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Neohemocytes

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NEOHEMOCYTES: HEMOGLOBIN-CONTAINING LIPID VESICLES

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of
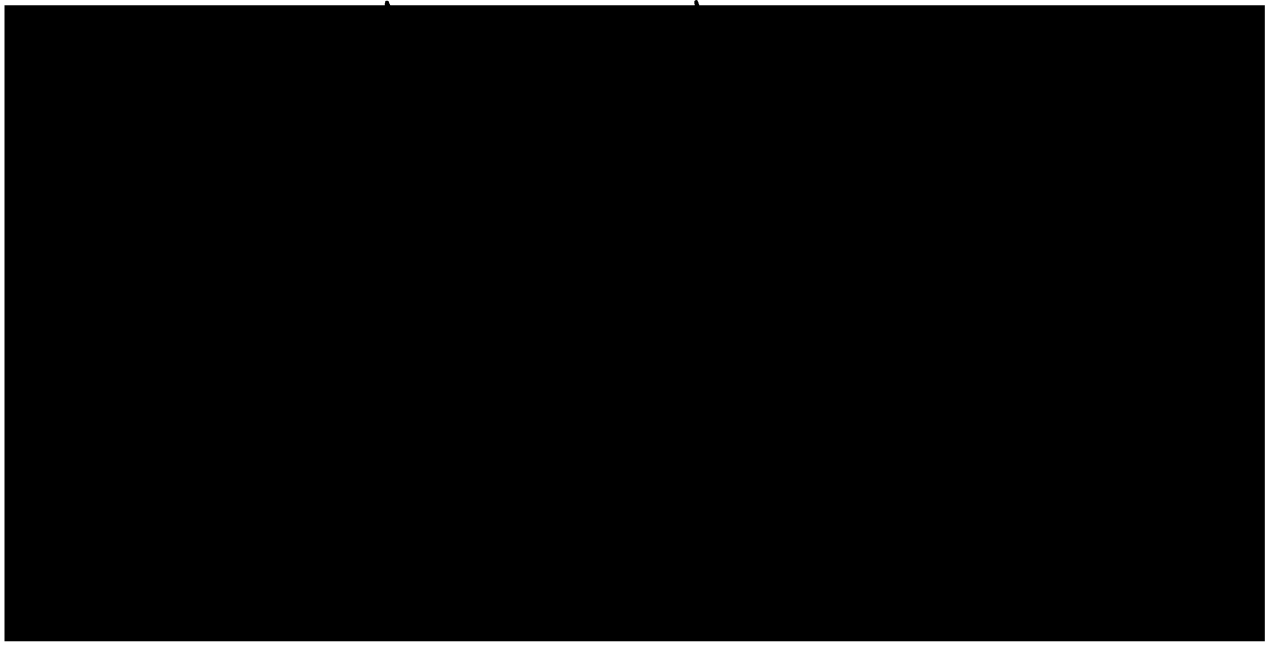
DOCTOR OF PHILOSOPHY

in

Pharmaceutical Chemistry

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

To My Father, Mother, Sister
and Wife

ACKNOWLEDGEMENTS

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Abbreviation	Meaning
ADP	adenosine diphosphate
ANSA	1-amino-2-napthyl-4-sulfonic acid
aPTT	activated partial thromboplastin time
ATP	adenosine triphosphate
CH	cholesterol
CyanmetHb	cyanmethemoglobin
DPG	2,3-diphospho-D-glyceric acid pentasodium salt
EM	electron micrograph
Hb	hemoglobin
HEPES	hydroxyethylpiperazine-N'-2-ethanesulfonic acid
^{125}I-BPE	radioiodinated p-hydroxybenzamidine phosphatatidylethanolamine
IODO-GEN	1,3,4,6-tetrachlor-3a,6a-diphenylglycouril
IHP	inositol hexaphosphate
IPP	inosital pentaphosphate
Km	Michaelis-Menten Constant
MLV	multilamellar vesicle
MetHb	methemoglobin
n	Hill number
NH	neohemocyte (s)
NH-30	NH prepared at 30 mosm
NH-30-300	NH prepared at 30 mosm and subsequently equilibrated with 300 mosm
NH-300	NH prepared at 300 mosm

List of Abbreviations

Abbreviation	Meaning
P	pyridoxal phosphate
P_{50}	the partial pressure of oxygen at which 50% of the Hb molecule is saturated with oxygen
PA	sodium dipamitoyl L-alpha-phosphatidate
PBS	phosphate buffered solution
PBS-30	30 mosm phosphate buffered solution
PBS-300	300 mosm phosphate buffered solution
PC	egg phosphatidyl choline
PT	prothrombin time
PTT	partial thromboplastin time
RBC	red blood cell
RES	reticuloendothelial system
SD	standard deviation
SUV	sonicated unilamellar vesicle

ABSTRACT

Prototypal artificial red cells, neohemocytes, were prepared by microencapsulating a concentrated (16 g/dl) solution of purified, nonantigenic human hemoglobin, Hb, and 2,3-diphosphoglycerate (pH 7.4) using a lipid membrane system. The membrane composition was phosphatidic acid, phoshatidylcholine, cholesterol and α-tocopherol in a molar ratio 1:4:5:0.2. The preparation procedure, a modification of the REV liposome procedure, produces no significant Hb denaturation and yields neohemocytes 0.2 to 1 μm in diameter. The transfusion suspension contained neohemocytes in a 0.9 g/dl sodium chloride solution including 3 g/dl bovine albumin and had an apparent hematocrit of 25. The Hb concentration was approximately 4 g/dl, P_{50} approximately 26 mm HG and the Hill number was approximately 2.1. Rats which were transfused with a control, physiologically compatible suspension of 4 g/dl Hb solution until their RBC hematocrit reached or dropped below 4, survived an average of only 4 hours. Rats similarly transfused with a neohemocyte suspension have completely recovered. Pathological evaluation of sacrificed animals revealed mild, reversible hyperproteinemia, probably due to the initial lysis of some neohemocytes.

