Alternative Technologies

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PART 1: ALTERNATIVE SOURCES OF BLOOD PRODUCTS

Introduction

Substitutes and alternative sources of therapeutic blood components are likely to eventually make the entire spectrum of human blood products obsolete, but it will be at least 3 to 5 years before any one of these new products becomes commercially available. Within the next decade, virtually all plasma derivatives will be synthesized using recombinant DNA technology. Substitutes for blood's cellular elements such as red blood cells (RBCS) and platelets will be much more difficult to develop, but present research activities, although in their infancy, suggest that all of the problems are likely to be resolved. The most probable order of availability is that alternative sources of plasma derivatives will be developed first, followed by red cell substitutes, and eventually by platelet substitutes.

As in the current blood industry, there is a division within the development process of alternative blood sources and substitutes. Most work in cellular substitutes has concentrated on an RBC substitute, with the recognition that such a product may really be an additional transfusion product or adjunct solution, rather than a replacement for RBCs. Most of the research is being performed by academic investigators or at military research institutes, although commercial organizations are also involved, ranging from plasma fractionation companies (e. g., Alpha Therapeutics) and large industrial concerns (e. g., Monsanto Corp.), to small biotechnology firms which have focused on specific preparations (e.g., hemoglobin synthesis).

In contrast, nearly all of the research on new sources of plasma derivatives is being conducted by biotechnology companies. There is substantial R&D in progress, in which the goal is to clone clinically useful plasma proteins utilizing recombinant DNA technology.

Impetus to develop blood substitutes and alternate sources comes from several factors. First, there has been increasing use of component v. whole blood therapy, which means that blood substitutes need only duplicate the specific property of the desired blood product. Second, recent advances in biotechnology, particularly in the field of recombinant DNA technology, have suddenly raised the prospect that alternative sources for blood products, particularly plasma proteins, can be attained. There is therefore a new technology which can be exploited for its potential for profitable commercialization.

Another major impetus to develop these products lies in their anticipated safety. Frequent blood recipients, such as hemophiliacs, invariably develop viral hepatitis, and nearly 10 percent of all blood recipients develop some posttransfusion viral hepatitis. Blood-transmitted AIDS is the most recent safety problem, and substitutes and alternative sources would avoid the problem of blood-transmitted infectious diseases.

The costs of transfusion may also be lower, because such requirements as special storage conditions and compatibility testing may be avoided. Although the price of a unit of blood currently ranges from about **$35** to **$60,** this charge does not include a hospital crosshatch and/or transfusion fee, which can often double the total charge for a blood transfusion. "Artificial blood" offers the hope that the direct cost of transfusions will be less. And with the plasma derivatives, current R&D activities are in many cases based on the hypothesis that these products can be produced at far less cost then the blood-extracted products they would replace.

Current availability of blood products also varies considerably as a function of the supply of donors. Shortages still occur in the summer

months and around the Christmas holidays. On-site, resuscitative solutions in large quantities are needed for civil disasters and battlefield casualties. Although U.S. hemophiliacs now have access to adequate supplies of Factor VIII, very few hemophiliacs in developing countries receive or have access to this treatment.

Blood substitutes may also be preferred over blood-derived products, such as for patients who for various reasons will not or cannot receive blood from other individuals; or in organ or tissue perfusion, where the greater oxygen delivery capacity of a blood substitute may better maintain tissue viability.

In sum, most of the impetus to develop alternate blood sources and substitutes is based on economic, safety, and availability considerations. None of these products has yet reached the marketplace, and developmental efforts are still at the stage of laboratory investigations or animal testing; clinical trials with humans have not begun except for oxygen-transport substitutes. Thus, it should probably be at least 3 to 5 years before the first of these products will be approved for human use.

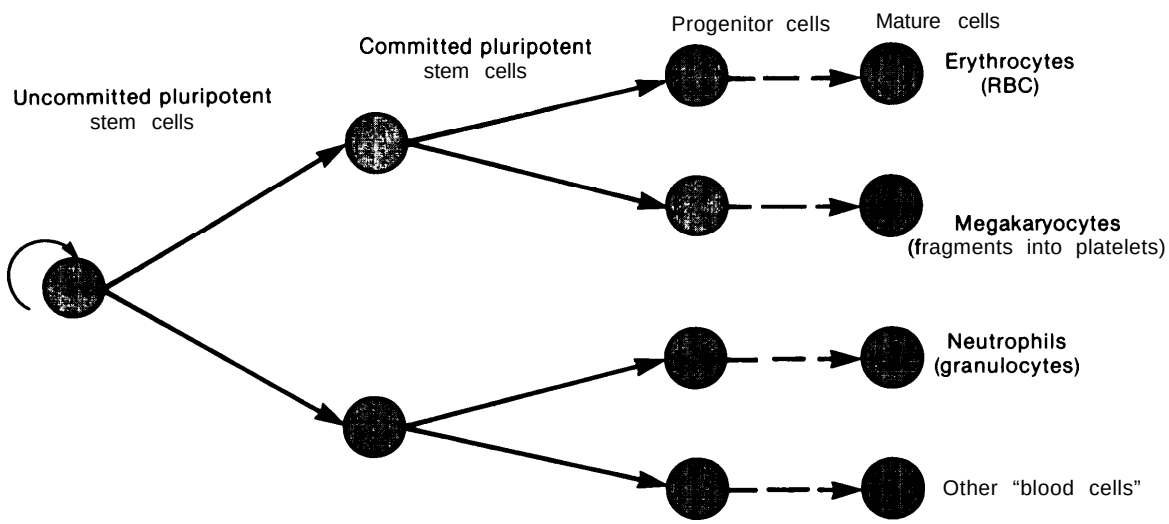
Stem Cell Culturing

Red blood cells, platelets, and granulocytes are mature blood cells that have a limited lifespan and are incapable of self-replication. Replacement of these cells takes place in the bone marrow, where less differentiated cells exist with capabilities to differentiate into different cell types. These "'stem cells" in the marrow possess both the ability to replicate (multiply) as well as to differentiate into specific hematopoietic cell lines (commitment). Figure 12 is a schematic diagram of stem cell replication and differentiation.

It is conceivable that stem cells could be grown in culture so that a continuous supply of cells for transfusion would become available. But at this time, stem cell culturing for the production of mature, transfusable blood cells is not possible.

Although it has been known for over a century that mature blood cells were derived from the bone marrow, it was not until 1961 that an assay was developed which proved that bone marrow cells are capable of forming colonies of hematopoietic progenitors (530). Other studies have conclusively shown that the colonies are derived from a single cell and that the responsible cell was a true multipotential stem cell (596). Having an extensive capacity for self-replication, stem cells comprise only 0.1 percent of the total cell population in the bone marrow. Stem cells also circulate in the blood stream, albeit in much lower concentration than in marrow. Thus, it is possible to obtain stem cells from two sites (marrow and

Figure 12.—Schematic Diagram of Cell Replication and Differentiation



SOURCE: R. Kahn, et al., *Alternate Sources,* 1984

blood), and their self-replicative capacity obviates the need for large numbers of cells to initiate a culture.

Stem cells have also been shown to differentiate and form colonies in tissue culture **(92).** Mouse stem cells that produced granulocyte progenitor cells and pluripotent stem cells have been cultured (159), and mouse bone marrow cultures have been induced to generate stem cells for more than a year (358), with documented red cell, granulocyte, and megakaryocyte (precursors to platelets) production.

The specific conditions needed in maintaining a long-term bone marrow culture vary with the species from which the cells are derived, but in general they include an optimum cell density, the proper nutrient medium, regular maintenance (i.e., changing the medium), and a specific incubation temperature. A number of investigators have successfully established long-term cultures of human bone marrow cells (135,213,266), but the culture methods developed are clearly suboptimal. The cultures survived for only 2 and 6 months; while hematopoietic differentiation was observed, it is uncertain whether there was significant stem cell replication. Differentiation also did not proceed along all cell lines. For example, mature, even functional, granulocytes have been produced (239), but mature megakaryocytes have not been seen.

The list of culture supplements is long and varies significantly between investigators (e.g., some use vitamins and others do not). Such culture supplements as fetal bovine serum, horse serum, and steroids have been used with very little knowledge of the active ingredient(s) or mechanism of action. The optimal temperature for growth is also unresolved. Some cultures require 330 C, while others are maintained best at 37°C.

Last, cultures of human stem cells have properties that cultures of mouse stem cells (which live far longer) do not, and it is uncertain whether the differences represent suboptimal conditions or are unique to the species used. For example, bone marrow cultures usually consist of a layer of cells adherent to the culture dish as well as a nonadherent layer. In the mouse system, stem cells are released from the adherent layer into the nonadherent layer, where they differentiate (158). This progression of stem cells from the adherent to the nonadherent phase is absent or significantly impaired in human cell cultures (135).

Future Directions

A considerable number of unresolved issues and problems must be addressed before stem cell culturing becomes an attractive method to produce transfusable blood cells. At the very least, much more needs to be learned regarding hematopoiesis in general. For example, there is little knowledge of the factors that tell the uncommitted stem cell to differentiate into progenitor cells, and then into the mature blood cell (124). At the very least, a complex series of cellular and environmental interactions govern the differentiation process (399,411). Some are known, such as the hormones erythropoietin for RBC production and thrombopoietin for platelet production, once progenitor cells have developed. Control mechanisms must be known in order to provide confidence that the cells produced are indeed normal, rather than products of an uncontrolled bone marrow malignancy. It will also be essential to determine those factors that govern stem cell replication as opposed to commitment, if mature blood cell production is to be sustained.

Even with greater understanding of the events governing hematopoiesis, several major problems remain. First, how will production be amplified from culture flasks producing millions of cells to production of sufficient numbers (10^{10} to 10^{12}) for transfusion? For example, the cumulative production of granulocytes in primary mouse marrow cultures reaches a maximum of 6×10^7 cells over a period of a year (10^6 cells / culture / week) (358), and therefore about 10,000 cultures would be necessary to generate 10^{10} granulocytes, or the number of cells found in one granulocyte transfusion product. It is conceivable, however, that maintenance of a culture for longer periods, accompanied by secondary culturing of the primary culture as well as by the addition of growth enhancement factors, could shorten production time considerably.

Second, how will the mature cells be harvested so that unwanted contaminants are eliminated?

Blood cells derived from a cell culture must be free of nutrient media, which contains proteins that can elicit an immune response in the recipient. Other supplemental factors (e.g., steroids) may also cause adverse reactions.

Third, will end-products of the culture be functional cells? It is likely that compatibility testing between "donor" and recipient will have to continue, thus negating the advantage of a universally compatible product, which other blood substitutes are likely to be. Last, there is evidence that long-term cultures will sometimes contain malignant cells (354). This would, at the least, require stringent precautions to purify the transfused product; it might prohibit use of those products altogether.

Stem cell culturing offers the potential of producing transfusable blood cells in a laboratory environment rather than from routine blood donations. This approach to blood cell production, however, is in its infancy; much more research is needed before an accurate assessment of its usefulness can be made.

Red Blood Cell Substitutes

Most of the research on substitutes for the cellular components of blood have focused on RBCs, with some research on platelet substitutes. Of the many different white cell populations, only the granulocyte is currently used for transfusion. As granulocyte transfusions are now used primarily for combating bacterial infections in an immunocompromised patient, the most practical substitute has been to administer antibiotics. The increasing availability of new, potent, and specific antibiotic preparations has contributed to the decline of granulocyte transfusions over the past few years, and development of other antibiotic preparations should obviate the need for granulocyte transfusions.

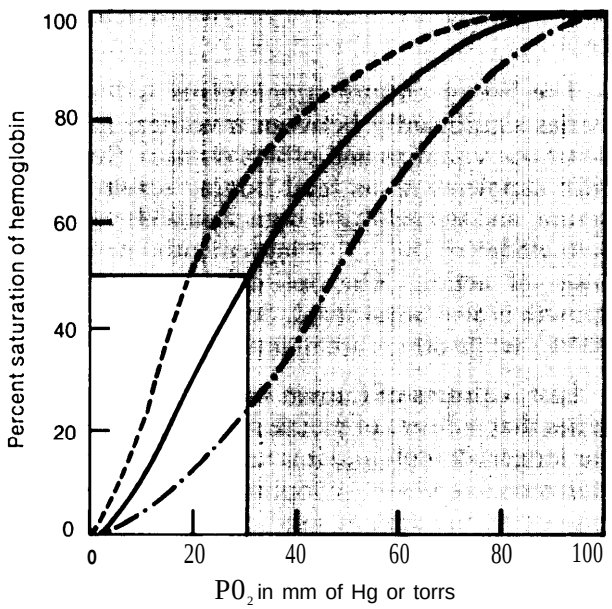
Function of Red Blood Cells

Red blood cells are biconcave discs that are, for all practical purposes, bags filled with hemoglobin. Each molecule of hemoglobin contains four polypeptide chains: two alpha chains and two beta chains. Each alpha chain has 141 amino acids and each beta chain 146 amino acids, and all four molecules comprise the globin portion of hemoglobin. Each of the four chains contains an iron atom to which oxygen binds in a reversible fashion. Thus, a hemoglobin tetramer binds four molecules of oxygen.

The four chains, which form a loose tetrameric structure, can readily dissociate to form two dimers. The formation of tetramers is favored when hemoglobin is contained within the red cell. When hemoglobin is free in solution, dimer formation is favored, which has poor oxygen binding characteristics.

The interaction between oxygen and hemoglobin in erythrocytes can be described by what is called an oxygen dissociation curve (fig. 13), which depicts the degree to which hemoglobin is saturated with oxygen. At so percent saturation (i.e., P_{50}), and under normal physiological conditions, the y-axis of the curve is near 26 mm of mercury, or 26 torr. When the affinity of hemoglobin for oxygen is increased and oxygen is therefore less able to dissociate from the hemoglobin molecule, the oxygen dissociation curve is shifted to the left and the P_{50} decreases. In other words,

Figure 13.—Oxygen Dissociation Curve



SOURCE Kahn, R., et al, *Alternate Sources,* 1984.

less oxygen is needed for 50 percent of the hemoglobin to be saturated, but oxygen is less able to be released from hemoglobin. Alternatively, if hemoglobin's affinity for oxygen is decreased and oxygen can more readily dissociate from hemoglobin, more oxygen will be needed for **50** percent of the hemoglobin to be saturated. This is represented by a shift in the oxygen dissociation curve to the right and an increase in the pressure of oxygen needed to saturate 50 percent of the hemoglobin with oxygen. A shift of the oxygen dissociation curve in either direction is unwanted, because the ability of hemoglobin to pick up oxygen in the lungs and to unload it in the tissues will be adversely affected.

The affinity of hemoglobin for oxygen is influenced by many factors. For example, pH, carbon dioxide levels and the intracellular molecule 2,3-DPG affect the position of the oxygen dissociation curve and hence the P_{50} value. A decrease in pH reduces the affinity of hemoglobin for oxygen and shifts the oxygen dissociation curve to the right. This change is referred to as the "Bohr effect" and is seen following substantial muscular action, which is associated with production of lactic acid. The decrease in pH helps in delivering oxygen to the exercising tissues. Carbon dioxide also binds to hemoglobin and, by displacing oxygen in the hemoglobin molecule, results in reduced affinity of hemoglobin for oxygen and a shift of the oxygen dissociation curve to the right.

Physiologically, however, the molecule **2,3-**DPG has the most important influence on P_{50} levels. It decreases the affinity of hemoglobin for oxygen (right shift) by shifting the equilibrium between the deoxygenated and the oxygenated forms of hemoglobin, It stabilizes the deoxygenated form, causing oxygen to be released. However, when the oxygen concentration is high—as it is in arterial circulation— the equilibrium is shifted toward the oxygenated form of hemoglobin, thereby negating the influence of 2,3-DPG. At low oxygen pressure (e. g., in tissues), 2,3-DPG displaces the oxygen on hemoglobin, causing oxygen to be released.

The function of hemoglobin can be altered in many ways that ultimately reduce its ability to transfer oxygen. For example, the iron of the heme group can be oxidized from the ferrous to the ferric form, producing methemoglobin, which is incapable of combining with oxygen. Such oxidization readily occurs to hemoglobin in solution, but protective effects within RBCs prevent this alteration from happening. Hemoglobin function also decreases when RBCs are stored, mainly because of a reduction of 2,3-DPG, thereby causing the P_{50} to decrease (left shift). Fortunately, 2,3-DPG levels are largely regenerated through metabolic processes within the first few hours following transfusion.

Although a left-shifted oxygen dissociation curve indicates a decreased capability of hemoglobin to unload oxygen, its occurrence does not necessarily mean that tissue oxygenation will be adversely affected. The body can effectively compensate for decreased oxygen availability by increasing cardiac output, heart rate, or the efficiency with which tissues can extract oxygen.

While oxygen is chemically bound to hemoglobin, it also is physically dissolved in plasma, but the concentration of oxygen in plasma is far less than in oxygenated hemoglobin, and plasma is normally an inefficient medium for oxygen delivery to tissues.

The ideal RBC substitute should have six properties: 1) an oxygen dissociation curve and oxygen-carrying capacity similar to that of intact RBCs; 2) be nontoxic and nonantigenic; 3) have good flow characteristics; 4) remain in the circulation for a long period of time; 5) have a long shelf life; and 6) be cost effective in comparison to present RBC transfusions.

