This was the completely dried, unwashed synthetic erythrocyte suspension.

EXAMPLE 11

200 Grams of synthetic erythrocytes, obtained in Example 8, were poured into a 1 liter flask. A vacuum pump was attached to the flask and vacuum pumped from the space above the mixture, at 4°C, until a sufficient amount of water was evaporated so that the

remaining mass could not flow. This was the partially dried, washed synthetic erythrocyte suspension.

EXAMPLE 12

50 Grams of the partially dried, unwashed synthetic erythrocyte suspension, obtained in Example 11, was subjected to additional drying, at 4°C using a vacuum pump, until the mass was dried so that the remaining mass flaked off the sides of the flask. This was the completely dried, washed synthetic erythrocyte suspension.

EXAMPLE 13

40 Ml of synthetic erythrocyte suspension in albumin solution, obtained in Example 2, containing 50% synthetic erythrocytes by volume was administered to a rat by a technique wherein an infusion pump was employed to effect simultaneous withdrawal of blood from the femoral artery and infusion of the synthetic erythrocyte suspension into the femoral vein. All 40 ml of suspension (approximately 250% of the rat's natural blood volume) was administered over a period of 3 hours. The rat survived the transfusion for more than 24 hours and eventually died of bacterial infection (septic shock).

We have described in the foregoing description certain modifications of our invention, but it is understood that our invention may be embodied in various forms and many changes may be made all within the spirit of the invention and the scope of the following claims.

1. A process for producing a synthetic erythrocyte preparation comprising, providing a stroma-free hemoglobin solution and a lipid composition, mixing said hemoglobin solution with said lipid composition, pressurizing said mixture to between about 500 p.s.i and about 10,000 p.s.i., and; passing said pressurized mixture through an orifice to thereby form the preparation of synthetic erythrocytes in which stroma-free hemoglobin is contained within membranes of lipid material, said preparation containing enough synthetic erythrocytes of a size greater or equal to 0.5 microns in diameter for said preparation to transport sufficient oxygen to sustain a mammal that is exchange transfused with said preparation.

2. A process in accordance with Claim 1 including lyophylizing said preparation to reduce the moisture content thereof to below about 50% by weight.

3. A process in accordance with Claim 1 including washing said preparation, filtering said preparation, and suspending said preparation in an albumin-containing saline solution to form a plasma-like suspension.

4. A dried synthetic erythrocyte preparation comprising erythrocytes having stroma-free hemoglobin encapsulated in lipid membranes, the moisture content of said erythrocytes being less than about 50 percent by weight, which synthetic erythrocyte preparation upon the addition of aqueous medium absorbs moisture to provide a synthetic erythrocyte preparation transfusable into a mammal.

5. A preparation in accordance with Claim 4 having sufficient hemoglobin encapsulated within said lipid membranes to provide a reconstituted synthetic

erythrocytes preparation which sustains a mammal exchange transfused therewith.