CHAPTER I INTRODUCTION

Blood substitutes fall into two categories: plasma expanders and blood substitutes which can transport oxygen and carbon dioxide. Substantial progress has been made in the use of plasma expanders and research has now produced several systems which can incorporate the advantages of a plasma expander along with the ability to transport oxygen and carbon dioxide.

Much can be accomplished with an agent which has the same oncotic pressure and osomotic properties of blood, and can mimic blood gas transport (Table I.1).

For example, an artificial blood substitute could be useful in sickle cell anemia if it could provide sufficient oxygen to avert the periodic crisis associated with the disease. This artificial substitutes should be universal in that one type would work for all people. This would avoid situations where inadequate supplies of a rare blood type would pose a problem. The agent would be free of hepatitis and could be used in surgical procedures where large volumes of blood were required. The patient's blood could be removed and reinfused after the surgery was over. Total body washout could be performed, thus removing toxic sub-stances present in the blood. For example, myocardial depressant factor is released into the blood when shock ensues,so it is possible that by doing a total exchange

Anemias
  Aplastic anemia
  Sicle cell anemia
  Cancer-associated anemia
Anaerobic infections
Blood loss
  Operative procedures
  Extracorporeal techniques
  Irreversible shock
Vascular occlusions
  Thrombosis
  Atherosclerosis
Toxicity screening
Chemo- and immunotherapy
Hormone transport and metabolism
Enzyme depletion and repletion
Studies on hematopoiesis
Studies on blood-brain barrier
Studies on problems such as sleep
Studies on plasma protein metabolism
New approaches to the study of circulatory
    physiology and hemodynamics
Rheology investigation
Research on lymph and lymphatics
Animal surgery and veterinary medicine
Organ perfusion and preservation
Total body washout for removal of toxins
    viruses, etc.

TABLE I. 1.   Potential uses of artificial blood substitutes
                (Taken from Geyer, 1975)

transfusion with an artificial blood substitute the blood volume could be restored while simultaneously removing myocardial depressant factor. In animal studies new substance toxicities could be evaluated in an environment that would preclude interaction with blood components. Toxicity could then be assessed in normal blood. The differences observed or lack thereof would provide an indication of the role whole blood plays in modulating toxicity.

Hormone transport, metabolism, and feedback can be very complex processes, one reason being the many factors present in the blood which may affect them. In a total washout transfusion these variables could be minimized (Geyer, 1975). Preexisting hormones or transport proteins could be removed from the circulatory system and selectively added back in order to assess their role. Enzymes could be removed and added in order to selectively assess their role in the vascular system.

Artificial blood substitues could be used in organ preservation, or in isolated organ perfusion (e.g., liver studies) to assess metabolic pathways independent of endogenous components normally present in blood. They could provide an ideal means to study circulatory physiology, for example, by varying viscosity, oncotic pressure, osmotic pressure and buffering systems. It could also be used as an universal source in animal surgery. There currently are no blood banks available for animals. Also for a rare species

one might not be able to find any compatible blood.

As can be seen there is a great need for the development of a useable blood substitute. It was these needs that provided the motivation behind this research.

Early Blood Substitute Research: Outside of using whole blood as a blood source most initial work revolved around developing plasma expanders. Plasma, dextran, albumin and other preparations have been used but their obvious drawback was their inability to transport oxygen. Over the years perfluorocarbons have been developed as blood substitutes, and a commercial product may soon be available through Green Cross Corporation. Unfortunately, patients transfused with a perfluorocarbon emulsion require an enriched oxygen atmosphere so that the emulsion can carry sufficient oxygen to the tissues (Baldwin et al., 1982). Also, there is some concern that perfluorocarbon emulsions may effect the microcirculation (Endrich et al., 1979). Even whole blood has its shortcomings in that it must be typed and cross-matched, it has a relatively short storage life of approximately 35 days, and it can transmit hepatitis.

A major advance would be made if an oncotically active protein solution capable of transporting oxygen could be developed. Significant progress has been made along these lines with the development of pure hemoglobin solutions. Initial work has been reviewed by Amberson (1937) over the period of 1859 to 1937 in which hemolyzed blood or

hemoglobin solutions were used. Sellards and Minot (1916) injected large quantities of dissolved human hemoglobin for the first time into human beings. Despite initial excitement, research interest decreased with reports stating the occurrence of renal damage and methemoglobin formation after the administration of the hemoglobin solution. These problems have largely been removed by improved purification techniques. However, there still remain the following difficulties:

(1) Hemoglobin is cleared very rapidly from the circulatory system having a $t_{1/2}$ of approximately 100 minutes. The rapid removal of the hemoglobin (Hb) is due to the dissociation of the Hb tetramer into monomers which are then small enough to be filtered through the glomeruli (De Venuto, 1979).

(2) The loss of large amounts of protein from the body causes an osmotic diuresis leading to a fluid imbalance.

(3) Hemoglobin by itself has a higher affinity for oxygen than the hemoglobin present in a red blood cell (RBC). The reason for this is that the RBC also contains 2,3-diphosphoglyceric acid (DPG) which facilitates the release of oxygen to the tissues. This facilitated release of oxygen lowers the P_{50} (partial oxygen pressure at which 50 percent of the Hb is saturated with oxygen) of the Hb. When DPG is not present the P_{50} is

higher. This results in a decreased mixed venous oxygen tension, which presumably reflects lower tissue oxygen levels, and is associated with systemic vasoconstriction. Probably the decrease in P_{50} results in redistribution of cardiac work, such that pressure work is favored at the expense of overall blood flow. Clearly, then, normal bulk oxygen transport can occur despite the decreases in venous oxygen tension and tissue oxygen tension. This is only possible because the Michaelis-Menten constant (Km) of mitochondrial cytochromes for molecular oxygen is very low; thus, mitochondria have the ability to use oxygen at near-normal rates down to very low tissue oxygen tensions (3 to 5 mm Hg). However, this decreased tissue PO_2 has profound effects on circulatory regulation in the absence of deficits in venous oxygen content (Hauser et al., 1982). For example, Nemoto (1978) has stated the importance of catecholamine metabolism in the CNS in the development of shock states and mentions that monoamine oxidase depends on molecular oxygen at relatively high tension to degrade CNS catechols. Also, Duling and Berne (1970) have suggested that peripheral vessels are directly responsive to oxygen tension.