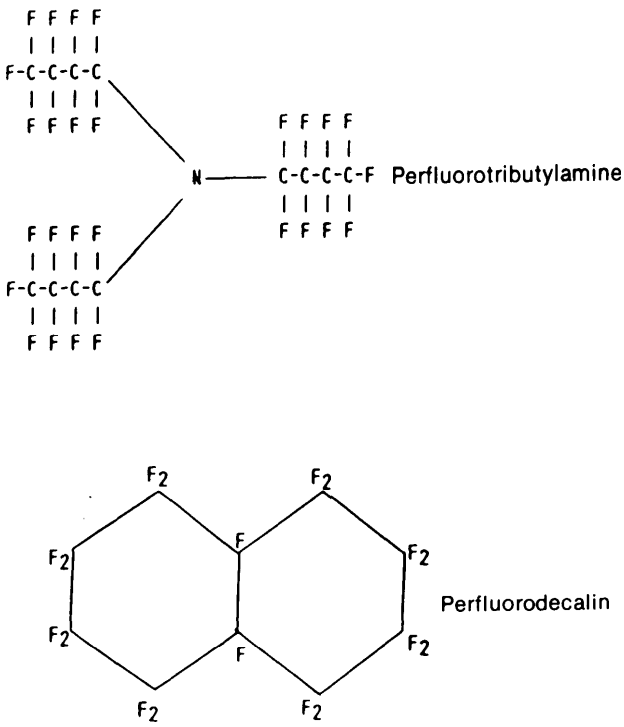
While no substitute as yet fulfills all of these criteria, four approaches to achieving the ideal substitute have been explored. One approach is to utilize a class of synthetic compounds called perfluorochemicals. Purified hemoglobin has also been chemically modified to prolong its circulation and enhance its oxygen binding/dissociation properties. A third approach has been to synthesize analogs of hemoglobin that, as in the native molecule, chemically bind oxygen. Last, investigators have attempted to assemble a red cell by encapsulating hemoglobin in lipid vesicles called liposomes.

Each of these substitutes has problems and drawbacks, some in common with one another and some unique to a particular substitute. Nevertheless, at least one clinically useful RBC-like preparation should become available by the close of this century.

Perfluorochemicals

Perfluorochemicals (PFC) are compounds in which all the hydrogen atoms have been replaced by fluorine atoms (fig. 14). They are chemically inert and not metabolized by the body. Unlike other RBC substitutes, PFC transports oxygen only in solution. Because the molecules of PFC do not strongly interact with one another, they have more room for gas to occupy the spaces between each atom and molecule. Thus, PFC can dissolve 40 to 70 percent oxygen per unit volume, almost three times the oxygen-carrying capacity of blood. It is this relatively high volubility of gases that make these compounds attractive as red cell substitutes.

Figure 14.– Perfluorochemicals



SOURCE: R. Kahn, et al., Alternate Sources, 1984

The concentration of physically dissolved oxygen in a PFC solution is linearly related to the concentration of the PFC in the solution. As the PFC concentration increases, its contribution to oxygen delivery and consumption also increases (235). However, the oxygen-carrying capacity of a PFC is also directly proportional to the concentration of oxygen in the environment. At room air oxygen levels, PFC will hold little oxygen; it therefore must be given in a high oxygen environment in order to be effective.

Pure PFCs, however, are immiscible with blood, and, without any alteration, would act as an embolus when injected into the bloodstream. Since PFCs are not easily converted to finely dispersed stable emulsions, they must be emulsified to produce a very low viscosity and surface tension. PFCs can be emulsified with surfactants known as Pluronics, and in this form they can be mixed with blood. Because PFC must be used in an emulsified form, formulation and preparation of emulsions are at the center of studies on these blood substitutes. The emulsifying agent most widely used in dispersing PFC is Pluronic F-68. The preparation has the appearance of either a clear solution or a suspension resembling diluted milk, and the size of the particles can be as small as 0.1 to 0.2 microns, or 1/70 the size of an RBC.

The emulsification process and the size of the particles produced are important for stability and potential toxicity. PFCs that are rapidly eliminated from the circulation do not form stable emulsions, and conversely, those PFCs that can be easily emulsified have been found to have long body retention times. Since the body does not metabolize PFC, those particles not excreted have been shown to aggregate in the liver and spleen.

In an effort to find a PFC that is both stable in emulsion and rapidly cleared from the body, there has been a systematic effort to screen a great number of PFCs for their utility as blood substitutes. In 1973, a combination of two PFCs were formulated, one with a short half-life in the body, and the other with a much longer half-life. It was this new PFC combination that was distributed by the Green Cross of Japan to its American subsidiary, Alpha Therapeutics, and used in the United States in recent patient studies. Called Fluosol-DA, it consists of 20 percent (weight to

volume) PFCs; perfluorodecalin (14gm / dl), with a half-life in the body of 7.5 days but with only a moderate emulsification capacity, and perfluorotripropylamine (6gm / dl), with a half-life of 65 days but with excellent emulsification.

Among perfluorocarbon preparations, Fluosol-DA is the most thoroughly studied and received the widest publicity. Besides containing the two PFCs and the emulsifying agent Pluronic F-68 in a balanced salt solution, hydroxyethyl starch is added to maintain blood volume and to enhance the flow characteristics of the solution (table 42) (219). The preparation has a circulation half-life of about 13 hours and a tissue half-life of 9 days as measured in animals (218).

Because they are biologically inert, PFCs contain no antigens, and therefore typing or cross-hatching is unnecessary. The emulsions may be frozen and refrozen, and they can be autoclave for sterility. They are also easily synthesized from readily available materials.

Other than as a substitute for red blood cells, PFCs have advantages in other areas of medicine. For example, carbon monoxide interferes with oxygen transport because hemoglobin's affinity for carbon monoxide is 250 times greater than its affinity for oxygen. In contrast, PFCs do not carry carbon monoxide at all, and PFC administration could provide oxygen to a carbon monoxide victim until the patient replaced his abnormal red cells. Another potential use for PFC relates to their small particles, which can easily penetrate vessels that have been constricted, such as in cerebral ischemia, myocardial infarction, or by abnormal blood cells (e.g., sickle cell crisis).

Table 42.—Composition of Fluosol-DA (20°/0)

Perfluorodecalin	14.0[a] %
Perfluorotripropylamine	6.0
Pluronic F-68	2.7
Yolk Phospholipids	0.4
Glycerol	0.8
NaC1	0.6
KCL	0.034
$MqCL_2$	0.02
$CaCL_2$	0.028
$NaHCO_3$	0.210
Glucose	0.180
Hydroxyethyl starch	3.0

[a]Composition expressed as percentage of weight to volume.

SOURCE: T. Mitsuno, H. Okyanagi, and R. Naito, "Clinical Studies of a Perfluorochemical Whole Blood Substitute (Fluosol-DA)," *Ann. Surg.* 195:60-69, 1982.

Uptake of oxygen by PFC in the lung depends entirely on the concentration of oxygen in the alveoli. PFC does not preferentially extract oxygen from the air as hemoglobin does, and the oxygen level in a PFC solution equilibrates with the oxygen level in the atmosphere. Since PFC preparations at ambient oxygen levels (air contains 21 percent oxygen) will carry very little oxygen, PFC therapy requires that the patient be given concurrent administration of 60 to 100 percent oxygen in order to deliver enough oxygen to the tissues.

PFC administration in a high oxygen environment is required because the highest achievable concentration of PFC in blood is about 20 percent; higher concentrations are unstable. Consequently, in order to efficiently utilize the oxygen carrying / delivery capacity of PFC, the patient must be exposed to the risks of a high oxygen environment, which can result in highly reactive oxygen metabolizes that can in themselves inactivate cellular enzymes, damage DNA, and destroy cell membrane architecture (195). The concentration of oxygen in inspired gas (i.e., the FiO_2) at which significant damage after short-term exposure (24 hours) is first seen is about 70 percent, which is only slightly greater than the minimal concentration of 60 percent oxygen that has been successfully used for PFC administration (233). Thus, the advantages of PFC must be weighed against the potential toxicity of the high oxygen environment in which they must be given. This cumbersome and costly requirement for PFC administration may prove to be the limiting factor in its use as a substitute for blood transfusion.

Animals have also been known to survive a total exchange transfusion if they inspired 100 percent oxygen without PFC infusion (233). This demonstrates that plasma can be a very effective oxygen carrier at high oxygen concentrations, and questions whether the benefits seen with PFC infusion resulted from the use of this material or were merely due to the animals' breathing a high concentration of oxygen.

Another disadvantage of PFC emulsions relates to the need to freeze them for storage because of the emulsion's instability. A surfactant that will make stable emulsions at room temperature with a variety of PFCs has recently been studied (123). The new emulsifier also enabled the PFC concentration to be increased to over so percent, compared to current PFC concentrations of 20 percent.

Clinical studies.—The first human volunteers were given the PFC emulsion, Fluosol-DA, in 1979. Initial clinical studies were performed in Japan and reported a high degree of safety and efficacy (272,384,413).

In April 1980, Alpha Therapeutics Corp., the American subsidiary of Green Cross of Japan, began clinical trials of Fluosol-DA in California. Seven severely anemic patients were given Fluosol-DA before surgery to determine its effectiveness in supplementing oxygen transport. When the patients breathed low levels of supplemental oxygen (slightly more oxygen than ambient air levels) the PFC carried only a small amount of oxygen, as expected, and had virtually no benefit. When patients received pure oxygen, their arterial oxygen content rose significantly and the PFC provided **24** percent of the oxygen consumed (537).

Japanese experience with Flurosol-DA and reports from some American investigators have generally given the substitute high marks for safety and efficacy. But the frequency of such reactions from Fluosol-DA administered to Japanese patients has been significantly less than reported in Americans. This could be due to a number of factors such as racial differences (e.g., lipoprotein levels differ between Japanese and Americans), different patient populations, medical practices, or monitoring procedures. Nevertheless, there is a definite reluctance to extrapolate the Japanese experience with Fluoso1-DA to American usage. Moreover, in the last 2 years an increasing number of reports have begun to document a variety of toxic affects attributed to its administration.

Adverse effects.—PFCs are chemically inert and do not appear to be degraded biologically. Their principal avenue of escape from the body is via the lungs and to a small extent through the skin. Because of the particulate nature of the emulsion, however, it was suspected, and later confirmed, that clearance also involved the reticuloendothelial system (RES). Both animal and human studies have shown that some of the material is retained by the liver and spleen for at least 2 years after a single administration (312). Small PFC particles coalesce intracellularly, but with time these larger particles undergo further change to become smaller again. Most droplets of PFC eventually disappear. The exact mechanism(s) by which PFCs move from the RES to the lung, or from the lung to the airway, is unknown at present. Macrophages (a type of scavenger white blood cell) may play a role, since they readily take up PFCs and could carry the material to the lungs.

Because the RES is involved in PFC clearance, it was anticipated that saturation of the RES would reduce the body's ability to clear other foreign substances, notably bacteria or viruses. This hypothesis was confirmed in mice challenged with a bacterial toxin simultaneous with PFC administration, in which the lethality of the toxin rose nearly eightfold (350). These results suggested that PFC administration would be contraindicated in patients who are likely to have infections (e.g., trauma patients) or a weakened resistance to infection.

Of greater concern, however, and as yet unaddressed, is the possibility of chemical carcinogenesis as a result of the long retention time. Although there is no data that would support such a hypothesis, the fact that these chemicals remain in the body for some time is disturbing, and the issue will have to be addressed before general use is permitted (197).

Early studies on the effect of PFC infusion occasionally noted pulmonary reactions, which were attributed to a variety of physical properties of the chemicals (122,218,502) that affected either platelets or plasma proteins (334). Recent evidence, however, suggests that alterations in the immune system may be the triggering event. The transient hypoxemia and pulmonary congestion seen upon Fluosol-DA infusion (537) is similar to the effect of infusing activators of the body's complement system. Stimulation of white blood cells is also often associated with pulmonary reactions. Thus, it seems likely that PFC emulsions cause

pulmonary dysfunction by activating complement and/or white cells. Release of a substance(s) from damaged leukocytes may then result in both leukocyte and platelet aggregation.

The first patient treated in the United States with Fluosol-DA developed a pulmonary reaction after receiving **30** milliliters of material over 15 minutes; no reaction occurred with a subsequent infusion after premeditation with steroids (572). Laboratory studies indicated that Fluosol-DA activated the complement system, thereby resulting in microphage aggregation, which was a reasonable explanation for the pulmonary reaction (chest tightness, shortness of breath) that was seen upon infusion. Further studies suggested that the component of Fluosol-DA responsible for the effect was the emulsifying agent, Pluronic F-68. This finding is not unexpected, since PFCs are very unreactive and, in emulsified form, are probably coated with the surfactant. It is therefore likely that any interaction with blood components involves the surfactant rather than the PFC itself.

The recent effect of another commercially available PFC emulsion on macrophages was studied and the material found to be cytotoxic to these cells (101b). Although the study was performed on cells kept in culture, where exposure to PFC is likely to be higher than in actual use, it reinforces concern over the effect of these compounds on the body's immune system. More evidence of toxicity is the finding that PFC emulsions can disrupt the phagocytic function of peripheral blood neutrophils and monocytes (574). These reports clearly suggest that further studies are needed to clarify the mechanism and consequences of the leukocyte dysfunction that are seen upon exposure to PFC preparations, and that more detailed evaluations of the effects of PFC on host immune defenses are also needed.

Concerns surrounding use of PFCs as blood substitutes prompted the Blood Products Advisory Committee of the FDA to disapprove Fluosol-DA for human use at one of its recent meetings (561). The committee heard presentations from the manufacturer of the product, including a summary of the results obtained from approximately 100 patients in the United States and Canada who were given Fluosol-DA under an Investigational New Drug (IND) permit issued by FDA. Although few reactions were noted in these patients, all of the subjects were premeditated with relatively high doses of corticosteroids.

The committee's concerns over the possible masking of immune reactions by steroid administration, the potential toxicity of the high oxygen environment in which the PFC was administered, the relative paucity of data regarding the effect of Fluosol's emulsant (Pluronic F-68) on the recipient's own blood cells and tissues, and the lack of convincing data that PFC administration to anemic patients was efficacious, were the key factors that led to the committee's decision (197,383).

Other emulsions having more favorable characteristics than Fluosol-DA (599) are being developed. Current PFC emulsions appear unsatisfactory for use as a blood substitute; their safety is far from established and their utility is hampered by the high oxygen environment in which they must be given, and a PFC that is stable as an emulsion at room temperature and that is quickly excreted needs to be developed.

Even if these issues can be resolved, it seems unlikely that clinicians will choose daily infusions of a PFC emulsion until the patient's red cell mass returns to normal over a red cell transfusion. The high oxygen environment requirement precludes use of PFC transfusions in the battlefield or at the site of an accident and would even discourage its use in a hospital setting. Resolution of the safety and efficacy issues may lead to use of PFCs for specific indications such as in patients with religious objections to blood transfusion, in carbon monoxide poisoning, sickle cell crisis, or cerebral ischemia. If its use is limited to these clinical situations, PFC emulsions will never have a significant effect on the blood transfusion industry.

Hemoglobin Solutions

Early studies on the use of unpurified hemoglobin solutions were disappointing in that their administration to animals and man were often associated with renal abnormalities and coagulation defects (10,56,93,251,453,454,498), although the effects appeared to be dose-related (175,224, 381,418,492).

Removal of RBC membrane fragments (i.e., stroma) from hemolyzed RBCs to produce "stroma-free" hemoglobin eliminated many of the renal and coagulation problems (74,75,125,455).

Stroma-free hemoglobin is produced by slowly lysing washed RBCs with a buffered solution of water (rather than unbuffered water), followed by high speed centrifugation and micropore filtration (424,454,455).

To further purify a hemoglobin solution, other investigators have altered the conditions of hemolysis, or the centrifugation and filtration steps (95,155,240,257,396,490).

Although it is possible to prepare at least limited quantities of a hemoglobin solution that has no known contaminants, questions regarding the safety of the preparation still remain. Coagulation abnormalities, renal dysfunction and alterations in cardiovascular function appear to be the most common effects (83,197), but do not always appear together. Liver necrosis is sometimes observed.

Severe hematologic alterations have occurred in the first few hours after infusion of purified hemoglobin in dogs, but not in monkeys and pigs (82). These results have raised many disturbing questions. Is it possible that contaminants below detectable levels can still cause adverse reactions? If so, how will the safety of a clinically useful preparation be determined prior to its infusion? Second, what animal species should be used for extrapolation to humans? Differences in response between species make interpretation of safety data confusing and the choice of animal model for experimentation extremely important. Variations in response within animal species have also been seen. For example, infusion of a presumably pure hemoglobin solution into a group of rabbits has caused severe, acute toxicity (even sudden death) in some of the animals, but no adverse effect in others.

Variations in the response to infusion of a hemoglobin solution into eight normal volunteers have been reported (477), and other investigators have noted unexplainable variations in the toxicity seen between preparations that were made using the same recipe (83). Such findings have recently prompted the Army Research and Development Command, the sponsor of the vast majority of research on hemoglobin solutions, to issue a request for proposals to obtain a supply of hemoglobin solution in sufficient quantity to study the nature of the interspecies variability, and to determine those factors which result in batch-to-batch variability.