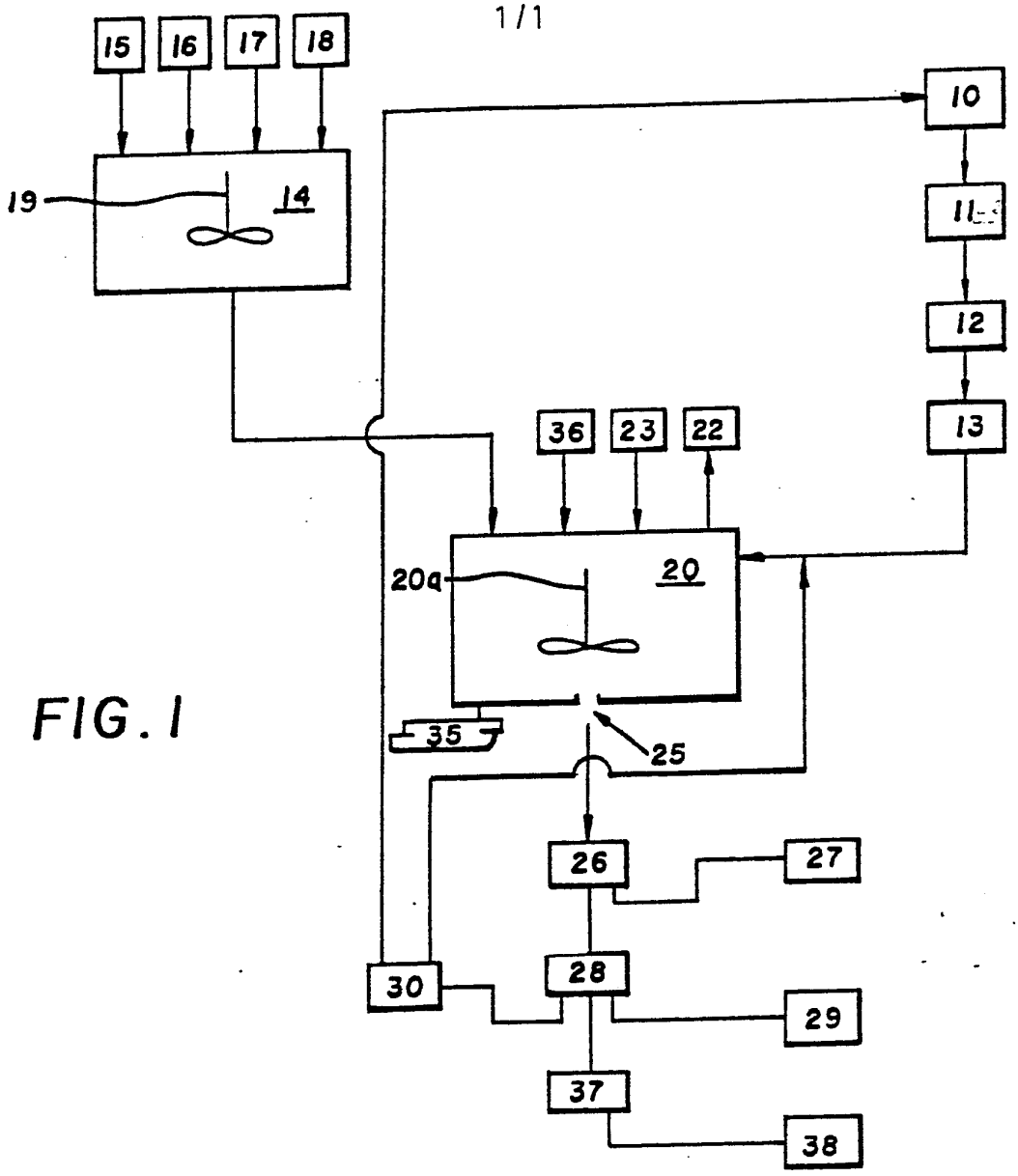

FIG. 1

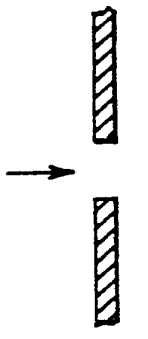

FIG. 2

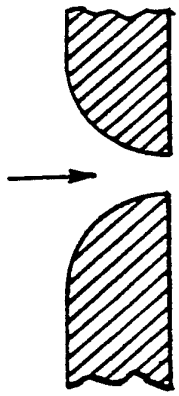

FIG. 3

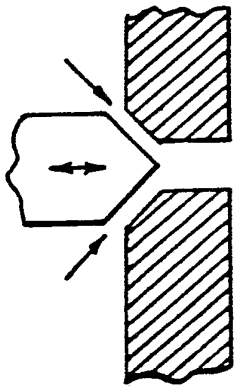

FIG. 4

INTERNATIONAL SEARCH REPORT

International Application No PCT/US82/00899

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

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

Int. Cl.[3] A 61K 37/00
US Cl. 424/101 ; 177

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
US	424/177 424/101

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

Chemical Abstracts: Vol. 76-95 Hemoglobin

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	US, A, 4,133,874 Published 09 January 1979 Miller	1-5
A	US, A, 4,061,736, Published 06 December 1977 Morris	1-5
A	US, A, 4,192,869, Published 11 March 1980 Nicolau	1-5
Y	Szoka, Ann. Rev. Biophys, Bioeng. Vol. 9 Published 1980 Pages 467,485+486	1-3

* Special categories of cited documents: [15]

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"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
05 October 1982	13 OCT 1982
International Searching Authority [1]	**Signature of Authorized Officer [20]**
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