(4) Practically, one can not give a free hemoglobin solution any more concentrated than about 7g/100ml since one would exceed the oncotic pressure normally present

in the circulatory system. This would result in hemo-
dilution. Since normal blood has 15g/100ml of hemoglo-
bin, one can see a severe limitation of free Hb solu-
tions. In order to deal with the high oxygen affinity
of Hb, researchers initially tried to add DPG to the
Hb and transfuse this solution. This failed because of
the short intravascular persistence of DPG (Sunder-
Plassmann et al., 1975). Benesch (1972) has shown that
pyridoxal phosphate (P) can be covalently bound to the
hemoglobin molecule permanently increasing the P_{50}. In
vivo experiments performed by Gould et al. (1980)
illustrated that an isolated shift in P_{50} results in an
isolated shift in venous oxygen tension. Thus Hb-P
appears to be better suited to minimize tissue hypoxia.

(5) Venous oxygen tension tends to be very sensitive to
hemoglobin concentration. Therefore, can further
improvement be obtained by increasing the hemoglobin
concentration while at the same time not increasing the
colloid osmotic pressure beyond physiologically accept-
able limits? This problem has been approached by
attempting to polymerize the hemoglobin molecule. This
can be accomplished by polymerizing pyridoxylated hemo-
globin using glutaraldehyde as a cross-linking agent
(Gould et al., 1980). This procedure gives a final Hb
concentration of 15 g/100ml with a P_{50} of 16 mmHg.
Eighty percent of the polyhemoglobin had a molecular

weight in the range of 120,000-600,000. The $t_{1/2}$ for Hb-P was 4.5 hours as compared to 3^a hours for polyhemoglobin. In total exchange transfusion in rats all rats (N=3) exchanged with polyhemoglobin survived at Hct below 3%. One notable problem with chemically modified hemoglobins is that they are perhaps less stable on long term storage than are native ones. The modification seems to place a strain on the molecule, particularly the heme pocket, altering steric stability. This led to an increased rate of methemoglobin formation, which can not carry oxygen.

Encapsulated Hemoglobin as a Possible Blood Substitute: Hb was first encapsulated by Chang (1964) when he succeeded in producing polymer microcapsules which contained Hb. These microcapsules had sizes ranging from $1\mu m$ to $100\mu m$ and thus, were too large to be used in a transfusion. Djordjevich et al. (1980) was the first to encapsulate Hb and DPG into multilamellar liposomes. The resulting hemoglobin containing liposomes (hemosomes) were claimed to have a P_{50} and hemoglobin concentration similar to that of whole blood. However, when these hemosomes were exchanged for the normal blood in a rat, a survival time of only 100 minutes was obtained. This result was achieved with only one animal. This raises serious doubts as to the usefulness of such a blood substitute since a rat whose blood is replaced with a 7 g/dl Hb solution will survive for six hours. Despite

these results hemoglobin containing liposomes have the potential to circumvent many problems other blood substitutes face.

For example:

(1) The coencapsulation of DPG allows for an elevated P_{50} much like that of whole blood.

(2) Both the Hb and liposomes of the right lipid composition have been shown to be nontoxic by themselves (Gregoriadis et al., 1976).

(3) Due to the large doses given, the reticuloendothelial system (RES) uptake can be saturated allowing a much larger fraction of the dose to remain in the circulation (Abra et al., 1981).

(4) The entrapped Hb would not be filtered through the glomeruli. Thus, the entrapped Hb should remain in the circulation for a longer time than does Hb.

(5) The resulting product is a universal blood substitute, that is, no typing or cross matching is required.

Even with the above advantages liposome encapsulated Hb could have its own drawbacks such as:

(1) Inhibiting the RES such that the body's defense against bacterial challenges is compromised.

(2) Even though Hb and liposomes by themselves may not be toxic their combination might be. For example the pres-

ence of possibly some of the Hb attached to the liposome's outer membrane might cause an immune response.

(3) The liposome's membrane might be impermeable to bicarbonate ion thus upseting the body's acid base balance.

With these considerations in mind, despite the limited success of Djordjevich, we felt lipid vesicles could be a useful carrier for Hb. This thesis describes our efforts in the preparation and quantification of a hemoglobin containing lipid vesicle (neohemocyte).

CHAPTER II   BACKGROUND   INFORMATION

Introduction to Hemoglobin Function: Hb transports hydronium ions and carbon dioxide in addition to oxygen. The binding of oxygen by hemoglobin is regulated by specific molecules in the Hb environment: namely hydronium ions, carbon dioxide, anions such as chloride, phosphate, and organic phosphate molecules. These regulatory molecules profoundly alter the oxygen binding properties of Hb although they are bound to sites on the protein that are far from the heme. These interactions are termed allosteric interactions and occur in many proteins. Hb exhibits three kinds of allosteric effects:

(1) The oxygen-binding curve of Hb is sigmoidal, which means that the binding of oxygen is cooperative, and is unlike myoglobin which has a hyperbolic oxygen-binding curve due to non-cooperative binding of oxygen (Figure II.1).