If presumably "pure" hemoglobin can cause adverse reactions, is it possible that hemoglobin itself can be toxic under certain conditions? Hemoglobin has been shown to activate the body's immune system (i.e. complement system), although it was possible that the preparation used was impure (589). It is also possible that the configuration of hemoglobin in solution is conducive to binding bacterial endotoxins, either in vitro (during preparation of the solution) or in vivo as the molecule passes through the gastrointestinal circulation (357). Unpublished observations (83) suggest that endotoxin may indeed be the primary factor contributing to the toxicity seen.

The fact that inter- and intraspecies variability exists emphasizes the need for standardization and complete documentation of the methods used to prepare the hemoglobin solution. Careful selection of the species used for testing and attention to the conditions of the experiment are also key issues. Unfortunately, the literature on this subject is, in general, much less thorough and exact. A wide variety of species under an equal variety of experimental settings have been utilized, with hemoglobin solutions whose preparation and properties are incompletely described.

Fate of infusion.—In addition to obtaining a nontoxic solution, other significant problems have been that: 1) free hemoglobin normally persists in the circulation only a short time with a half-life of only 2 to 4 hours (75,87,455,477); 2) free hemoglobin has a significant oncotic effect; and 3) its affinity for oxygen is unacceptably high.

The short circulation time of free hemoglobin is due to the tetrameric form of the molecule easily dissociating into dimers and monomers, with rapid clearance of these subunits by the kidney. Hemoglobin also readily binds to the plasma protein, haptoglobin, which is then rapidly cleared

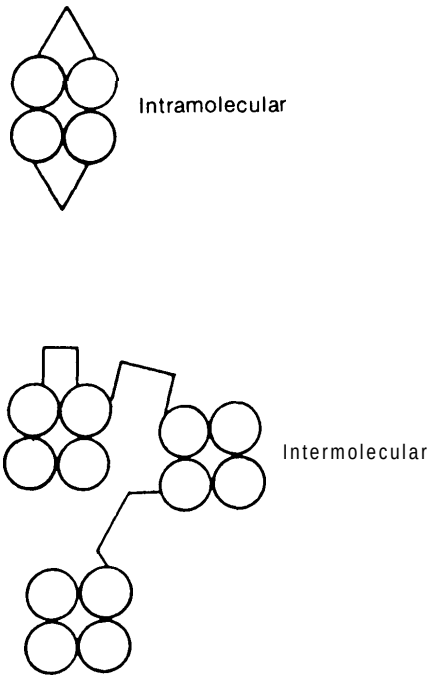
by the reticuloendothelial system, primarily the liver (101a,212). If the amount of hemoglobin transfused exceeds the haptoglobin binding capacity, unbound hemoglobin is filtered through the kidney, where it is reabsorbed. If the filtered amount exceeds the reabsorptive capacity of the kidney, hemoglobin then appears in the urine.

Free hemoglobin's significant oncotic effect severely limits the maximum amount that can be given. While the concentration of red cell enclosed hemoglobin in blood is 14g/dl, only 7g/dl of free hemoglobin can be administered without exceeding the normal oncotic pressure of plasma. Increasing the amount infused to achieve the oxygen-carrying capacity of the blood lost will raise the risk of volume overload in the recipient. Therefore, significantly less oxygen transport capacity is attainable with hemoglobin in solution compared to an equal volume of blood, and it cannot be used as a 1:1 replacement fluid.

Several approaches have been taken to stabilize the hemoglobin molecule and thus slow its disappearance from the circulation, as well as to decrease its oncotic effect so that more can be given. One way is to crosslink (or "modify") hemoglobin molecules (386) either intermolecularly or intramolecularly (fig. 15). The former type of crosslinking produces hemoglobin of high molecular weight, while the latter stabilizes the tetrameric form of the molecule. Although the intravascular half-life using either method is prolonged (to 15 to 30 hours) and the oncotic effect of the polymer becomes similar to that of hemoglobin in RBCs, the oxygen affinity of modified hemoglobin remains extremely high, and therefore its ability to deliver oxygen is still greatly impaired.

Another potential problem with many modifying agents is that hemoglobin molecules become crosslinked both intra- and intermolecularly, resulting in a wide assortment of hemoglobin polymers of different sizes. Thus, batch-to-batch reproducibility of a preparation is extremely difficult, and the ability to determine which polymers, if any, are undesirable is a difficult task. Recently, however, crosslinking agents have been found that result in well-characterized, reproducible polymers of hemoglobin. One such agent is the adenine nucleotide, adenosine triphosphate (479).

Figure 15.-Crosslinked Hemoglobin



SOURCE: R. Kahn, et al., *Alternate Sources,* **1984**

Another new crosslinking agent is the chemical 3,5 dibromosalicyl-bis-fumarate (540), which is nontoxic, inexpensive, and capable of crosslinking hemoglobin at specific sites in a reproducible manner, and thus is attractive for high-volume production of a modified hemoglobin solution.

Another approach to stabilize hemoglobin is to attach it to a larger molecule which persists in the circulation for longer periods (113,525,526). Hemoglobin has been attached to the sugar, dextran, with longer circulation times, but the relatively high viscosity of the complex severely limits the amount that can be given (113,525,526).

Another major drawback of native hemoglobin is its high affinity for oxygen. Hemoglobin itself has a P_{50} of 13 to 18 torr, whereas the red cell P_{50} is 26 to 28 torr (234,396). This difference is mainly due to the intracellular molecule 2,3-DPG, which modulates oxygen affinity by binding to hemoglobin. This binding stabilizes hemoglobin's conformational state and enhances oxygen delivery (67,423).

Much research has centered on the search for molecules that could substitute for 2,3-DPG. Unfortunately, 2,3-DPG itself cannot be used because of its reduced binding efficiency to hemoglobin in solution **(238).** The most widely used, and most successful, attempts to normalize the P_{50} of hemoglobin solution have made use of the chemical, pyridoxal 5'-phosphate (P-5-P). This compound is an organic phosphate analog of 2,3-DPG (67,241,377) and has been shown to decrease the oxygen affinity of hemoglobin to essentially the P_{50} of RBCs (i.e., **26** to **28** torr). P-5-P functions by irreversibly binding to the beta chains of hemoglobin near the binding site of 2,3-DPG (68,69).

To achieve a P_{50} approximating that of RBCs and a longer circulation time, many investigators have both pyridoxalated and crosslinked hemoglobin. Pyridoxalated-polymerized hemoglobin does not have the oncotic pressure restrictions of unmodified hemoglobin, and it can be administered in a concentration of 14 to 15 gm/dl, the same hemoglobin concentration as in whole blood. The solution could be administered as a one-for-one replacement of blood, and the oxygen content per unit volume of circulating fluid would be unchanged. Because of these features, pyridoxalated crosslinked hemoglobin has been widely adopted as the preparation of choice, and it is the formulation currently being tested in animals and being proposed for future scale-up studies.

However, the P_{50} of such a preparation is really a composite P_{50}, since not all the hemoglobin will be pyridoxylated and crosslinked in a given preparation. Polymerized hemoglobin and unmodified hemoglobin are also eliminated by different mechanisms (89), and the contribution of each to any toxicity that is seen is difficult to resolve. Future studies are likely to focus on methods to prepare homogeneous preparations of modified hemoglobin, which could then be used to more easily determine the etiology of any toxic effects. The true benefits of transfusing a hemoglobin solution are difficult to assess, because, as mentioned previously, cardiovascular and tissue compensatory mechanisms occur when the concentration of hemoglobin in blood is moderately decreased. This does not imply that hemoglobin solutions are of little value, but rather that the clinical efficacy of one preparation over another will be very difficult to prove, and testing in animals will have to closely simulate the clinical condition in humans, for which its use is intended.

Other problems.—Three other potential problems with hemoglobin solutions as a blood substitute are related to the source of the hemoglobin, how it will be stored, and whether the infused material will invoke an immune response with the formation of antibodies in the recipient.

Hemoglobin solutions are currently being made from outdated human blood. If all the outdated blood in the country could be channeled into hemoglobin solution production, only approximately 600,000 units would be available (or 5 percent of the 12 million units collected).

An alternate source for hemoglobin is through recombinant DNA technology. The hemoglobin molecule has been cloned (see later discussion) and therefore it is possible that sufficient amounts could be prepared to satisfy all hemoglobin solution production requirements. Alternatively, it is conceivable that an animal hemoglobin could be substituted for the human molecule. Crosslinked and pyridoxalated bovine hemoglobin has the equivalent circulation time and oncotic pressure as its human counterpart and has a more favorable P_{50} than does human hemoglobin (156).

Injections of bovine hemoglobin into rabbits have resulted in antibody formation (185), and it seems likely that antibody formation would occur in humans. Even if only one "unit" were given, the ability to track such recipients and insure that they would not receive another unit at a later date would be very difficult. Whether human hemoglobin given to humans will be proven non-immunogenic is also unknown, and it is possible that modifying the configuration of the molecule upon crosslinking may enhance its immunogenicity.

A final concern regarding hemoglobin solutions is their stability during storage. Refrigerated or frozen storage may be suitable in a hospital environment, but is not satisfactory for use as an emergency resuscitative fluid, particularly in the battlefield. The most suitable form of the product for this purpose is as a freeze-dried powder that could be kept at room temperature. It is not certain that long-term storage of clinically suitable

freeze-dried hemoglobin can be achieved. But other equally complex, large proteins have been successfully freeze-dried and reconstituted without loss of function, and there is no reason to assume that the ability to freeze-dry and reconstitute a functional hemoglobin preparation will be any more difficult to resolve.

Clinical studies.—Although nearly all the work done on hemoglobin solutions has utilized animal models, there have been published reports in which no more than a few milliliters of a hemoglobin solution was transfused into humans (10, 93,109,175,212,224,333, 381,415,418,430,477, 492). All but the most recent report (477) used a crude preparation of hemoglobin that was uncharacterized. All of the studies were performed on volunteers who had normal cardiovascular parameters, and in all of the reports adverse effects were noted.

In the most recent study (477), a microporefiltered hemoglobin solution was administered to eight volunteers, all of whom experienced cardiovascular, renal and coagulation abnormalities lasting several hours. Another unpublished study using presumably pure hemoglobin was sponsored by the Biotest Corp. (88). The study began in the late **1970s** but was terminated prematurely after the first two volunteers who received the unmodified preparation developed severe side effects and had to be hospitalized. Although the etiology of the reactions was thoroughly investigated, no explanation was uncovered.

Since 1978, ***no*** clinical trial has been initiated in the United States. The Army Research and Development Command hopes to initiate a clinical study in 3 to 5 years, but the starting time is dependent on whether the present safety issues can be resolved (83). Ability to reproducibly prepare large batches of a homogeneous hemoglobin solution is a fundamental requirement for such a study to begin.

As a substitute for blood, the major shortcoming of hemoglobin solutions is their failure to circulate for an appreciable period. Current research so far appears unlikely to generate a molecule whose circulation time anywhere near approaches that of an RBC. Thus, hemoglobin solutions may be limited to use as an emergency resuscitative fluid.

The anticipated cost of the product will also be an important consideration. It is impossible to reasonably predict at this time what the dollar cost will be, since scale-up beyond laboratory use has not been accomplished. However, it has been estimated that preparing a unit of hemoglobin (equivalent in function to a unit of blood), may cost as much as $100 (83). If this estimate proves correct, the price would be significantly greater than the present price of a unit of whole blood. Thus, cost might be a drawback to its acceptance for civilian use. (The military is inclined to be less concerned with cost because of the emotional/ emergency circumstances in which such hemoglobin products would be transfused.)

If the hemoglobin used in the preparation were derived from recombinant methods, the final cost of the product might be far less than if the starting material were human blood. This is because the purification of human hemoglobin from whole blood is a multi-step process with a 30 to 40 percent loss of starting material. However, cost issues at this point remain secondary, since the major hurdles of safety and efficacy have not been passed. Whereas the newer hemoglobin preparations have adequate retention times and oxygen transport properties for their intended use, the etiology of the sporadic adverse reactions seen in animals remains an enigma. The antigenicity of the solutions will have to be evaluated and, at worst, restrictions on the frequency of infusion may have to be put in effect.

Successful large-scale reproducible preparation and storage of these solutions have yet to be accomplished. The fact that polymerized preparations are composed of many molecular species poses major obstacles to characterizing just what will be transfused, how reproducible each batch will be, and what properties of a hemoglobin preparation are undesirable. Nevertheless, it seems likely that a hemoglobin solution will become available for transfusion eventually, and the Army's Medical Research and Development Command hopes to begin clinical trials of a hemoglobin solution in 3 to 5 years.

Synthetic oxygen binding chemicals. -Rather than use hemoglobin as an oxygen-carrying blood substitute, attempts have been made to synthesize organic chemicals that reversibly bind oxygen. Some compounds have been synthesized that do, indeed, bind oxygen reversibly (9,58,59). However, none of the compounds demonstrate a P_{50} that is appropriate for clinical application, nor have they been used in a biological system. If, however, one such compound should show promise, its small size would necessitate binding it to a larger molecule in order to achieve adequate circulation time.

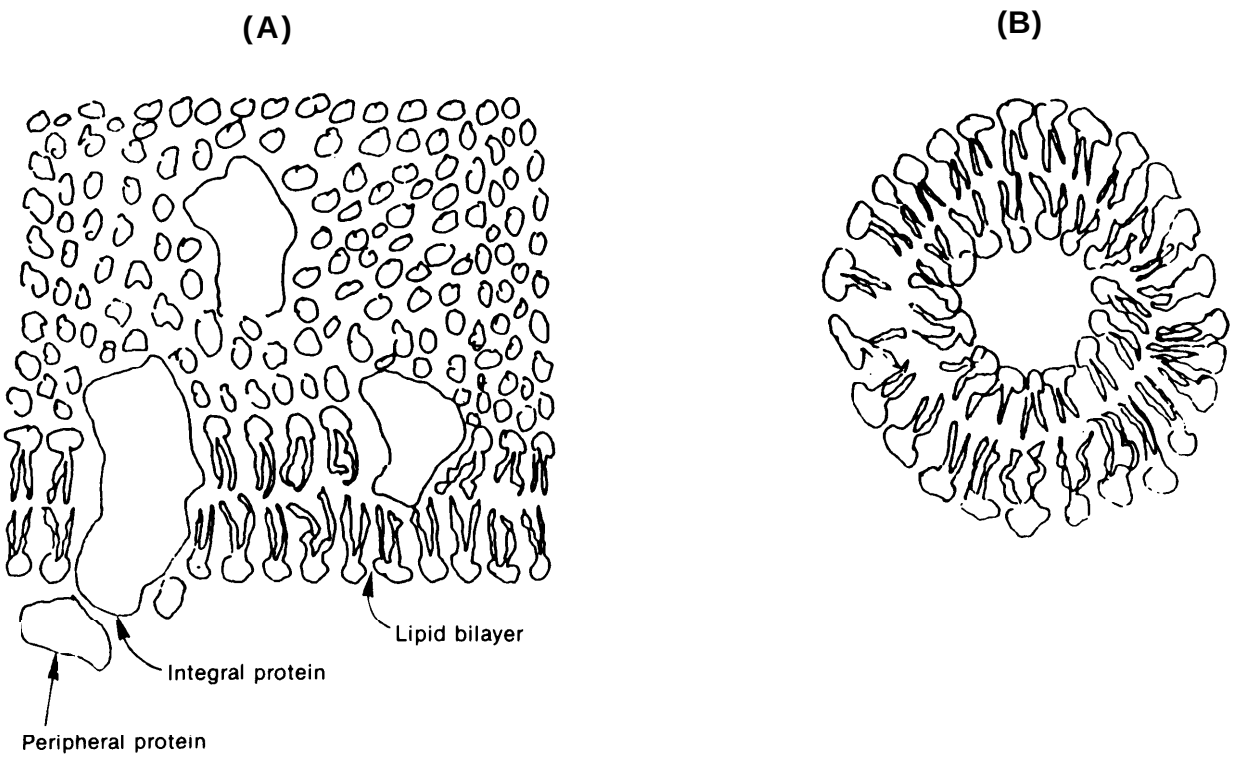
It is likely, however, that availability of recombinant-made human hemoglobin will obviate the use of an organic analog. Moreover, it seems certain that the former development will succeed before a synthetic compound can be made and successfully complete all testing. Hence, research in this area is unlikely to yield a blood substitute of practical significance within the confines of a practical span of time.

Liposomes

Another approach to preparing an RBC substitute involves encapsulation of hemoglobin into liposomes. Liposomes are closed spheroidal vesicles which contain an aqueous phase. The internal volume is separated from the outside by a thin matrix composed of lipid molecules arranged in a bilayer structure as in a normal cell membrane (fig. 16). The composition of the aqueous internal compartment reflects the solution in which the liposomes are formed and can provide for entrapment of a wide variety of substances if so desired. Liposomes can be prepared from a single type of phospholipid, a mixture of phospholipids, or mixtures of phospholipids and neutral lipids; e.g., phosphatidylcholine plus cholesterol. Since these molecules are found in all mammalian cell membranes, liposomes are usually non-immunogenic to the host.