(2) Hydronium ions and carbon dioxide promote the release of oxygen from Hb which is important in enhancing the release of oxygen in metabolically active tissues such as muscle. Conversely, oxygen promotes the release of hydronium ions and carbon dioxide in the alveolar capillaries of the lungs. These allosteric effects generated by hydronium ions, carbon dioxide and oxygen are known as the Bohr effect.
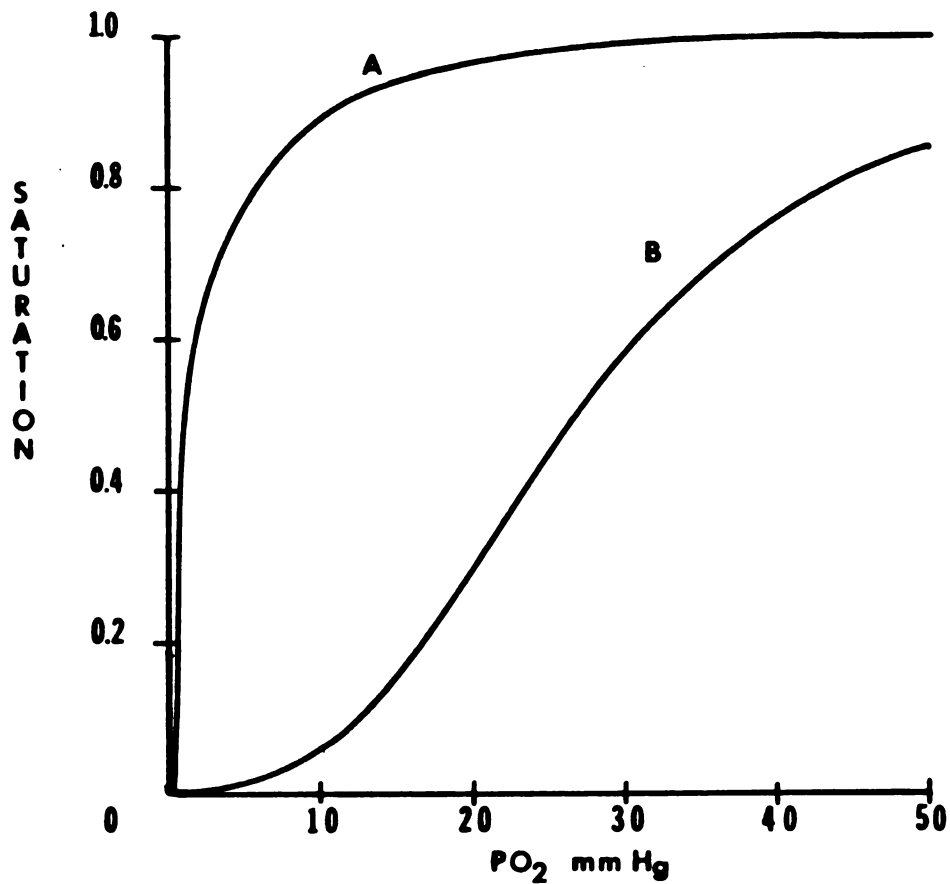
FIGURE II.1. Saturation of myoglobin (A) and hemoglobin (B)
as a function of the partial pressure of oxygen.
Myoglobin binds oxygen noncooperatively whereas
hemoglobin binds oxygen cooperatively. Adapted
from Stryer (1975).

(3)   The affinity of Hb for oxygen is further regulated by 2,3-diphosphoglyceric acid (DPG), adenosine triphosphate (ATP), other organic phosphates, chloride, and phosphate. These molecules all lower the oxygen affinity for Hb.

Allosteric Changes in Hemoglobin: Hemoglobin can be dissociated into four constituent chains $(\alpha_1, \alpha_2, \beta_1$ and $\beta_2)$. Hemoglobin's allosteric properties arise from interaction between these subunits. X-ray crystallographic studies have shown that oxy and deoxyhemoglobin differ markedly in quarternary structure. In oxyhemoglobin, the carboxyl terminal residues of all four chains have almost complete freedom of rotation. In contrast, these terminal groups are anchored in deoxyhemoglobin by noncovalent electrostatic interactions (called salt bridges) between oppositely charged groups. Deoxyhemoglobin also has four other electrostatic interactions between oppositely charged amino acid side chains not present in oxyhemoglobin. Thus, deoxyhemoglobin is more constrained than oxyhemoglobin.

Deoxyhemoglobin is designated as being in the tense (T) state and upon oxygenation the Hb molecule goes to the relaxed (R) state. This process is illustrated in Figure II.2. As oxygen progressively binds to Hb the salt bridges present in deoxyhemoglobin are increasingly weakened and finally broken allowing subsequent oxygen molecules to bind more readily, transforming the Hb molecule into the oxy or R

T STRUCTURE

ELECTROSTATIC
INTERACTION

WEAKENED ELECTROSTATIC
INTERACTION

R STRUCTURE
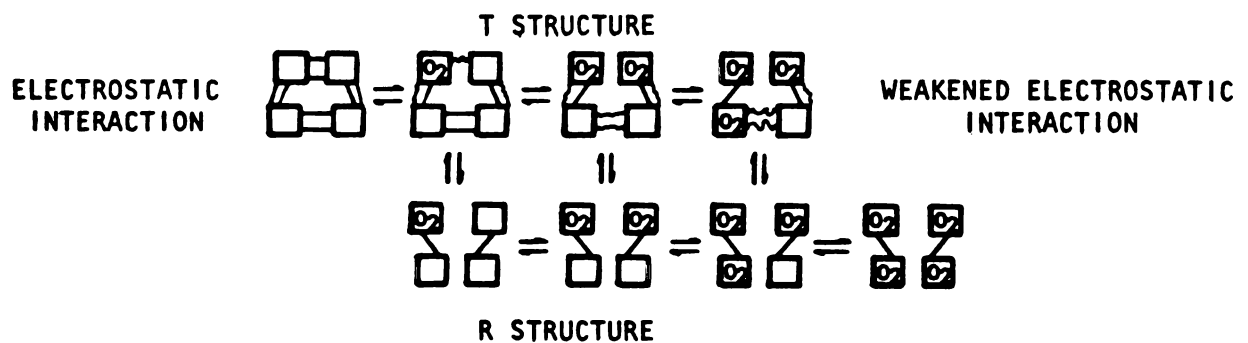
FIGURE II.2. Hemoglobin which does not have any oxygen bound
to it is in the tense (T) state. As the partial
pressure of oxygen is increased, oxygen begins
to bind to the hemoglobin. This weakens the
electrostatic interactions holding the hemoglobin
molecule in the T state. This decrease in
electrostatic interaction allows the hemoglobin
molecule to go to the relaxed state. Adapted
from Perutz (1978).

state. These interactions are the essence of the coopera-
tive effect of oxygen bonding to Hb.

In transition from oxy to deoxyhemoglobin, movement
along the $\alpha_1\beta_2$ contact region occurs (Figure II.3). Contact
between the two dimers has two stable conformations, one for
the T structure and one for the R structure. On transition
between the T and R structures the dimers move from one
position to the other. The dimers are stabilized by hydro-
gen bonds formed between amino acid chains attached to
opposing faces of the dimers.

The trigger for these allosteric changes is movement of
the iron atom after binding of oxygen (Figure II.4). The
iron atom in deoxyhemoglobin is 0.75 $\overset{o}{A}$ away from the plane
of the porphyrin, displaced toward the F8 proximal his-
tidine. The reason for an out of plane location of iron in
the deoxyhemoglobin is that the electron density distribu-
tion around the iron nucleus prevents movement into the por-
phyrin ring. Upon binding of oxygen to the sixth ligand
position of the iron, the electron density is such that the
iron-oxygen complex can move into the ring. This is the
primary structural change underlying all the allosteric
interactions in Hb. The movement of the iron atom upon
oxygenation or deoxygenation is transmitted to the histidine
attached to the iron. Since the histidine is connected to
the rest of the amino acids in the monomer, the motion of
the iron atom is translated into structural changes at the

T STRUCTURE   R STRUCTURE
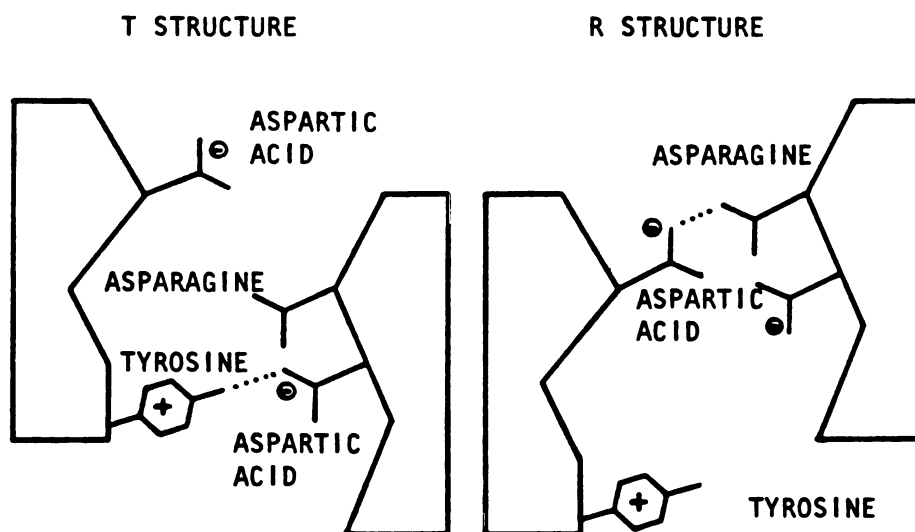
FIGURE II.3. As the hemoglobin molecule changes from the tense (T)
state to the relaxed (R) state, the hemoglobin molecule
is stabilized by alternative sets of hydrogen bonds
formed between adjacent amino acids. Adapted from
Perutz (1978).
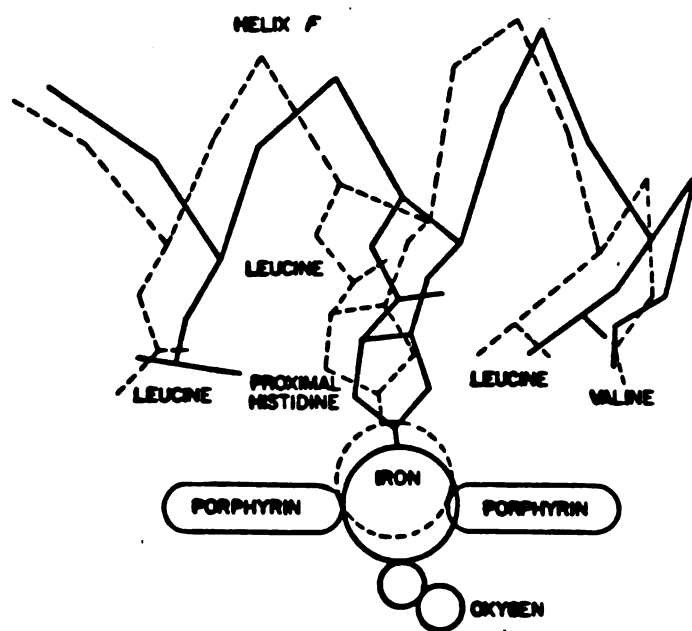
FIGURE II.4. The transition from the tense (T) state to the relaxed (R) state due to the movement of the heme iron into the porphyrin ring. Adapted from Perutz (1978).

interfaces between subunits. The binding of oxygen at one heme site is thereby communicated to parts of the molecule which are far away.