The properties of liposomes can be somewhat controlled by utilizing different lipids and/or different methods of preparation. For example,

Figure 16.—Liposomes



SOURCE: R. Kahn, et al., *Alternate Sources*, 1984.

whether the liposome will be multilamellar (i. e., vesicles composed of a series of alternating lipid layers separated from each other by an aqueous compartment) or unilamellar, or whether they are large or small, can be determined by the method of preparation. The net surface charge of the liposome can also be manipulated by the types of phospholipids used.

As an alternative to liposomes, vesicles can also be formed from synthetically made organic molecules (183). Since these vesicles contain only man-made molecules, their functional groups can be easily altered. They can be made to remain stable for months and have an extremely long shelf life. The size and permeability of small molecules can also be designed in the manufacture of the vesicles. The ability to control these properties make synthetic vesicles an attractive alternative to lipid containing vesicles but, at present, little work has been done with synthetic vesicles as biologically relevant macromolecules.

Preparation techniques. -Liposomes have been used in a variety of ways, such as model membrane systems for studying the mode of action of certain membrane-bound proteins, as carriers for enhancing the pharmacological activity of drugs, or to study the functional incorporation of macromolecules into cells. In all liposome preparations, special attention is given to the following characteristics: 1) captured volume (volume enclosed by given amount of lipid); 2) efficiency of the liposomes to encapsulate material; 3) ability to reconstitute cell membrane derived proteins; and 4) control of vesicle size.

Liposomes as synthetic red blood cells.–Although the overwhelming majority of research and development on liposomes has focused on their utility as a research tool to study cell membrane function, or as carriers of drugs, there has been a limited amount of work using liposomes as a blood substitute preparation. Hemoglobin has been successfully encapsulated into lipid vesicles at concentrations equal to that found in RBCs (160,177,276,285). The general approach has been to encapsulate concentrated hemoglobin solutions into liposomes made by mixing phospholipids and cholesterol. The liposomes contained no proteins (either inside or within the lipid bilayer) other than the encapsulated hemoglobin and, therefore, did not cause immune reactions (285).

The functional characteristics of the liposomal RBC substitutes were very similar to those of native red cells. In studies in which 2,3-DPG was also encapsulated, incorporation of 2,3-DPG resulted in an oxygen dissociation curve similar to that of whole blood (160,276). Liposomes with 2,3-DPG typically showed P_{50} values equal to 28 torr in one study (160,285) and 26 torr in another (276). Thus, with respect to their ability to carry oxygen from the lungs to the tissues, liposomes appear to be at least equal to other artificial blood substitutes. Unfortunately, they disappear rapidly from the circulation, with a half-life of only 5 hours (177).

Although data on storage of these liposomes are not extensive, they suggest the potential value of this technique. Suspensions of encapsulated hemoglobin were stable when frozen and stored at –20° C. (160). The liposomes showed very little lysis and had P_{50} values similar to those before freezing.

Many investigators have performed exchange transfusions with rats using liposomal suspensions (160,276,285). However, it should be noted that a less than 95 percent exchange transfusion in animals can be tolerated simply by administering a colloid or crystalloid solution.

A different approach to preparation of vesicles has been taken by Davis and Asher (149). They recently patented a process whereby stroma-free hemoglobin was encapsulated in a polymerized hemoglobin membrane. The crosslinked hemoglobin membrane was permeable to oxygen and impermeable to hemoglobin. The vesicles were capable of reversibly binding oxygen and had diameters of less than 4 microns. Moreover, they were reported to be stable under conditions of normal blood flow. However, data supporting these claims have not been published, and the physiology of this liposomal preparation is unknown.

Although the above studies are **certainly encouraging**, many problems remain. First, the problem of rapid liposome removal from the circulation must be resolved before this substitute

will find widespread acceptance. Several factors which govern removal of lipid vesicles from the circulation are: 1) irreversible binding of vesicles to tissues; 2) lysis of vesicles by plasma lipoproteins; and 3) clearance by the RES. Of these, removal by the RES (i. e., liver and spleen), is by far the dominant process **(310,541).** All studies reporting on the distribution of liposomes after intravenous injection have found that the liver is the site of liposome retention (111,309,449,514). Approximately 50 percent of the injected dose is found in the liver within a few hours after administration. The mechanism of uptake is not completely understood but may be related to the fact that the liver contains capillaries which allow small molecules to penetrate.

The rapid uptake of liposomes not only results in shortened liposome lifetime but also can saturate the RES and result in reticuloendothelial blockage. Thus, before liposomes can function as RBC substitutes, methods for limiting their uptake by the RES must be developed.

Recently, natural and synthetic glycolipids have been inserted into the liposomal membrane and tested for their ability to alter the final disposition of the liposomes. Incorporation of these glycolipids decreased uptake by the RES **(lo4, 292,359,597).** However, many glycolipids are antigenic and thus would not be suitable for insertion into a liposome that will be transfused. Alternatively, there are non-antigenic glycolipids that may also retard rapid liposome clearance. Hunt and Burnette (1983) have attached inert carbohydrates to lipids and incorporated the glycolipids into liposomes. They claimed to have reduced RES clearance and binding of liposomes to tissues, although complete studies were not published. Thus, it would seem that addition of inert carbohydrates may be one way to retard rapid liposome clearance.

The potential toxicity of a liposomal preparation is also of great concern. Initial studies (160, 177,276,285) indicated that hemoglobin-filled liposomes were non-toxic when infused into rats or rabbits. However, these studies did not address the long-term effects of the liposomal preparation, and toxicity of liposomes could potentially arise either from impurities present in the hemoglobin preparation, the lipids themselves, or impurities introduced in the encapsulation procedure.

Acute toxicity following administration of liposomes may not be seen, but adverse reactions may arise if the contents of the liposomes escape. Encapsulation of a toxin will essentially hide the entrapped material until the liposomes degrade, either by storage for long periods of time prior to use, or when they are exposed to destructive elements in the circulation (e.g., lipoproteins). There are, however, several approaches to prevent this potential problem. The most direct is to identify the impurities in the liposome preparation and eliminate them, which could be difficult and costly. A second approach is to design liposomes which retain their encapsulated material when stored or injected into the circulation. These approaches are not mutually exclusive but will likely have to be engineered into the liposomal preparation.

Lipids used in construction of the liposome are another source of potential toxicity. Although in vivo toxicity of liposomes is thought not to be a concern if proper care is taken in isolation and storage of the lipids, one study has shown that liposomes caused damage to the central nervous system in mice **(4).**

Another potential source of concern is contaminants in the lipids that can affect liposomal integrity. Changes in the structure of lipid molecules inevitably occur with time and can result in accumulation of lipid breakdown products in the preparation. These contaminants can facilitate liposomal degradation and loss of their encapsulated contents. To avoid this problem, the lipids will have to be purified prior to use. There does exist, however, a naturally occurring class of lipids (branched-chained dialkyl ethers) that are not subject to such breakdown **(324).** Another alternative would be to use synthetically made molecules that are not subject to these effects (183).

It will also be important to examine the effects of blood and blood constituents on liposomal disruption. It is known that serum lipoproteins can interact with phospholipid vesicles (393) resulting in their disruption **(427,458,524).** Since the loss

of liposome integrity appears to be due both to the binding of plasma components to the liposomes and to exchange of vesicle components with plasma lipid constituents, any modification of liposomes that will prevent these events should increase their ability to retain entrapped material.

One such modification is the addition of cholesterol, which has been shown to dramatically decrease the permeability of liposomes in the presence of serum, plasma, or whole blood (311). Inclusion of specific, naturally occurring phospholipids has also been shown to increase the stability of liposomes in the presence of plasma proteins (261). These results are encouraging, but no detailed study has yet been carried out to determine the effect of plasma or whole blood on the long-term stability of modified liposomes.

As is desired for all RBC substitutes, red cell-like liposomes should have a long shelf life. Many investigators have examined the stability of several liposome preparations for their ability to entrap and hold ***drugs*** (361,522). All preparations, however, show some disruption after liquid storage, and storage of liposomes for extended periods is likely to result in some loss of membrane integrity.

A possible solution to many of the problems discussed above (e.g., toxicity, stability, and storage) may lie in the use of synthetically made vesicles, or so-called "membrane mimetic systems" (183,184) formed from synthetic surfactants. They can be stabilized by polymerization, which effectively seals the membrane surface. The polymerized vesicles are appreciably more stable than liposomes and have a shelf life of several months. Moreover, the size of the vesicles and their permeability properties can be engineered as desired. Although utilization of these vesicles has become a significant area of research in chemistry, biological applications have not been explored.

Last, scale-up to prepare large amounts of a liposomal preparation for transfusion using existing technology could be a formidable task. First, it will require isolation of large quantities of lipids and hemoglobin. The latter will undoubtedly be obtained by recombinant DNA technology (see following section). The majority of lipids could be isolated from crude preparations of egg lipids at a reasonable cost. However, it is known that liposomes which contain only these lipids would be cleared rapidly, and thus special masking lipids would have to be included in the final product.

Because the relationship between liposome composition/structure and clearance is not fully understood, it is very uncertain what the final product will contain and cost. But from the studies reviewed above, it is likely that some type of synthetically constructed lipid will be necessary. For example, a single synthetic surfactant may eliminate the need for purification and storage of lipids. Production of surfactants is also easier and less costly. Although no data have been collected concerning the potential toxicity of these preparations, it is entirely conceivable that the desired properties of stability and safety could be engineered into the surfactant's structure. This approach, however, is speculative; many years of basic research will be required before testing in humans can begin.

The second major problem in scale-up *relates* to preparation of the vesicles. Many of the methods for encapsulating materials were developed for drug delivery. Applications involved in drug therapy require relatively small amounts of vesicles compared with the large volume requirements for a blood substitute. In fact, difficulty in obtaining significant amounts of liposomes is likely to be the reason that extensive animal studies have not yet been reported with existing preparations.

At the outset, therefore, only those preparative techniques which can be easily scaled-up are likely to be considered. However, at the present time no suitable large-scale encapsulation procedure has been devised or tested to provide confidence that mass production of liposomes (or synthetic vesicles) can be achieved.

In sum, the use of encapsulated hemoglobin as an RBC substitute has received relatively little attention, particularly in comparison to the work on PFCs and hemoglobin solutions. While the technology is still in its infancy, encapsulation appears to be the approach most likely to lead to a blood substitute that has all the properties of an RBC. Certainly, major obstacles remain—particularly the toxicity, stability, and storage of such a preparation. But studies on liposomes or

synthetic vesicles, while relatively new, are accelerating at a rapid rate, and technological advancements in this area will undoubtedly find solutions to the problems that remain. Even so, it is clear that many years of laboratory research will be required before clinical studies will be appropriate.

Platelet Substitutes

Under normal conditions platelets circulate in the blood for approximately 10 days as disk-shaped, formed elements, and do not adhere to other cellular elements, the vascular endothelium, or to themselves. But within a few seconds after injury to a vessel, platelets adhere to the exposed collagen surface. Such platelets become "activated" and release their internal constituents, resulting in a growing aggregate of platelets, and the simultaneous occurrence of clot formation. This growing mass literally plugs the hole in the damaged vessel wall. Thus, platelet function can be described in terms of the following reactions: 1) attachment at the site of the injury (adhesion); 2) aggregation of platelets to each other; and 3) release of substance(s) that facilitate blood coagulation.

Adhesion to a cut vessel wall is a complicated process that has been shown to require plasma factors as well as key platelet membrane molecules. Aggregation of platelets to each other is mediated by numerous exogenous platelet agonists such as thrombin and adenosine diphosphate (ADP). Each of these agonists has one or more receptors on the platelet surface. Binding of the agonist unmasks specific sites for plasma fibrinogen on the platelet membrane (70,355), which is thought to somehow serve as the "bridge" between platelets. Platelet secretion is also initiated by binding of agonists to the platelet membrane. Last, platelets have been implicated in the activation of several coagulation factors such as factor IX, factor X, and prothrombin (298,380,602).

Because of the complex role of platelets in the arrest of bleeding, it follows that the biochemistry involved in platelet function is also quite complex, and the mechanism(s) by which collagen, ADP, thrombin and other agonists result in platelet adhesion, aggregation and secretion are not completely understood. Thus, it is impossible at this time to fashion an artificial platelet which will mimic the entire physiology of the platelet. Nevertheless, two approaches have been explored as means to develop a suitable platelet substitute.

Platelet Fragments

The fact that platelets will clump to each other to form a plug and thereby seal broken vessels has been known for some time. Although the mechanism for this effect is still being unraveled, it might be hypothesized that intact platelets are unnecessary to achieve plug formation. Thus, it is not surprising that some early investigators examined the efficacy of transfusing platelet fragments. In one study (314) freeze-dried platelets were given to children who required platelet transfusion. Although substantial improvement was noted in certain coagulation tests, the clinical efficacy of the preparation in controlling bleeding was equivocal.

Another study by the same group utilized platelets disrupted by freezing and thawing, and again a transitory control of bleeding was seen (313). These somewhat encouraging results, however, were challenged by animal studies in which irradiation was used to create a clinical condition that warranted platelet transfusion. Three independent, well-designed studies employing this model showed that neither freeze-dried nor fragmented platelets were of value in control of bleeding (187,263,288).

These studies seem to have put to rest the notion that platelet fragments could adequately substitute for the intact cell. But in 1983 McGill and colleagues (365) examined the ability of platelet fragments to prevent bleeding in platelet-deficient rabbits. Their studies found that fragments appeared to have the same adhesive qualities as did intact platelets. In studies directed toward understanding platelet function in general, others observed that platelet membranes would interact with "activated" platelets in vitro but not with unstimulated platelets (446). Many investigators, however, remain skeptical that these recent observations warrant reopening this approach to obtaining a suitable platelet substitute. No clinical studies in humans using platelet fragments have

begun or are even contemplated, and further studies by McGill in this area have been discontinued (366).

Even if such platelet fragments were effective, there would remain the question of the utility of such a preparation. The fact that platelet outdating is substantial in this country (i.e., greater than 10 percent of the units collected) implies that there is no chronic shortage, and the recently improved shelf-life of the component (from 3 days to 5) has greatly alleviated inventory problems that arise over weekends and holidays. Moreover, platelet fragments would still carry the risk of infectious disease and other adverse effects associated with traditional platelet transfusions. Thus, it is very unlikely that this substitute will ever see clinical use.

Liposomes as Platelets

Because of our understanding of the role many surface membrane constituents play in platelet function, it maybe relatively easy to reconstitute one or two of these functions into liposomes. In fact, some progress in this area has very recently been achieved.

One of the platelet membrane glycoproteins which is thought to play a critical role in platelet adhesion has been reconstituted into lipid vesicles (499). When liposomes containing this protein were incubated with a plasma factor that is also important in platelet adhesion, liposome agglutination occurred, although the reaction did not entirely mimic that seen with normal platelets. In other studies the platelet membrane glycoproteins required for fibrinogen binding to intact platelets have been reconstituted into phospholipid vesicles (57,421). Both of these studies incorporated isolated platelet membrane glycoproteins into phospholipid vesicles and showed that the vesicles bound specifically to intact platelets in vitro. These data show that it is possible to reconstitute at least part of the platelet membrane constituents necessary for platelet function in vitro, although in no sense are the liposomal preparations used in these studies suitable for clinical use.

The fact that individual activities can be reconstituted, at least in this primitive fashion, offers promise that many activities can be reconstituted in a single liposome. All the necessary proteins would have to be reconstituted correctly. Since many platelet functions are interdependent, and since a completely reconstituted artificial platelet must also function in a similar fashion, the interactions between molecules must also be reconstituted. Although this might appear to be an impossible task, several cooperative membrane activities have been reconstituted in a stepwise fashion (294,456). Therefore, at least in theory, it should be possible to reconstruct many platelet functions.

However, virtually all of the problems discussed in relation to liposomes as artificial red cells are also relevant for development of liposomes as platelet substitutes. In addition, these liposomal preparations could be highly antigenic, since the reconstituted membrane proteins will be exposed on the liposome surface. Of concern is the observation that some cell membrane proteins have been found to be more antigenic when reconstituted into liposomes than they are in their native state in the cell membrane (300,495). However, the antigenicity of certain proteins can be modified by the lipid composition of the liposome (81), and it may also be possible to modify the reconstituted glycoproteins (e.g., remove the glyco- or carbohydrate portion) in such a way that they are no longer antigenic but still carry out their normal function. Neither of these approaches, however, has been studied so far.

It is impossible to estimate all of the difficulties that may arise for mass production of such a preparation. Very few of the membrane proteins critical to platelet function have been identified, much less isolated. However, when this information becomes known, production of significant amounts of the required proteins will no doubt make use of recombinant DNA technology. The rate-limiting step, however, as in the case of liposomal red blood cells, may be in the ability to generate significant quantities of liposomes.

Since membrane proteins will have to be incorporated into the lipid bilayer, the only suitable method known is the detergent removal procedure (57,421,499), which is very time-consuming and likely to be too expensive for mass production. New preparation procedures will likely need to be developed. Thus, it will be several years, at least, before a serious attempt will be made to construct a liposomal platelet substitute.

PART 2: ALTERNATE SOURCE FOR PLASMA PRODUCTS– USE OF RECOMBINANT DNA TECHNOLOGY

Introduction

Except for the oxygen-carrying function of RBCs, the physiological functions performed by the cellular and humoral components of blood are mediated largely through proteins. These proteins may act in a relatively specific and independent fashion, as in the case of antithrombin III and C1 esterase. Alternatively, a single species such as albumin may play a number of roles, alone or in conjunction with other factors, in maintaining homeostasis. Finally, a variety of proteins can interact in complex patterns to collectively carry out a function (e.g., coagulation).