Effect of Organic Phosphates and Anions on Oxygen Affinity: Several organic phosphates (DPG, inositol pentaphosphate (IPP), inositol hexaphosphate (IHP), and ATP) and anions, such as chloride and phosphate, decrease oxygen's affinity for Hb. In all cases these negatively charged species undergo electrostatic interactions with positively charged amino acid residues on one monomer chain causing adjacent chains to be more constrained. These interactions favor the T state and thereby causes a decrease in oxygen affinity. In particular, Figure II.5 shows the mode of binding of DPG to human deoxyhemoglobin. DPG interacts with three positively charged groups on each β chain. On oxygenation, DPG is excluded because the central cavity becomes too small. Specifically, the gap between the H helices of the β chains becomes narrowed. Also, the distance between the α-amino groups increases from 16 to 20 $\overset{o}{A}$, so that they can no longer make contact with the phosphates of DPG. To a first approximation the binding of oxygen and DPG to Hb is mutually exclusive, therefore, the binding of Oxygen to Hb can be represented by:

$$Hb-DPG \quad + \quad 4O_2 \quad \rightleftharpoons \quad Hb(O_2)_4 \quad + \quad DPG$$
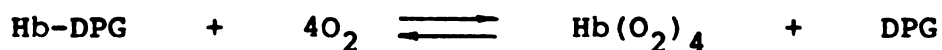
As one might expect from the pKa's of the amino acids interacting with DPG (histidine pKa=6.5, terminal amino
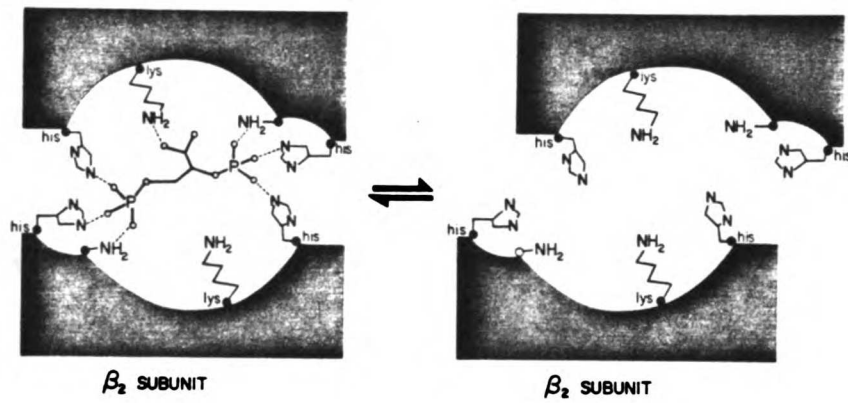
FIGURE II.5 DPG interacts electrostatically with the amino
acid side chains present in the central cavity
of the hemoglobin molecule. When the DPG is
released there is a conformational change of
the hemoglobin molecule. Adapted from Goodford
(1980).

pKa=8), the binding of the DPG is pH dependent. Binding is optimal only in the pH range of 6-7.7 (Garby et al., 1971). Udkow et al. (1973) suggested that both the oxy and deoxy forms of Hb bind DPG at pH values lower than pH 7.2. Possibly at these lower pH values the binding of DPG may not be restricted to the central cavity since other portions of the globular protein may now carry sufficient positive charge to interact with DPG. This binding might have some effect on the oxygen affinity but not as much as if had the DPG interacted within the central cavity.

Experiments by Benesch et al. (1969) have shown that the same lowering of oxygen affinity is obtained using either the D or L form of DPG. This result suggests that the electrostatic interactions within the central cavity are rather nonspecific, which may, in part, explain why other organic phosphates such as ATP, IHP, and IPP also cause a marked drop in oxygen affinity. Experiments by Benesch et al. (1969) have also shown that chloride by itself can cause shifts in oxygen affinity equivalent to that obtained by DPG provided a high enough concentration (0.5M) is used. Amiconi et al. (1981) showed that other anions besides chloride and phosphate will lower oxygen's affinity for Hb. Apparently all of the organic phosphates and other anions compete for the DPG binding site (Udkow et al. 1973, Amiconi et al. 1981). Following binding of an appropriate anion a maximum decrease in oxygen affinity is eventually reached

which is unaffected by the addition of other anions.

The Effect of Hydronium Ion and Carbon Dioxide on the Release of Oxygen: In rapidly metabolizing tissues, such as contracting muscle, large amounts of carbon dioxide and acid are produced. Their accumulation in the capillaries promotes the release of oxygen from oxyhemoglobin. This process is one way that the higher oxygen needs of metabolically active tissues are met. Conversely, in the alveolar capillaries of the lungs, increasing oxygen concentrations drive off hydronium ions and carbon dioxide, which results in the effective metabolic elimination of these products. These linkages are known collectively as the Bohr effect.

About two protons are taken up for every four molecules of oxygen released, and two protons are liberated when four oxygens are taken up. This mechanism is the key to carbon dioxide transport. Because of the limited solubility of carbon dioxide in plasma, it can not be transported to any great extent by plasma, but it can be rendered more soluble by combining with water to form a bicarbonate ion and a proton. In the absence of Hb this reaction would soon be brought to a halt by an excessive increase in pH. However, deoxyhemoglobin acts like a buffer removing protons and shifting the reaction toward the formation of soluble bicarbonate. In the lungs the process is reversed. As oxygen binds to Hb, hydronium ions are generated, driving carbon dioxide out of solution so that it can be exhaled. The

reaction between carbon dioxide and water is catalyzed by carbonic anhydrase, an enzyme in red blood cells.

There is a second less important mechanism for transporting carbon dioxide. Carbon dioxide binds more readily to deoxyhemoglobin than it does to oxyhemoglobin so that it tends to be taken up by Hb when oxygen is liberated and released when oxygen is bound. Carbon dioxide reacts only with uncharged amino groups, and so the amount of carbamate formed depends on pH and the pKa of these groups.

$$ie. \quad R-NH_3^+ \rightleftharpoons R-NH_2 + H^+$$

$$R-NH_2 + CO_2 \rightleftharpoons R-NHCOO^- + H^+$$
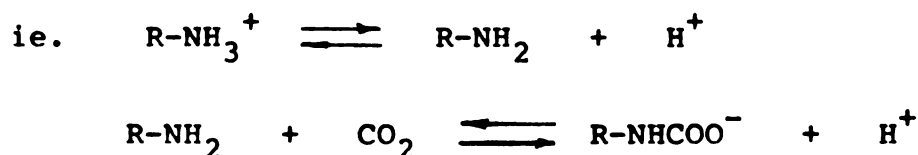
The pKa values of the α-amino groups are between 7 and 8 so that an appreciable fraction of these groups is ionized, and therefore, is capable of forming carbamino-compounds.

The two mechanisms of carbon dioxide transport are antagonistic: for each molecule of carbon dioxide bound to deoxyhemoglobin either one or two protons are released, retarding the conversion of other molecules of carbon dioxide to bicarbonate.

Red Blood Cell Function: The major function of the red blood cell (RBC) is the transport of Hb, which in turn carries oxygen from the lungs to the tissues. RBCs also contain carbonic anhydrase, which catalyzes the reaction between carbon dioxide and water, increasing the rate by a factor of

about 250 times. The rapidity of this reaction makes it possible for blood to react with large quantities of carbon dioxide and thereby transport it from the tissues to the lungs. It is important to note that Hb is an excellent buffer (as is true for most proteins), and, as a consequence, RBCs are responsible for 70 percent of the total buffer capacity of whole blood.

Cell Oxygen Requirements: In mammals essentially all carbohydrates are converted to glucose before they reach the cell; proteins are converted to amino acids, and fats converted to fatty acids and glycerol. Glucose, fatty acids, and amino acids enter the cell where they react with oxygen to produce ATP and the metabolic byproducts carbon dioxide and water. ATP provides the energy needed for membrane transport, synthesis of molecules, and mechanical work in the cell. Because of the properties of respiratory enzyme systems, when the cellular PO_2 is more than 3 to 5 mmHg, oxygen availability is no longer a limiting factor in the rates of these reactions. Therefore, only a very low PO_2 is required for normal intracellular chemical reactions to take place. The primary rate limiting factor is the concentration of adenosine diphosphate (ADP) (Guyton, 1981).

Cells are rarely more than 50 microns away from a capillary. Diffusion of oxygen over such a distance is normally not rate limilting for metabolism. Occasionally,

cells are located farther from capillaries and in these cases the rate of oxygen diffusion may be too low to maintain a cellular PO_2 of 3-5 mmHg. In this case diffusion, not ADP, is the rate limiting factor.

The total amount of oxygen available each minute for use in any given tissue is determined by the quantity of oxygen transported in a given volume of blood that is also available for release to tissues, and the magnitude of the blood flow. If blood flow falls too low or the amount of oxygen available for release is too low, the amount of oxygen available for metabolism can fall below the minimum value of 3 to 5 mmHg.

Thus, four main factors can control the rate of cellular metabolism: ADP, diffusion, blood flow, or oxygen availability.

Transport of Carbon Dioxide in the Blood: Carbon dioxide diffuses out of the cells in gaseous form but not to a significant extent in the bicarbonate form because the cell membrane is far less permeable to bicarbonate than to the dissolved gas. On entering the capillary the chemical reactions illustrated in Figure II.6 occur very rapidly.

(1) Transport in the dissolved state: Assuming the typical PCO_2 values of 45 mmHg for venous blood and 40 mmHg for arterial blood, there is approximately 0.3 ml of carbon dioxide dissolved in each 100 ml of blood. This

CAPILLARY

CO$_2$ transported as:

1. CO$_2$ = 7%

2. HbCO$_2$ = 23%

3. HCO$_3^-$ = 70%
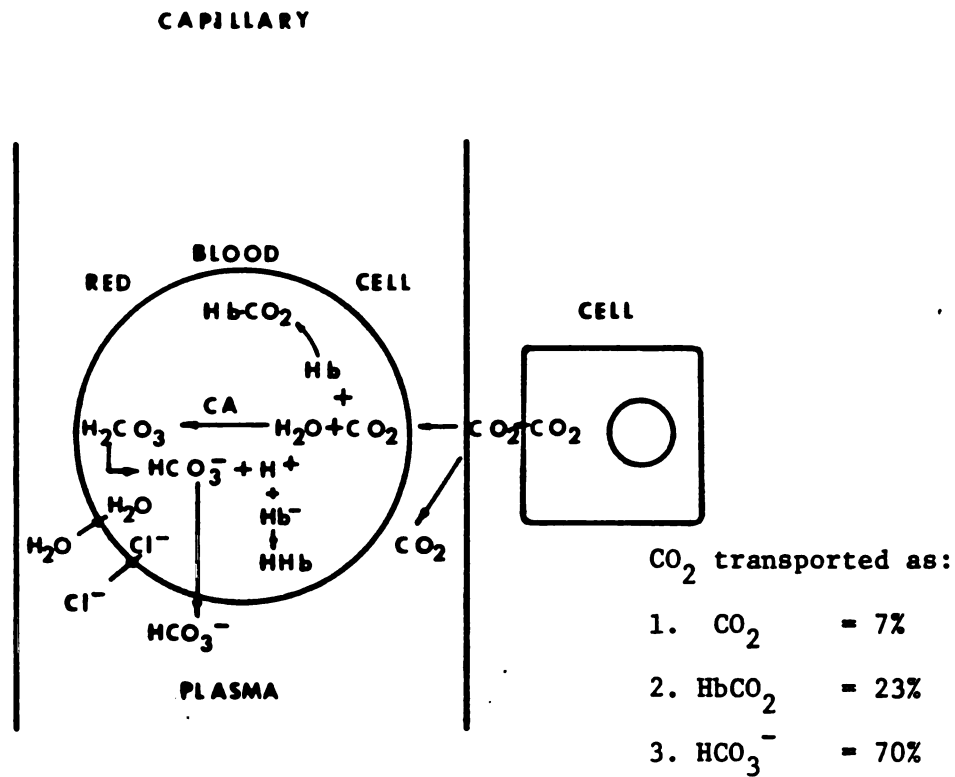
FIGURE II.6.   Carbon dioxide produced by the cells is transported
in the circulation either as free carbon dioxide
bound to hemoglobin or as bicarbonate ion. Adapted
from Guyton (1981).

represents about 7 percent of all the carbon dioxide normally transported.