All plasma proteins of therapeutic value have the potential of being produced by microorganisms carrying the genetic coding for the primary structure of the specific protein. The ability to construct a microorganism that can produce a useful product is possible because of recombinant DNA techniques that allow specific segments of DNA to be isolated and inserted into a bacterium, or other host, in a form that will allow the DNA segment to be replicated and expressed as the cellular host multiplies. The DNA segment is said to be "cloned" because it exists free of the rest of the DNA that, with it, constituted the genome of the organism from which it was derived.

A DNA clone can be prepared in a number of ways, but generally the process involves linking the desired DNA segment to a second piece of DNA known as the vector. The one feature all vectors have in common is that they are replicated independently inside a cell. The vectors commonly used for gene cloning in bacteria are of three basic types: 1) plasmids; 2) derivatives of the bacterial virus known as lambda; and 3) genetic hybrids constructed from plasmids and lambda, called cosmids. The vector chosen to prepare a gene clone will depend upon two primary considerations: 1) the size of the DNA segment to be cloned; and 2) whether or not the protein coded by the DNA is to be expressed (produced).

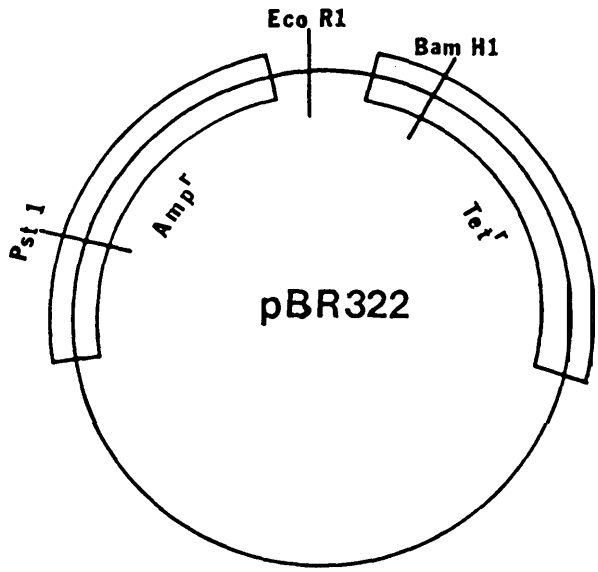
Plasmids used in cloning technology represent modified forms of DNA molecules that occur naturally in bacteria or yeast and generally confer a selective advantage to a cell harboring them (146). In the common bacterium E. coli, naturally occurring plasmids conveying resistance to antibiotics such as penicillin and tetracycline have been described (343,379,523). Another naturally occurring E. ***coli*** plasmid, known as Col E1, carries a gene coding for a protein that is secreted into the environment and inhibits the growth of other bacteria.

All plasmids have certain features in common. For example, their presence in a cell confers a selective growth advantage. In addition, they carry a specific sequence of DNA nucleotides that serves as a recognition signal for the host enzymes responsible for DNA replication. These enzymes will therefore not only replicate the chromosome as the cell multiplies, but will also replicate the plasmid molecule (and any foreign DNA linked to it).

The most commonly used plasmid for cloning foreign genes in E. ***coli*** is a derivative of the Col E1 plasmid known as pBR322 (84,85,86,91,519). The characteristics of pBR322 have been selected to optimize the plasmid for cloning DNA fragments up to approximately 5 kilobase pairs (kb) in length. The plasmid is efficiently replicated in the commonly used laboratory strains of E. coli, with multiple copies of the plasmid being present in a single bacterial cell (91). The pBR322 plasmid is diagrammed in figure 17 and can be seen to contain separate genes that confer resistance to the antibiotics penicillin (e.g., ampicillin) and tetracycline for the host cell.

The penicillin resistance gene codes for an enzyme known as beta-lactamase, which alters the structure of the penicillin molecule so as to inactivate it (11). The gene conferring resistance to tetracycline codes for a membrane protein that blocks transport of the drug to the interior of the

Figure 17.—Structural Map of the pBR322 Plasmid DNA Molecule



The entire plasmid is 4362 base pairs in length and exists in a circular form as shown. The relative location of the genes coding for tetracycline (tet^R) and ampicillin (amp^R) resistance are shown, as are the sites for the restriction endonucleases, Pst-1, Eco R1, and Bam H1.

SOURCE: R. Kahn, et al., *Alternate Sources*, 1984.

cell, where it exerts its inhibitory effect (523). Thus, E. coli cells harboring pBR322 will multiply on nutrient media containing penicillin and tetracycline, whereas cells not carrying the plasmid will die under these conditions. This difference is exploited in the cloning process.

One of the hallmark. discoveries that has enabled molecular geneticists to splice DNA in a precise and premeditated fashion was the discovery of enzymes called restriction endonucleases (352,385). These enzymes recognize specific sequences of nucleotide bases in DNA and cleave the molecule within that sequence. To date, numerous restriction endonucleases have been identified from a large variety of sources, each with its own nucleotide base recognition sequence (352,385). Thus, a desired segment of DNA can be specifically excised from a larger piece of DNA if sites for particular restriction enzymes are known to flank the desired segment.

As shown in figure 17, pBR322 has several restriction enzyme sites that cleave the molecule once and have been used routinely for inserting pieces of foreign DNA. In addition to the single cleavage sites for restriction enzymes such as Pst-1, Barn H1 and Eco R1, pBR322 has numerous sites for a wide range of other enzymes that "cut" the plasmid molecule more than once (519).

Although pBR322 has been used extensively for cloning, a number of plasmids that are custom-tailored for a specific purpose have been constructed from pBR322 and other segments of DNA derived from a bacterial and/or bacteriophage genome. A modified plasmid may be desirable for a number of reasons, but most often they are used so that the information encoded in a cloned gene will be expressed by the bacteria carrying the gene. Several "expression vectors" have been constricted which are custom-tailored to express the protein product encoded in any piece of DNA inserted into the vector in a specific way (94,258,441, 573,581).

Other commonly used vectors for cloning foreign DNA are genetically modified derivatives of the lambda virus. As was stated previously for plasmids, lambda is a naturally occurring bacterial virus that infects ***E. coli*** cells (513). Once the cells are infected, their capabilities are commandeered by the virus such that viral particles are synthesized to the exclusion of other host macromolecules. Mature viral particles accumulate inside the cell until it finally lyses, at which time a large number of phage particles are released into the environment to repeat the process upon contact with other bacteria.

The modifications of lambda phage necessary to make it a useful cloning vector primarily require excising from the phage that part of DNA which is not involved in its growth cycle (586). The DNA excised from the natural genome is then replaced with fragments of foreign DNA. Replication of the cloned DNA is achieved by infecting a culture of ***E. coli*** cells with the modified lambda.

The one significant advantage to the use of a lambda vector for gene cloning is that very large DNA fragments can be cloned. Whereas most plasmids can accommodate only 3 to 5 kb of foreign DNA, lambda vectors can accommodate up to 20 kb of it (77,338,586). The main disadvantage to cloning with lambda is that the expres-

sion of the protein encoded in a eukaryotic (higher-order organism) gene is generally not efficiently expressed.

A third cloning vector, known as a cosmid (131,268,269) is actually a genetic hybrid between lambda virus and a plasmid. As with lambda, the primary advantage of cloning with cosmids is that very large pieces of foreign DNA (up to approximately 30 kb) can be cloned (269). Cloning in cosmids also suffers from the same disadvantages as cloning with lambda.

Choice of a particular vector to be used to clone a desired DNA fragment will depend ultimately upon what the investigator wants the cloning system to do. If the goal is to study the structural organization of a gene on a chromosome, the vector of choice would probably be one of the derivatives of lambda phage or a cosmid. Cloning with these systems would enable a large segment of a chromosome containing the entire gene and possibly flanking DNA to be studied in a single isolate. If, however, the aim of a cloning procedure is to engineer a cell to produce a protein product, cloning with a plasmid designed for expression of foreign DNA is the system of choice. Since the goal of cloning genes coding for therapeutically useful plasma proteins is to produce those proteins, the discussion that follows will focus upon cloning with plasmids rather than with the other vectors mentioned.

Eukaryotic genes, as they exist in the chromosome, are generally fragmented into regions of coding and non-coding DNA called exons and introns, respectively. In bacteria, genes do not consist of exons and introns but rather of a single coding sequence of DNA. If mammalian chromosomal DNA is cloned into a bacterial cell, the information will not be properly expressed, because the bacterium cannot decipher the complex exon/intron code of the mammalian DNA. In order to resolve this problem, messenger RNA is used to enzymatically synthesize a complementary DNA copy.

During the expression of a chromosomal gene in a mammalian cell, the information encoded in the separate exons is ultimately spliced together into a messenger RNA (mRNA) molecule, The mRNA is then transported from the nucleus to the cytoplasm, where it is translated into the amino acid sequence of the protein molecule (579).

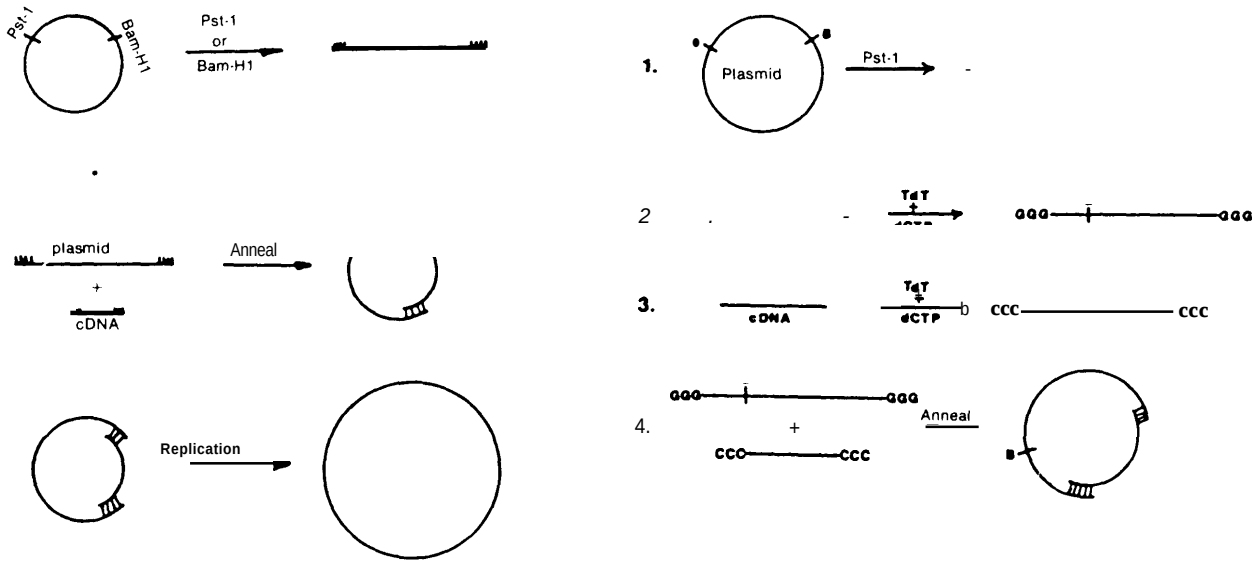
Messenger RNA occupies a unique position in the flow of information from the nucleus of a cell, since it contains the entire coding sequence of the gene. Messenger RNA isolated from a mammalian cell can be used to program the synthesis of a double-stranded complementary DNA copy of itself in vitro, In many cases, cloning a complementary DNA (cDNA) copy of mRNA is the method of choice when cloning a gene that is to be expressed by a bacterial host. Since the cDNA form of a mammalian gene lacks the complex exon/intron organization of the nuclear DNA, it can be deciphered by a bacterium as if it were a bacterial gene.

The synthesis of cDNA from mRNA relies on use of an enzyme, isolated from avian tumor viruses, known as reverse transcriptase (60,528). This enzyme, under proper conditions, will synthesize a single-stranded DNA molecule that is the complement of an mRNA template. The single-stranded cDNA product can then be used in conjunction with another enzyme, DNA polymerase, to make a double-stranded cDNA molecule (353, 471), which can then be inserted into the vector. One method of insertion can be accomplished by chemically modifying the vector DNA and the cDNA so that the ends of both molecules are "sticky" (375,480). If the vector and modified cDNA are mixed, the sticky ends of both species can bind together, and the result will be the formation of a single, circular recombinant molecule (fig. 18) (126).

Another way to link cDNA to the vector is to attach a series of guanosine residues to the free ends of the vector DNA. Cytosine residues are also attached to the ends of the cDNA; when the vector and cDNA are mixed, the base pairing property of guanosine with cytosine allows the molecule to anneal into a single recombinant plasmid (573) (fig. 18).

The procedures for inserting foreign DNA into a plasmid do not yield a uniform product. When sticky ends of plasmids are generated, a certain percentage of the plasmids recircularize and thus do not recombine with the DNA to be cloned. It therefore becomes important to be able to dis-

Figure 18.—Common Methods **for Inserting Foreign DNA Into the pBR322 Plasmid**



A. Insertion by annealing of sticky ends on plasmid and foreign DNA. In the example shown, the plasmid and foreign DNA have been modified with restriction enzymes to produce sticky ends. The two DNAs are then mixed together in the proper ratio and allowed to anneal. With a single replication of the recombinant molecule, the foreign DNA is recombined permanently into the plasmid.

B. Insertion by dG:dC tailing. Residues of guanosine and cytosine are added to linear plasmid and foreign DNA, respectively, using the enzyme terminal transferase (TdT). The dG and dC tails are complementary resulting in the recombination of the foreign DNA into the plasmid.

SOURCE: R. Kahn, et al., *Alternate Sources,* 1984

criminate between plasmid molecules that have cloned inserts and those that do not. A sensitive method to screen for plasmids with inserts involves exploiting the presence of restriction enzyme sites located in the middle of the plasmid genes that code for resistance to the antibiotics penicillin and tetracycline (fig. 17). In general, when a piece of foreign DNA is inserted into a gene coding for resistance to an antibiotic, the gene is inactivated. Thus, foreign DNA inserted into the gene coding for penicillin resistance will inactivate that gene, and a penicillin-resistant bacterium will become sensitive to the drug. However, under these conditions the gene coding for tetracycline resistance will continue to function unaffected.

Inactivation of a gene following the insertion of foreign DNA provides a quick way to screen a large number of bacteria for those that harbor plasmids with foreign DNA inserts. If the DNA has been inserted into the gene coding for penicillin resistance, recombinant plasmids will no longer be resistant to penicillin but will continue to be resistant to tetracycline. Plasmids which do not contain DNA inserts should have resistance to both antibiotics. In practice, the plasmid mixture is added to bacterial cells that have been made permeable to DNA molecules and, following a brief incubation, the cells are plated on ***medium*** containing tetracycline. Under these conditions, bacteria that did not take up any DNA will not grow because they have no resistance to tetracycline, whereas bacteria that took up plasmid, with or without a DNA insert, will give rise to colonies.

An inoculum from each colony that grows on the tetracycline-containing plates is then tested for its ability to give rise to a colony on medium containing penicillin. Only those colonies that originated from bacteria that took up plasmid without inserts will grow on penicillin, and thus are discarded. In this example, colonies that grow

only on tetracycline and not penicillin represent bacteria that received recombinant plasmids harboring a foreign DNA insert.

Uptake of recombinant DNA molecules by competent *E. coli* bacteria (or other organisms) results in the appearance of colonies on agar plates within 24 to 48 hours of incubation. The collection of colonies (known as a library) will contain a representation of the cloned genes to which the bacteria were originally exposed, and therefore one or more screening protocols must be devised to identify the colony(ies) harboring the DNA sequence of interest.

In some cases, identification of the desired clone can be easily accomplished, as in the cloning of cDNAs coding for hemoglobin (353), or albumin (336). In these cases, the cDNAs were synthesized from mRNA extracted from the cell type primarily responsible for synthesizing the protein (i.e., reticulocytes and liver cells, respectively). In reticulocytes, most of the message present in the mRNA code is for hemoglobin; thus most of the bacterial colonies of the library will harbor cDNA that codes for hemoglobin. The result is that relatively few colonies will have to be screened before a cloned hemoglobin gene is identified. In most cases, however, the cDNA library will contain cDNA clones synthesized from a heterogeneous mixture of mRNAs, and thus the colony harboring the desired gene clone will be identified only after a large number of colonies have been screened.

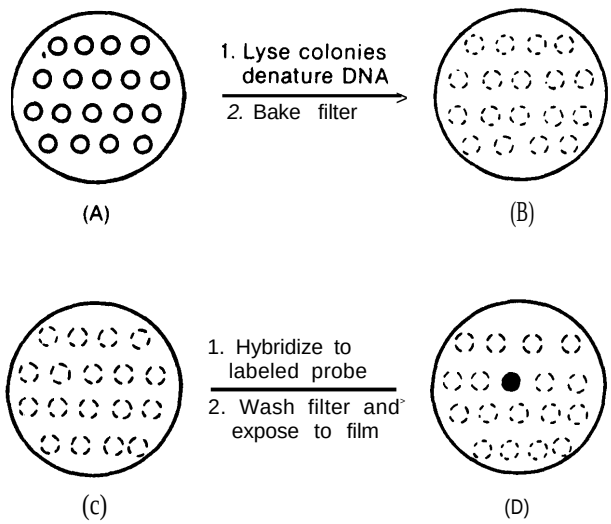
Many methods are available to screen a library of colonies. Colony hybridization (247) is one method capable of examining hundreds of bacterial colonies quickly and easily. This method essentially involves growing colonies on nitrocellulose filter paper soaked in medium, lysing the cells and fixing the released DNA to the filter paper (fig. 19). Thus, a collection of DNA spots the exact size and shape of the colonies from which they were derived is left on a baked filter. The filter is then incubated with a buffered solution containing a nucleic acid probe that has been radioactively labeled.

Since the recombinant clones on the nitrocellulose are denatured, (i.e., the two strands of the DNA molecule have been separated) the single strands are immobilized on the filter and are capable of pairing to labeled complementary nucleic acid molecules present in the hybridization buffer. Colonies that hybridize to the probe can be easily detected by exposing the filter paper to X-ray film; positive colonies will appear as black dots on the developed film (fig. 19).

Maniatis et al (353) purified the mRNA coding for hemoglobin from reticulocytes and then hybridized labeled mRNA to a cDNA library. The success of this particular approach was due to the large amount of hemoglobin mRNA that was easily purified from reticulocytes. Success in identifying cDNA clones coding for albumin have also been reported using this technique (542). Once again, purification of the mRNAs was possible because of their large abundance in a particular tissue.

Identification of cDNA clones synthesized from less abundant mRNAs has also been possible using hybridization techniques. One approach that has repeatedly proven effective in identifying clones involves hybridization of a gene library to a chem-

Figure 19.—The Filter Hybridization Technique of Grunstein and Hogness, 1975



In this protocol bacterial colonies are grown on nitrocellulose filters that have been placed atop nutrient agar plates (A). The colonies are lysed and the DNA sticks to the filter (B). Baking the filter *in vacuo* results in the irreversible binding of the DNA to the filter in a form that is capable of hybridizing to a radioactive probe (C). DNA on the filter that hybridizes to the probe will become radioactive and expose the overlying area of the X-ray film (D).

SOURCE: R. Kahn, et al., *Alternate Sources*, 1984.

ically synthesized oligonucleotide probe (516b). Synthetic oligonucleotides of virtually any length and of a predetermined sequence can be easily and reliably synthesized by a variety of chemical means (516b). The longer the probe, the more specific it will be in hybridizing only to the clone of interest.

If the amino acid sequence of a limited part of a protein is known, a nucleotide sequence that will translate into that amino acid sequence can be deduced. Synthetic oligonucleotides have been used to identify gene clones for several plasma proteins. Edlund et al. (170) and Prochownik et al. (448) identified the genes coding for plasminogen activator and antithrombin III, respectively, using this methodology. The successful cloning of these and other genes emphasizes the effectiveness of synthetic oligonucleotides as hybridization probes for clone identification.

If the amino acid sequence of the desired protein is not available and its messenger RNA is in short supply, an antibody that reacts with the protein may be used for clone identification. In this technique, the antibody reacts with the antigenic site(s) on the protein synthesized by the bacterial colony harboring the clone gene. Immunological screening involves growing colonies on nitrocellulose filter paper and lysing the cells in a manner that will allow the cellular proteins to stick to the paper (258). The filter is then incubated with radiolabeled antibody and exposed to X-ray film. Any colony synthesizing immunoreactive protein will show up on the film as an exposed spot and thus presumably harbors the gene of interest. Immunological screening has been used to identify recombinant clones coding for the proteins proinsulin (94,573) and tropomyosin (258).

The advantage of the immunological screening approach to clone identification is that antibodies are more easily generated than oligonucleotides. Furthermore, colonies identified with this protocol are synthesizing the protein from the cloned gene in a correct fashion. The disadvantage to this approach relates to the requirement that the bacteria must express the cloned genetic information correctly and must synthesize enough of the protein to be easily detected by an antibody. These requirements may not be met by some ***colonies*** harboring the correct gene sequence, but not expressing the protein correctly or in sufficient amounts.

Once the desired gene clone has been isolated, it can be used in a number of ways. Since the ***gene*** is cloned, it can be produced in virtually any amount and in a pure form for study or further modification. For example, if the colony harboring the gene does not express the protein coded by it, the gene may be excised from the vector and reinserted into another vector that will allow the protein to be expressed.

Of all the applications of gene cloning technology, perhaps the most widespread use is destined to be in mass production of useful proteins. The hope is that existing protein products can be made more efficiently, more cheaply, and more cleanly by gene cloning than by current methods. Before these ambitions can be realized, however, several hurdles must be passed. The first involves applying the techniques discussed previously to identify the desired gene clone and to get the product of the gene expressed by the cellular host. As will become apparent, this step is probably the easiest task to surmount. Second, depending upon the product, some modification may be required to convert a precursor form of the protein, which may be inactive, into the activated form. The third requirement involves putting the recombined gene into a host that will be suitable for growth on an industrial scale. Finally, the protein product must be purified to meet safety and efficacy standards set by Federal regulatory agencies.

Insulin is a protein that has undergone all of these steps and is now marketed by Eli Lilly (Indianapolis, IN). It was one of the first pharmaceutically useful proteins for which the gene has been cloned and the protein expressed. Since the A and B polypeptide chains which comprise insulin are relatively short and the complete amino acid sequence of each was known, it was a straightforward task to chemically synthesize oligonucleotides that would separately code for each chain (227). The oligonucleotides were linked to the E. coli gene for an enzyme, beta-galactosidase, using a modified form of the pBR322 plasmid. The beta-galactosidase gene facilitated the expression

of the insulin gene linked to it. Thus, insulin chains were synthesized in the form of a fusion product to beta-galactosidase. In other words, one long protein chain was composed of a portion of the beta-galactosidase protein and the entire insulin chain linked together end to end.

Ingeniously, the genetic code for the amino acid, methionine, was attached ahead of the code for the first amino acid of both the A and B insulin chains. Methionine can be destroyed by the chemical, cyanogen bromide, and thus this construction enabled the insulin polypeptides to be readily cleaved from beta-galactosidase. Since neither the A nor B insulin chain contains a methionine residue, the protein was not affected by cyanogen bromide cleavage.

The scheme to produce biologically active insulin was to isolate the A and B chains from the recombinant strains harboring the respective genes and then mix the polypeptides together. In the presence of mild chemical treatment, the chains were found to associate together correctly and the net result was pure, biologically active insulin (227). Subsequent clinical trials of recombinant insulin have shown the recombinant hormone to be as safe and effective as natural insulin in its physiological effects (303). However, the cost of the recombinant product is about twice the price of the hormone extracted from bovine/porcine pancreas, from which it has conventionally been derived.

Another recombinant product currently undergoing clinical trials is the antiviral agent, interferon. Interferon actually refers to a collection of three small proteins (known as alpha, beta and gamma) that apparently affords protection to cells from viral infection and some forms of cancer. Before it was produced by recombinant methods, interferon was isolated from lymphocytes, but the yield and purity was poor. Using recombinant methods, however, strains of ***E. coli*** harboring the alpha interferon gene have been developed that synthesize large amounts of interferon (153,228, 237,398). Preliminary results suggest comparable efficacy and side effects for the synthetic molecules as compared with the natural material isolated from lymphocytes (579). In addition to alpha interferon, the successful cloning of beta and gamma interferon has also been reported (153,154,237).

Although it should be clear from the foregoing discussion that pharmaceutically useful proteins can be produced in recombinant organisms, it should be noted that in each case a certain amount of serendipity was involved. For example, the techniques used to produce recombinant-made insulin can be used in only a limited number of cases. Since neither the A nor B chains of insulin contain the amino acid methionine, the cyanogen bromide treatment had no effect. However, if the protein did contain methionine, it would be cleaved by the treatment at each methionine site, resulting in a fragmented product that would probably be inactive.

Another potential problem that fortuitously was irrelevant for insulin or alpha interferon production is that neither protein needs modification after its synthesis before it can function. One common modification of proteins after their initial synthesis is the attachment of carbohydrate, termed glycosylation. Should glycosylation be a requirement for normal function, the product of recombinant bacteria will have to be glycosylated in some way in order to produce an active product.

One of the obstacles facing the biotechnology industry is the production scale-up of a recombinant product and its subsequent large-scale purification. To this end, strains of bacteria are continually being sought that synthesize a desired protein efficiently and in large amounts. The current maximum yield of product appears to be about one gram of protein per liter of ferment. With regard to the isolation of the desired protein from the bacteria, current methods generally involve treatment of the bacteria to release the protein. Treatment can include complete lysis of the bacterial cell or a milder procedure that makes the cell wall leaky. In either approach, a crude preparation of the protein is initially recovered.

Impurities of the preparation may not be very important, as in the case of industrial enzymes. Alternatively, one common contaminant of the lysed bacteria is cell wall fragments, which are pyrogenic (fever-causing) in humans. Other contaminants may be antigenic in humans and thus

elicit an antibody response upon repeated therapy with the product. Any product to be infused into a human will have to meet FDA standards for purity and safety. Thus, novel procedures capable of substantial scale-up may have to be devised.

Because it is easier to purify a protein from culture medium than it is from a bacterial lysate, it would be advantageous if a recombinant-made protein were secreted into the medium. One of the shortcomings of E. coli as a host for cloning is that this bacterium does not normally secrete anything into its environment. This precludes harvesting the product from spent culture medium. Currently, a great deal of research is being done to modify E. ***coli so*** that it will secrete recombinant products.

Alternatively, other bacterial species such as ***Bacillus subtilis*** maybe suited for scale-up, since this bacterial species already secretes a large amount of its own proteins into the external environment. Thus, the secretory machinery already operational in ***B. subtilis*** may be more easily exploited for more efficient production of plasma proteins. The fact that this feature of ***Bacillus*** strains has been exploited in the manufacture of industrially used proteins provides encouragement that the organism can be made to secrete recombinant plasma proteins.

Purification of protein products from recombinant organisms may be accomplished in a variety of ways. In many cases the purification scheme will depend in large part upon the biochemical properties of the protein to be isolated. One of the more specialized and successful methods available makes use of monoclinal antibodies (see subsequent discussion of “immunoglobulins” for an explanation of this new technology). If a monoclinal antibody produced against the recombinant protein is coupled to a solid matrix, it is possible to pass a crude mixture of proteins containing the protein of interest over the matrix, and only the protein recognized by the antibody will be retained. The desired protein can then be recovered by a variety of methods (72,587). This approach has proven successful for the large-scale, one-step purification of interferon from recombinant bacteria (428).

Application of Recombinant DNA Technology to Large-Scale Production of Plasma Proteins

The proteins currently recovered from the fractionation process are albumin, factor VIII, prothrombin complex and immunoglobulins. Other proteins with potential clinical application cannot be recovered in high yield from plasma or can be recovered only at the expense of another product. In addition, transfusion of many plasma fractionation products carries the ***risk*** of ***infectious*** disease, such as viral hepatitis and AIDS. It is for these reasons that recombinant DNA technology is believed to hold much promise. The gene sequences coding for a number of plasma proteins have been cloned, but numerous problems must be resolved before this new industry can effectively compete with conventional plasma fractionation.

The recombinant DNA industry will also likely attempt to clone genes coding for newly identified, clinically useful plasma proteins. This will probably be the easiest task facing genetic engineering technology, however, since the techniques discussed in the previous sections have proven so successful in the isolation of virtually any desired gene.

A major challenge facing gene cloning technology is scaling up production sufficiently to meet demand. It is likely, therefore, that large-scale production / purification capabilities must be available before genetic engineering can meet even the U.S. demand for plasma products. For some plasma products such as albumin, thousands of kilograms will have to be synthesized and purified. For products such as Factor VIII, such large amounts will not be required, but purification methods that will not inactivate this highly unstable molecule will have to be developed. Thus, scale-up will involve challenges in both the production of a recombinant plasma protein and its subsequent purification.

An additional problem to resolve will be to design biological systems for modifications after genetic expression for those plasma proteins that need such modifications to be functional. If the gene coding for a plasma protein that needs to

have a carbohydrate molecule attached (i.e., glycosylation) is cloned in a bacterial cell which is incapable of doing so, a means of properly glycosylating the protein once it is purified from the recombinant bacteria must be devised. Alternatively, the gene may be cloned into yeast or mammalian cells which, under proper circumstances, may correctly glycosylate the protein. While glycosylation is perhaps the major form of post-translational modification, other modifications are sometimes necessary for protein activity.

Each plasma protein will, therefore, have its own specific problems. In the discussion that follows, the general status of cloning the genes that code for the plasma proteins of demonstrated or potential clinical value is reviewed.

Albumin. -Albumin is the major single protein species in plasma, with a normal concentration of approximately 70 mg/ml (371). It performs many functions, including maintaining the oncotic pressure of blood and serving as the carrier molecule for fatty acids and other small molecules in plasma **(467).** Albumin is synthesized in the liver and is the major protein product of that organ. The large amount of albumin synthesized by the liver is reflected in that tissue's abundance of albumin-specific mRNA, averaging 5 to 10 percent of the total mRNA in liver (474). With such a high concentration, it has been a fairly straightforward task to clone the cDNA that codes for albumin.

The gene for human albumin is probably the first plasma protein gene to be cloned, and numerous recombinant DNA companies have publicly claimed to have both recombinant yeast and bacterial strains carrying the human albumin gene (table 43). In addition to the companies listed, there are likely to be others that have all or part of the albumin gene cloned.

One company, Genentech, Inc., has also published the detailed construction of a recombinant E. ***coli*** that produces albumin (336). In these experiments, the albumin gene was linked to the DNA that regulates the utilization of the amino acid, tryptophan, in E. coli. In other words, the human gene was under the control of a segment of bacterial DNA that responded to the concentration of tryptophan in the medium. Under conditions of tryptophan starvation, the tryptophan genes as well as the albumin gene would be activated.

Table 43.-rDNA Companies That Are Working on Cloning the Gene for Serum Albumin

1. Genentech, Inc., San Francisco, CA (in conjunction with Mitsubishi Chemical Industries, Japan)
2. Biogen, Inc., Cambridge, MA
3. Genex, Boston, MA (in conjunction with Green Cross of Japan and Kabi of Sweden)
4. Speywood Laboratories, England
5. Genetics Institute, Boston, MA (in conjunction with Baxter-Travenol)
6. Chiron Corp., Emeryville, CA

SOURCES: D. Clark, personal communication, 1984; and Marketing Research Bureau, Inc. (1982).

The majority of the albumin synthesized by the recombinant bacteria was found to be biochemically and immunochemically identical to native human albumin (336). However, the complex molecular configuration proposed for natural albumin (96,168) was not evaluated in the recombinant protein. Thus, the degree to which the natural and recombinant albumin molecules are identical in any way other than amino acid sequence and immunologic similarity remains unclear at this time. Interestingly, the recombinant E. ***coli*** of Lawn et al. (336) appeared to synthesize a small amount of albumin that was smaller in size than the native product, and may have represented incompletely synthesized albumin. Thus, not all the protein synthesized by this particular strain is comparable to native albumin.

The authors of the Genentech report do not give specific numbers for the rate with which albumin is synthesized by their bacterial strain(s). Rather, they state that the strain(s) make albumin at a "modest" rate (336). Other biotechnology companies claiming to have the cloned albumin gene have not published the biochemical properties of their product, its rate of synthesis by the host cell, or other technical information—all of which is regarded as proprietary.

In brief, it appears that the biotechnology industry has conducted the necessary initial research and development for production of recombinant albumin, and recombinant albumin production appears to be at the scale-up phase of production. But most companies appear to have this project

on a back burner for the present, given the considerations discussed below.

The first obstacle to be faced in producing albumin by rDNA technology is whether the technology can produce enough protein to meet present needs, and in a cost-effective manner. Currently, most developed countries use between 100 and 400 kg of serum albumin per million population per year (62). In the United States in 1982, approximately 87,500 kg of serum albumin was used (356), or a rate of use of approximately 387 kg/million population/year. To address the feasibility of producing sufficient albumin to meet U.S. demand (as well as the additional 57,5oo kg of albumin that was exported in 1982), an estimate of how much albumin could be synthesized by "state of the art" recombinant strains of bacteria or yeast is needed.

Although no rDNA company will divulge synthetic rates for individual proteins due to the proprietary nature of such information, it is general knowledge that in current expression systems the synthesis of a cloned gene product can constitute 10 to 50 percent of the total protein synthesized by the cell. The maximum achievable rate may vary significantly, however, for individual proteins (186,305,569). In general, the larger the protein the less efficiently it is synthesized by ***E. coli.***

To determine how much fermentation will be necessary to meet the U.S. consumption of albumin, two important assumptions must be made: 1) that **50** percent of the cellular protein synthesized by an optimized strain of bacteria will be albumin (in the optimized strains of E. ***coli*** producing human insulin, 50 percent of the cellular protein was insulin); and 2) that it will be possible to obtain 10 wet weight grams of bacterial paste from a liter of fermented culture (which, in fact, can readily be done). Of the 10 grams harvested, about 15 percent (1s grams) will be protein (34o). Thus, 0.75 grams of albumin could be obtained per liter of fermented bacteria (i. e., 1.5 g protein/liter of fermented culture x 50 percent of total protein being albumin).

Extending the calculation further: 87,500 kg of albumin equals 87.5 x 10^6 grams of protein. Dividing 87.5 x 10^6 grams by 0.75 per liter of culture obtains 116.7 x 10^6 liters (or about 31 x 10^6 gallons) of fermented culture per year that will be necessary to produce sufficient albumin to meet U.S. demand. In order to supply the worldwide market, 193 x 10^6 liters (or about 51 x 10^6 gallons) of fermented culture will have to be produced.

Production capabilities already in place in this country could ferment this volume easily. For example, a typical bakers' yeast production facility may have 6 to 1230,000 to 40,000 gallon fermenters in operation (286). These fermenters are capable (using yeast) of producing about 1-2 x 10^6 liters of ferment every day. Thus, if a recombinant strain of E. ***coli*** producing albumin at 0.75 g/liter of ferment and growing at a rate comparable to a yeast cell were given to such a production facility for albumin production, U.S. needs for albumin could be met within about 80 to 100 days of routine production. Worldwide albumin supply could be produced within a year.

The ease with which the albumin supply may be produced will, of course, depend upon the validity of the assumptions made. Once again, the assumptions were that a strain of ***E. coli*** is available that produces albumin at a rate comparable to the current rate of recombinant insulin production, and that this strain will multiply efficiently in a fermenter to yield 10 grams of cells per liter. In addition, it is assumed that the time necessary tore-seed a fermenter (i.e., the turnaround time) is comparable for yeast and bacteria. The first assumption regarding the albumin production rate is dependent upon the efficiency of the expression vector. But even if the maximum biosynthetic rate for recombinant albumin production were one-tenth that observed for insulin, the world market for albumin could be satisfied within a year by several companies with fermentation facilities comparable to those of Anheuser Busch in St. Louis.

Others, using more conservative numbers for the rate of recombinant albumin synthesis, arrived at a quite different conclusion; namely, that the current rDNA industry is not equipped to produce and process sufficient albumin to keep pace with worldwide, or even nationwide, demand (167). They estimated that 12.5 grams of albumin fractionated from plasma that currently sells for $25 will cost anywhere from $40 to $80 if pro-

duced by recombinant DNA technology. In their calculations, the rate of albumin production is assumed to be 10 percent of the bacterial protein, or a yield of about 100 mg/liter of ferment, compared to the previous calculation of sO percent and 750 mg/liter.

Their conclusion, however, is based primarily upon processing considerations. For example, they assume that 50 gallons of water will be needed to process each liter of plasma by standard fractionation techniques. Based on the yield of recombinant albumin per gallon of water used, and extending water usage to an annual rate, they conclude that 10 billion gallons of water would be needed to produce the albumin that is currently fractionated from plasma with only 50 million gallons of water. They estimate that the cost of the additional water and disposing the effluent from the processing would greatly increase the cost of the product.

However, the entire contents of the fermenter will not have to be processed. Processing recombinant E. coli is not the same as plasma fractionation. First, the bacteria need to be separated from the media in which they are growing. Separation is achieved by continuous-flow centrifugation. This technique is commonly used in the fermentation industry and has the capacity to centrifuge cells from 30,000 gallons of ferment in about 2 to 4 hours (286). Thus, only the cell paste which is obtained from the fermenter is processed. The amount of water used, therefore, will be much less than in conventional plasma fractionation.

Since it costs only about $400 to produce a ton of bacteria or yeast in a fermenter (169) and 2,100 tons of cells will be needed to produce the world market of albumin, the costs incurred in the scale-up of albumin (or any other plasma protein) are likely to come from purifying the protein rather than from producing it.

Initial scale-up for recombinant products, however, is likely to be quite expensive. Although Eli Lilly will not divulge the scale-up costs for recombinant insulin, it has been reported that their costs were $7o million over expectations (584). This has resulted in an average of 50 to 55 cents per dose for recombinant insulin, whereas the price for mixed bovine and porcine insulin averages 28 to 35 cents and for highly purified porcine insulin **44** to 52 cents.

Perhaps the greatest difficulty in producing clinically acceptable recombinant albumin is the purification of the protein of bacterial contaminant. Several biochemical techniques to fractionate protein on the basis of size maybe useful. Ultrafiltration is one such technique, in which a filter of controlled pore size retains molecules above a certain size while allowing smaller molecules to flow through. Thus, if albumin is significantly larger or smaller than the contaminants, it can be easily separated from them on a large scale by filtration. However, for a membrane filter to effectively separate two molecules, their respective molecular weights must differ by tenfold. Thus, in practice, ultrafiltration may prove incapable of purifying recombinant albumin. Alternatively, purification of albumin on a column containing a resin that separates molecules by size is a further refinement of the size-separation technique, although not so easily scaled-up.

Recombinant albumin may also be purified using techniques that exploit its affinity for specific molecules. For example, albumin is known to bind fatty acids, so chromatographic resins that are “fat-like” in their chemistry may prove useful. Likewise, electrostatic attractions between albumin and oppositely charged molecules (i.e., ion exchange chromatography) may prove effective for purification. In fact, this technique is currently being used by some plasma fractionates in the purification of albumin (119). All of these methods can be scaled-up for industrial production, and should any one prove ineffective, a combination of two or more may purify recombinant albumin to an extent sufficient to meet safety requirements.

Scale-up in other countries maybe more cost-effective. Japan, for example, has considerable experience in bioprocessing technology (549). In addition, there are extensive fermentation facilities in Japan, and many such corporations have ties with U.S. rDNA companies. Furthermore, the Japanese government has targeted biotechnology as a key industry and has provided tax incentives and other subsidies to bioprocessing. Thus, it may be cost-effective to produce high-volume prod-

ucts like albumin in Japan, and import the product to the United States.

Finally, the cost considerations discussed above were based on the rate with which the protein product is synthesized and how the product is recovered from the recombinant organism. It is very likely that further R&D will result in more efficient protein production, thus reducing the cost of the final product.

One possible method to increase the efficiency of production involves use of other strains of bacteria, such as ***Bacillus subtilis,*** or yeast. ***B. subtilis*** is a common, non-pathogenic, soil bacterium that has been used industrially for large-scale production of proteins used in detergents and in the processing of corn starch. The annual production of these proteins by ***B. subtilis*** ranges in the thousands of tons. ***B. subtilis*** has attracted the interest of the rDNA industry because of its great biosynthetic capability. When grown under suitable conditions, it can produce up to 10 to 15g/liter v. 1.5 gram/liter for E. coli (186,265). In addition, products made by ***B. subtilis*** are excreted into the medium rather than accumulated intracellularly, as is the case with E. ***coli.***

Thus, if the gene coding for albumin were expressed in ***B. subtilis,*** the yield would be far greater than with E. ***coli,*** and the product would accumulate in the medium rather than in the cells. This would facilitate subsequent purification and processing and possibly continuous fermentation without re-seeding. To date, however, the use of ***B. subtilis*** for production of recombinant proteins is still several years behind the E. ***coli*** system.

Expression of recombinant albumin in yeast also lags behind the ***E. coli*** system, yet offers certain advantages for scale-up. Yeast, like ***B. subtilis,*** have been grown industrially for years, and considerable experience exists in large-scale bioprocessing techniques. In addition, the wet weight yield of yeast in a fermenter is about 2.5 times the wet weight yield of ***E. coli.*** Since current strains of recombinant yeast can accumulate protein to a level comparable to E. coli (569), the yield of recombinant protein should be increased 2.5 times per liter of ferment.

Also, yeasts have the capacity to secrete certain recombinant proteins into the medium. For example, a group from Chiron Corp. (543) synthesized the gene coding for the hormone urogastrone, and were able to get the gene expressed in a yeast system. In their report, the protein accumulated intracellularly to a level of approximately 30 mg per liter of non-fermented yeast culture. The Chiron group has now extended its early work and has linked the urogastrone gene to a gene coding for a yeast protein that is secreted into the medium (214). Thus, there is successful exploitation of a natural process in yeast, resulting in secretion of the recombinant protein.

Success of the secretion process also depends upon the individual properties of the recombinant protein, with small proteins being more efficiently secreted than larger ones. The secretion rate for the small hormone urogastrone, for example, is in the range of 20 mg per liter of ferment. But according to industry spokemen, a protein as large as albumin would not be secreted by this mechanism. Thus, different methods will have to be developed for successful secretion of albumin and other larger proteins by yeast cells. But even if the protein is not secreted, yeast offers an attractive approach to producing albumin in a very cost-effective manner.

One potential disadvantage to use of the yeast system in which the recombinant product accumulates intracellularly is the difficulty ***en***countered in breaking yeast cells open to recover the recombinant protein. The yeast cell wall is very resistant to treatments such as detergent, osmotic shock and even sonic disruption, thus complicating initial processing procedures. Furthermore, the harsh procedures employed to break open the cells can have a denaturing effect on the biologic activity of the protein to be isolated.

In summary, current technology employing ***E. coli is*** capable of producing recombinant albumin to meet worldwide demand. Attention is now focused on the purification of recombinant albumin and whether this can be accomplished in a cost-effective manner. It is possible that sophis-

ticated purification techniques will be efficacious but not cost-effective. Alternatively, albumin may be produced in a more cost-effective manner in other cloning systems (e.g., ***B.*** subtilis) where removal of hazardous contaminants may be less of a problem. However, these systems are not as well developed as is ***E. coli,*** and more research and development will be required. However, the ability to produce albumin by recombinant technology in a cost-effective manner will probably be achieved; exactly when this will happen is uncertain.

Coagulation Proteins. —Blood coagulation involves a large number of plasma proteins, at least one tissue protein, phospholipid membrane surfaces, calcium, and platelets (148,287). Coagulation of blood can occur via one of two mechanisms, known as the intrinsic and extrinsic pathways. The end result of both pathways is the conversion (activation) of the plasma protein prothrombin to thrombin, which then converts the soluble plasma protein fibrinogen to an insoluble fibrin clot.

Factor VIII.—Currently, most Factor VIII is obtained by fractionating fresh-frozen plasma. Factor VIII is generally quantitated in "units of activity" and in the United States in 1982, approximately 500 million units of Factor VIII were prepared by plasma fractionation (356). The 500 million units of Factor VIII have been estimated to constitute only about 280 grams of purified protein.

The Factor VIII molecule itself appears to consist of at least two proteins (63,178,339,378). One of these is known as Factor VIII:von Willebrand's factor (VIII: vWF). Deficiency of this protein is the underlying cause of the bleeding disorder known as von Willebrand's disease. The other component of the Factor VIII complex (VIII:C) appears to be a glycoprotein with an apparent molecular weight of around 200,000 (178,287). It is VIII:C that is lacking in hemophilia A patients and the molecule that the rDNA industry hopes to produce using gene splicing techniques. The world market for recombinant Factor VIII:C has been estimated to be as high as $2 billion (255).

The site of synthesis of factor VIII:C is not conclusively known, but recent evidence points to the cells lining the hepatic sinusoids (516a). The normal circulating concentration of factor VIII:C is approximately 100 nanograms per milliliter of plasma, and the protein is very unstable. Information regarding the molecular nature of factor VIII:C has been slow to accumulate. Consequently, the approach that has been taken to obtain recombinant-made Factor VIII:C has been to synthesize an oligonucleotide probe based on the amino acid sequence of the molecule, which was then used to screen bacteria containing human DNA (535). Using this technique, segments of human chromosomal DNA that code for portions of factor VIII:C have been identified, and the approach has been to splice these segments together to construct the complete factor VIII:C gene. A list of the companies claiming to have cloned at least a portion of the factor VIII:C gene is shown in table 44.

The clones identified in this manner were constructed with chromosomal DNA, and, therefore, the factor VIII:C gene exhibits the exon/intron gene structure characteristics of mammalian chromosomal DNA, which are not present in bacterial DNA. Recall that bacteria are incapable of properly expressing genes that consist of exons and introns. Thus, investigators will have to splice together the regions of the factor VIII:C gene that actually code for the protein in a bacterial expression vector in much the same way as were the cDNAs for albumin and interferon discussed earlier.

While a factor VIII:C gene spliced together from the separate gene segments may be necessary for expression of the gene by bacteria or yeast cells,

Table 44.-Companies Working on Cioning the Gene Coding for Factor Viii

1. Genentech, Inc., San Francisco, CA (in conjunction with Speywood Laboratories)
2. Genetics Institute, Boston, MA (in conjunction with Baxter-Travenol, Deerfield, IL)
3. Biogen, Cambridge, MA (in conjunction with Feijin in Japan and Kabi in Sweden)
4. Armour Pharmaceuticals, Kankakee, IL
5. Integrated Genetics, Boston, MA
6. Chiron Corp., Emeryville, CA

SOURCES: D. Clark, personal communication, 1984; Marketing Research Bureau, Inc. (1982); *Biotechnology Newswatch*, May 7, 1984.

an intact chromosomal gene with its exon/intron organization may be suitable for expression by mammalian cells. In fact, techniques exist for the efficient introduction of foreign DNA into a variety of cultured mammalian cell lines (585), and results from several studies have shown that the genetic information coded in a clone of human chromosomal DNA can be properly expressed by a mouse cell line. Because this approach may prove feasible, virtually all the rDNA companies working on cloning factor VIII:C are considering mammalian expression systems.

Several problems will be encountered in synthesizing factor VIII:C by rDNA methods. First, the molecule is fairly large in size (i.e., molecular weight of approximately 200,000). To date, the largest human protein efficiently made by bacteria has been albumin, which has a molecular weight about a third that of factor VIII:C (i. e., 68,000 vs. 200,000). But in the E. coli strain used to synthesize albumin as described by Lawn et al. (336), a significant proportion of the albumin made was smaller in size than the native molecule. These results suggest that when large recombinant proteins are produced by E. ***coli,*** incompletely synthesized proteins may also appear. Cloning the factor VIII:C gene in yeast or mouse cells may overcome this problem. In addition, it is possible that only a portion of the factor VIII:C molecule is required for the activity of the entire molecule, so DNA coding for the smaller fragment alone might be cloned and efficiently expressed in a bacterial system.

Another potential problem is that factor VIII:C is a glycoprotein, and it is still unclear whether or not the associated carbohydrate is important for procoagulant activity **(600).** If carbohydrate is important for coagulant activity, the factor VIII:C molecule synthesized by recombinant bacteria somehow has to be glycosylated before it is clinically effective. Alternatively, the factor VIII:C gene could be cloned and expressed using hosts capable of glycosylating proteins (e.g., yeast or mammalian cells).

One final problem to be faced by the rDNA industry in producing factor VIII:C will be the inherent instability of the molecule. Factor VIII:C is extremely sensitive to proteases (enzymes that degrade protein), and recombinant organisms synthesizing factor VIII:C will have to be engineered to have low levels of protease. Furthermore, since disrupting virtually any cell results in the release of proteases, it would be desirable for the recombinant organism to secrete the molecule into the medium.

Because of our lack of knowledge regarding the precise molecular biology of factor VIII:C, it is unclear how difficult or cumbersome it will be to produce the recombinant-made product. Thus, it is impossible to estimate its potential costs. But availability of the product and its safety with regard to infectious diseases are also important, and recombinant factor VIII:C should be a far better product than the plasma derivative. Since the worldwide market for factor VIII:C has been estimated at only 280 grams, recombinant organisms synthesizing factor VIII:C, even inefficiently, should have little problem producing this amount.

Several rDNA companies have publicly announced that recombinant factor VIII:C will soon be available. Genetics Institute, for example, has claimed that it hopes to begin testing recombinant factor VIII:C in about 2 years (216). Genentech has announced that it had cloned the entire Factor VIII:C gene and inserted it into mammalian cells, which then made and secreted Factor VIII:C into the culture medium. The gene was found to consist of 26 exons separated by introns and consisted of about 200,000 nucleotides, of which about 9,000 comprised the exon segments. Laboratory experiments showed that the product, about 4 times larger than albumin, was biologically active (484). These findings were published in November 1984 (226,536,570,592). The next steps to be undertaken include increasing the amount of Factor VIII:C produced by the cell line, scale-up, purification and producing a homogeneous product, and pre-clinical and clinical testing.

Factor IX.—Factor IX is another plasma coagulation factor whose congenital deficiency results in the bleeding disorder known as hemophilia B (148,287). Although less common than hemophilia A, hemophilia B can be equally severe, and treatment is by infusion of Factor IX complex (404). Factor IX complex actually consists of several procoagulant proteins, including Factors II, VII, IX and X. Thus, it can be used for

treatment of the congenital deficiency for each of these factors as well as for other hemorrhagic conditions (404). Like plasma-derived Factor VIII, however, treatment with Factor IX complex presents an increased risk of infectious disease.

The market for Factor IX is fairly limited because of the relative rarity of hemophilia B and an ample supply of Factor IX from plasma fractionation **(78).** A recent survey showed the Factor IX market to be only approximately $13 million in 1982, and that reflected a 15 percent gain over 1981 (356). The Factor IX market is not as great as for Factor VIII ($13 million v. $51 million in the United States), and some analysts believe the synthesis of recombinant Factor IX is not financially practical at this time (78). Nonetheless, several rDNA companies as well as academic laboratories have actually cloned the gene coding for Factor IX (115,290,323).

Factor IX consists of a single polypeptide chain of approximately 55,000 molecular weight (148, 287), containing 26 percent carbohydrate (148). It is synthesized in the liver in an inactive form and subsequently modified by glycosylation and by conversion of 12 molecules of glutamic acid to gamma-carboxyglutamic acid, a vitamin K-dependent process (571).

Upon activation in the coagulation process, Factor IX is cleaved into three polypeptide chains of 29,000, 16,000 and 9,000 molecular weight (MW) (287). The 9,000-MW fragment contains most of the carbohydrate associated with the entire Factor IX molecule, while the 16,000-MW chain appears necessary for its activation. The site for Factor IX coagulation activity is located on the 29,000-MW species (287). Circulating concentration of Factor IX is approximately 2 to 4 mg/ml (115).

The Factor IX gene clone was identified from a cDNA library generated by using mRNA extracted from human liver. An oligonucleotide probe was chemically synthesized based upon the known amino acid sequence of Factor IX. These oligonucleotides were subsequently radioactively labeled and used as specific hybridization probes against the liver cDNA library (115,290,323). The cDNA clone isolated by Kurachi and Davie (323) contained the entire coding sequence for Factor IX. In addition to the identification of cDNA clones, Choo et al. (115) have cloned the actual chromosomal DNA coding for Factor IX.

Production of recombinant Factor IX protein should be less difficult than making Factor VIII:C. The size of the polypeptide (i.e., 55,000 MW) is within the current limits of commonly used expression vectors. Furthermore, a report detailing the cloning of the Factor IX gene has already been published (323) and other clones have been constructed by some rDNA companies (535). Finally, an organism producing even a moderate amount of Factor IX should be capable of yielding sufficient amounts to meet market demands. However, the limited market and the availability of an ample supply of Factor IX fractionated from plasma, have together slowed the impetus to develop a recombinant Factor IX in favor of the more profitable Factor VIII:C molecule.

Production of recombinant Factor IX will not, however, be an altogether easy task. In the first place, the molecule is a glycoprotein, and it is not yet known how the activity of Factor IX is influenced by the associated carbohydrate. As with Factor VIII:C, it may be necessary to clone the Factor IX gene in a yeast or mammalian host capable of correctly glycosylating the protein. However, a large part of the carbohydrate associated with Factor IX is attached to the 9,000-MW fragment, which is cleaved from the molecule as it is activated (323). Thus, the carbohydrate portion of Factor IX may not function in coagulation. If this is the case, glycosylation of recombinant Factor IX may not be necessary to obtain a useful molecule.

A second, more challenging, obstacle to the production of functional recombinant Factor IX will involve the other major post-translational modification of the protein that normally occurs in the liver, the conversion of several N-terminal glutamic acid molecules of Factor IX to gamma-carboxyglutamate residues by a vitamin K-dependent enzyme system (416). This enzyme system is lacking in bacterial and yeast hosts (571). It may therefore be necessary to modify these glutamic acids by chemical means in order to produce a functional Factor IX molecule.

In summary, it does not appear that a great deal of industrial interest has been generated toward production of recombinant Factor IX. The lack of interest appears to result from the smaller market for the product as well as an abundant supply of Factor IX concentrate from plasma fractionation. Thus, development of recombinant Factor IX is likely to occur after the development of recombinant Factor VIII:C. Although the production of recombinant Factor IX will probably require some post-expression modification, chemical rather than enzymatic reactions may be used to accomplish that goal.

Other Coagulation Factors .—There is currently a great deal of scientific and medical interest in all of the other protein factors that participate in coagulation. As a result, virtually all of the coagulation factors have attracted the attention of rDNA companies. Should a market for these proteins develop, the genes for these additional factors will probably be identified using oligonucleotide probes synthesized from known stretches of the amino acid sequence of these proteins. Industrial development of these products is likely to occur after development of Factor VIII:C and Factor IX.

Plasma Enzyme Inhibitors. —There are numerous proteins in plasma whose physiological role is to inhibit proteases. Although these inhibitors are not currently fractionated from plasma on a commercial basis, they have gained widespread attention for their potential as therapeutic agents. The plasma proteins of interest are alpha 1-antitrypsin, anti-thrombin III, and C1-esterase inhibitor (496).

Alpha 1-antitrypsin. Alpha 1-antitrypsin inhibits the enzyme, neutrophil elastase, a potent protease that degrades structural proteins (335). In addition to inhibiting elastase, alpha 1-antitrypsin is capable of inhibiting a number of other proteases, including trypsin, chymotrypsin, collagenase, thrombin, kallikrein and plasmin (335). The inhibitor appears to act by combining with the protease to form a stable, inactive complex consisting of equal amounts of protease and inhibitor.

Alpha l-antitrypsin is a glycoprotein and is synthesized in the liver. The protein consists of a single polypeptide chain of approximately 50,000 MW (139) with three carbohydrate side chains (370). Its normal plasma concentration ***is*** about 2 mg/ml, and a low circulating level of the protein is often associated with chronic obstructive pulmonary emphysema and infantile cirrhosis of the liver (335). In essence, the continuous action of proteases on structural tissues, particularly in the lung and liver, results in autodigestion of the tissue. Alpha 1-antitrypsin inhibits this self-destructive process. At present more than 30 genetic variants of alpha 1-antitrypsin have been identified (138).

Preparations of alpha 1-antitrypsin are not currently available for routine use but are being evaluated in certain experimental protocols. The protein can be prepared from plasma using one of several methods. Polyethylene glycol precipitation has been used to prepare a crude concentrate of alpha 1-antitrypsin which retains some biological activity (210). Alpha 1-antitrypsin concentrates have also been made by further processing of the remaining plasma after the usual plasma fractions have been extracted (102). The product can be pasteurized to inactivate potential infectious contaminants.

The potential market for alpha 1-antitrypsin is uncertain. As of 1982, only 2.5 percent of the plasma fractionated in the United States was devoted to preparation of all inhibitor products (i.e., alpha 1-antitrypsin, anti-thrombin III, Cl-esterase inhibitor, etc.) (356). Patients suffering from a deficiency of the inhibitor require maintenance therapy, but the deficient condition is fairly uncommon. It is possible, however, that alpha 1-antitrypsin may play a role in the pathophysiology of other diseases, such as acute respiratory distress syndrome. Alpha l-antitrypsin therapy is currently being evaluated for its effect on such patients. Should the inhibitor be of benefit for this disease, its use could become quite common (496).

The gene coding for alpha l-antitrypsin has been cloned by at least one laboratory (323). This group synthesized an oligonucleotide probe based upon the known amino acid sequence of the molecule and used the oligonucleotide probe to screen a cDNA library prepared from liver mRNA. A

cDNA clone was identified that coded for the C-terminal region of the molecule (323). Given that Kurachi and co-workers reported on their work in 1981, a full length cDNA clone has most likely been isolated, and one company, Chiron Corp., reportedly has the entire gene coding for alpha 1-antitrypsin cloned in both yeast and E. coli (569).

In the yeast system, 20 percent of the total protein inside the cell during fermentation is alpha 1-antitrypsin. By performing a simple calculation, one can deduce that every liter of ferment of this yeast strain would yield almost 1 gram of alpha 1-antitrypsin, which is the concentration of the molecule in approximately one unit of whole blood. While the Chiron Corp. claims to have a strain of yeast synthesizing the protein, the company would not provide information regarding the efficacy of the recombinant product. Alpha-1-antitrypsin has been given "orphan drug status" by the FDA (193), and this designation will hasten the FDA review process and provide manufacturers with tax credits to offset development costs.

Since alpha 1-antitrypsin is a glycoprotein, it is possible that the associated carbohydrate is necessary for activity. The yeast host should be capable of glycosylation, but Chiron Corp. would not divulge whether or not its yeast expression system glycosylated the molecule correctly, if at all. Therefore, many of the glycosylation problems discussed earlier for the coagulation proteins may also be relevant to the synthesis of functional alpha 1-antitrypsin.

Antithrombin III. Among members of a family of plasma proteins with antithrombin activity, anti-thrombin III (ATIII) is the most potent and physiologically relevant inhibitor of the procoagulant protease, thrombin. In addition to inhibiting thrombin, ATIII significantly inhibits many other proteases involved in coagulation (496). The mechanism of ATIII inhibition, like that of alpha 1-antitrypsin, involves the binding of protease to inhibitor to form an inactive complex (114,448). The rate of inactive complex formation is greatly enhanced by heparin. This finding has led to successful use of ATIII in conjunction with low doses of heparin to prevent post-operative thrombosis (304).

Increasing awareness of the clinical importance of ATIII has also come from descriptions of hereditary deficiencies of the molecule. The frequency of congenital ATIII deficiency is approximately 1:3500 (410). Most cases of the inherited disorder are characterized by ATIII levels at approximately 50 percent of normal; levels less than 25 percent of normal are rare. Yet, families with even 50 percent of normal ATIII levels have a hyperactive clotting system.

A transient deficiency in ATIII levels can also be acquired, especially in patients with venous thrombosis from a number of causes, including chronic liver or kidney disease. ATIII depression also occurs in women taking oral contraceptives (496). Such women have an increased propensity for thrombosis, and the ATIII deficiency is felt by many to be the underlying cause. However, this theory is not firmly established.

Several varieties of ATIII concentrates are available commercially, but as was mentioned in the discussion for alpha 1-antitrypsin, 1982 plasma fractionation statistics indicate that production of inhibitor concentrates constituted only about 2.5 percent of the total plasma fractionated (356). But in reviewing the literature concerning ATIII, it becomes apparent that a much more significant market may exist for the product.

ATIII is a glycoprotein and member of the alpha-globulin family of plasma proteins. It has a molecular weight of 55,000 to 60,000 (1,114, 512). Much of the amino acid sequence of the protein is known (426). As a result, an oligonucleotide probe to identify the ATIII gene has now been prepared in several laboratories, with both the cDNA coding for the entire protein sequence and the entire chromosomal gene having been cloned in E. coli (114,448,512). The ATIII gene has not yet been cloned in any host other than E. ***coli.***

Although there has been no report of a recombinant cell that synthesizes ATIII, it should not be difficult to engineer such a cell, and several rDNA companies including Genex and Genentech have expressed interest (119,280). ATIII activity is present in stored blood (279), which suggests that the ATIII protein is a relatively stable molecule. Problems associated with production of recombinant ATIII should not present any unique

challenge, other than the potential need for glycosylation of the protein.

C1 Esterase Inhibitor. C1 esterase inhibitor is a protein that inhibits the enzyme of the same name. Deficiency of the inhibitor results in prolonged esterase activity, with swelling of the surrounding tissue (496). The most serious consequences of C1 esterase deficiency can be angioedema of the upper respiratory tract, with the potential for suffocation.

The inhibitor can be extracted from fresh-frozen plasma and is effective in treating acute episodes of angioedema (496). Androgen (male hormone) analogs are also successful in stimulation Cl esterase levels in deficient patients, and therefore the widespread use of C1 esterase inhibitor concentrates will probably never be realized. The gene coding for C1-esterase inhibitor has not yet attracted the attention of the rDNA industry and has probably not been cloned.

Plasminogen Activators.—Plasma contains an enzyme system capable of digesting the fibrin in blood clots, thus leading to clot dissolution. One component of the system consists of a family of proteins collectively known as plasminogen activators **(116).** Plasminogen activator (PA) converts plasminogen to plasmin, which then degrades the fibrin network of the clot to form soluble products (130). The system is elegantly specific in that the activating effect of PA on conversion of plasminogen to plasmin is dependent upon the presence of fibrin. Thus, the anticoagulant effect of the system is limited to the immediate site of a clot. This is in contrast to the more widespread effects of other clot dissolution agents such as streptokinase and urokinase, which are widely used clinically. In some cases the nonspecificity of these drugs can cause serious bleeding problems due to the digestion of fibrinogen as well as fibrin (7).

Because of the site specificity of PA, production of the protein by recombinant methods has received considerable attention. Recent commercial interest in PA has come as a result of advances in two areas of research. First, it was shown that the infusion of 7.5 mg of a purified PA induced the dissolution of a 6-week-old thrombus without concomitant bleeding problems (580). Second, the PA used in this study was harvested from a melanoma cell line that overproduces PA and secretes it into the medium (461). This PA was found to be identical to the PA isolated from normal tissue (461). The 7.5 mg of PA used to treat this patient (at a cost of about $2,000) was harvested from about 75 liters of spent culture medium from the melanoma cell line (7).

PA is composed of a single polypeptide chain (578), and has a molecular weight of 70,000 (170,422). It is presumed that PA is the normal vascular regulator of clot dissolution and is synthesized and released into the circulation by vascular endothelial cells in response to the proper stimuli. The amino acid sequence of much of the molecule has been determined (578). Research on PA has been hampered by its extremely low concentration in blood, tissue extracts, and cell culture medium. However, since the amino acid sequence of the protein was known, it was relatively easy to synthesize an oligonucleotide probe for screening a cDNA library prepared from mRNA extracted from the melanoma cell line that overproduces PA (170,422).

Furthermore, Pennica et al. (1983) of Genentech, Inc. have cloned the entire coding sequence for PA and have engineered an E. coli cell to produce recombinant PA. In addition to Genentech, Inc., Integrated Genetics, Abbott Laboratories, Biogen, Collaborative Research and HEM Research have all been involved in PA research and gene cloning (119). Currently, Collaborative Research and Abbott Labs market PA synthesized by the melanoma cell culture method (582). It is likely that both companies have the gene sequences for PA cloned as well. It has been estimated that recombinant PA can be synthesized at l/200 to 1/500 the cost of the product secreted by the melanoma cell line (7).

The future market for PA in the United States is estimated to be 400 million to 2 billion dollars (215). Because of the clinical potential of PA, and the difficulty incurred in purifying it from natural sources, recombinant PA is likely to have little competition for a potentially expanding clinical market. Initial results of Pennica et al. (422) suggest that a biologically active recombinant product can be produced by E. ***coli*** and optimiz-

ing the level of expression in the recombinant strain should be straightforward.

Immunoglobulin.The plasma fractionation industry currently isolates immunoglobulin fractions from plasma of donors hyperimmunized against known antigens. In 1970, production of immunoglobulins was significant, accounting for some **10,000** kg of total production **(404).** Of this, approximately 900 kg was immune globulin of known titer and specificity. As of 1982, the market for immune globulin had not expanded and, in fact, may have decreased slightly. Immunoglobulins are used for therapy and prophylaxis of tetanus, poliomyelitis, and other viral agents, including hepatitis (356,404).

Production of immunoglobulin using methods other than plasma fractionation will probably not utilize recombinant DNA technology. Instead, cell fusion techniques, originally described by Kohler and Milstein (318), will enable hybrid cell lines to be constructed that secrete antibodies of known specificity. A clonal population of such hybrid cells is known as a hybridoma, and the antibody product of the cell is said to be "monoclinal" because the antibodies are identical in structure and reactivity. Antibody-secreting cell lines (hybridomas) are routinely prepared in numerous academic and industrial laboratories. In fact, the number of new companies founded to produce and market hybridoma technology is almost equal to the number specializing in gene cloning. However, the major focus of the industry to date has been in the development of monoclinal antibodies as diagnostic reagents.

Construction of a hybridoma that secretes a useful antibody essentially involves the physical fusion of an antibody secreting lymphocyte isolated from an immunized host with a myeloma tumor cell line that multiplies rapidly in cell culture. The fusion process can occur simply by mixing the cell types, but is greatly facilitated in the presence of the chemical, polyethylene glycol. The resulting hybrid cells exhibit the properties of the two parents; namely, secretion of a specific antibody from the lymphocyte and capacity for unlimited growth from the myeloma. A mixture of hybridomas resulting from a fusion can be screened to select the hybrid that secretes the desired antibody. Once identified, the hybridoma is capable of providing an inexhaustible amount of the antibody.

To date, essentially all hybridomas are constructed using mouse or rat lymphocytes and mouse myeloma cells. The limited application of hybridoma technology to development of diagnostic reagents has resulted from this fact, since administration of a rodent antibody to humans would result in potent immunization against that antibody. The use of hybridomas employing human lymphocytes and human myeloma cells has been slow to develop. However, several reports have appeared claiming to have produced human hybridomas (321,414a,431), and human monoclinal antibodies reacting with tetanus toxoid have been produced using the cell fusion technique (223,332,414a). Unfortunately, a human myeloma cell line capable of producing stable hybridomas has not yet emerged. Therefore, human hybridoma technology has not yet developed sufficiently for the isolation of a wide variety of clinically suitable monoclinal antibodies.

Human hybridomas have also been produced through viral transformation of peripheral blood lymphocytes with Epstein-Barr virus (EBV) (515). The antibody-secreting lymphocytes are converted to leukemia cells with EBV, and therefore grow in culture yet still secrete their antibody product. Although viral transformation has been used to construct hybridomas, those produced using this technique reportedly do not secrete large amounts of antibody, and production is unstable (98,414a).

Replacement of plasma fractionation by hybridoma technology as a source of immunoglobulin is not likely to occur in the next few years. Although some useful human hybridomas have been developed, the industrial scale-up of antibody production is still in its infancy. One company, Celltech in Great Britain, has pioneered the scale-up of monoclinal antibody production, but their experience is primarily with diagnostic antibodies used for tissue typing or imaging of tumors.

Although most hybridoma companies are concentrating on the mass production of mouse an-