(2) Transport of carbon dioxide as bicarbonate ion: Carbonic anhydrase catalyzes the reaction between carbon dioxide and water forming carbonic acid in the RBCs which readily dissociates into hydronium and bicarbonate ions. Most of the hydronium ions then combine with Hb. In turn bicarbonate is transported to plasma while chloride ions diffuse into the RBCs to maintain charge balance. This mechanism accounts for the transport of 70 percent of all the carbon dioxide transported to the lungs. At the lungs these reactions are reversed releasing gaseous carbon dioxide into the lungs.

(3) Transport of carbon dioxide in combination with Hb: Carbon dioxide binds reversibly with the neutral amino termini forming carbaminohemoglobin. About 23 percent of carbon dioxide is transported in this way.

Acid-Base Regulation: Slight changes in hydronium ion concentration can cause marked alterations in the rates of intracellular chemical reactions, some being depressed and others being accelerated. For this reason hydronium ion concentration must be maintained within a narrow limit. Life cannot be maintained for more than a couple of hours if the pH drops to 7.0 or rises to 8.0. To prevent acidosis or

alkalosis, several special control systems are available:

(1) All body fluids have acid-base buffering systems that immediately combine with any acid or alkali and thereby prevent excessive changes in hydronium ion concentration. The three major buffering systems of body fluids are bicarbonate, phosphate and proteins. Bicarbonate is not a powerful buffer for two reasons. First, the pH of extracellular fluids is about 7.4 while the pKa of the bicarbonate buffer is 6.1 (maximum buffer capacity occurs when the pH equals the pKa of the buffer). Second, the concentrations of carbon dioxide and bicarbonate are relatively low. Yet, despite this fact, the bicarbonate is as important as other buffers in the body because the concentrations of carbon dioxide and bicarbonate can be regulated, carbon dioxide by the respiratory systems and bicarbonate by the kidneys. As a result, the pH of the blood can be shifted up or down by the respiratory and renal regulatory systems. The pKa of dihydrogen phosphate under physiological conditions is 6.8. However, its concentration in extracellular fluids is only one twelfth that of bicarbonate. The most plentiful buffers in the body are proteins of the cells and plasma. They exist both intra- and extracelluarly at relatively high concentrations, and have amino acid residues with pKa values close to 7.4. All the buffer systems inside the cells help to buffer

the extracellular fluids as well. About 75 percent of all the chemical buffering power of the body fluids is inside cells and most of this results from the intracellular proteins. However, except for RBCs, the slowness of the movement of hydronium and bicarbonate ions through cell membranes often delays, for several hours, the ability of the intracellular buffers system to compensate for extracellular acid-base abnormalities.

(2) If the plasma hydronium ion concentration does rise measurably, the respiratory center (in the medulla oblongata) is immediately stimulated to increase the rate of breathing. As a result, the rate of carbon dioxide removal increases, causing the pH to return to normal. Conversely, if pH falls too low, the respiratory center becomes depressed, alveolar ventilation decreases, and the pH begins to return to normal. Unfortunately, respiratory control can not return the pH to its normal value of 7.4, because as the pH returns toward normal, the stimulus that has been causing either increased or decreased respiration will itself decrease. In effect, respiratory regulation of acid-base balance is a physiological buffer. The buffer capacity of the respiratory system can be one to two times that of all the chemical buffers combined.

(3) When plasma pH changes, the kidneys excrete either an increasingly acid or alkaline urine. As a consequence

plasma pH is maintained in the normal range. The kidney accomplishes this adjustment by incomplete titration of hydronium ions against bicarbonate, leaving one or the other of these to pass into urine. If there where a sudden 0.4 unit shift in pH the kidneys would be able to return the pH of body fluids to normal in one to three days. Though this process is slow, it continues acting until the pH returns to normal. Therefore, the real value of the renal mechanism for regulating pH is not its rapidity of action, but rather its ability to neutralize completely any excess acid or alkali that is either generated in or introduced into body.

Effect of Low PO_2 on Blood Flow, Ventilation and Vasculature: Whenever the availablity of oxygen to tissues decreases, tissue blood flow through the tissues increases. As the arterial PO_2 falls to about 25 percent of normal, tissue blood flow increases about three-fold; that is, the blood flow increases almost enough, but not quite, to make up for the decreased amount of oxygen in blood. Each peripheral tissue has some control over its own blood flow.

Low blood PO_2 values will not normally increase alveolar ventilation significantly until the alveolar PO_2 falls to about one-half its normal value. Respiration is mainly controlled by the extremely powerful PCO_2 and pH feedback control mechanisms. The reason that a low PO_2 has almost no acute effect on respiration is that if increased ventilation

occurs to elevated alveolar PO_2, carbon dioxide is blown off, which reduces PCO_2 and decreases pH. A lower PCO_2 and a decrease in pH both inhibit respiration. As a result these mechanisms keep decreased oxygen from causing a marked increase in ventilation until PO_2 falls to 20 to 40 mmHg, a range incompatible with life for more than a few minutes. Yet, under some abnormal conditions the PCO_2 and pH increase at the same time that the arterial PO_2 decreases. In this case all three of the feedback mechanisms support each other and the PO_2 mechanism then exerts its full share of respiratory stimulation, sometimes becoming even more potent as a controller of respiration than are the PCO_2 and pH mechanisms.

The Reticuloendothelial System (RES): In this discussion the RES will include only the fixed macrophages. The fixed macrophages are localized in the blood sinuses of the liver, lung, spleen, and bone marrow. These fixed macrophages clear particulate matter from the blood by phagocytosis (Benacerraf et al., 1957; Biozzi et al., 1965; Dobson et al., 1952). The macrophages in the liver and spleen account for approximately 85 percent to 95 percent of the total intravascular phagocytic activity (Saba, 1970). In particular the RES has been shown to be involved in:

(1)   Clearance from blood of inert and metabolizable foreign collodial and particulate matter (Benacerraf et al., 1957; Biozzi et al., 1965; Benacerraf et al., 1958;

Biozzi et al., 1953).

(2) Clearance from the blood of worn out endogenous tissue debris (Vannotti et al., 1957; Boyden et al., 1963).

(3) Antigen uptake and possibly antigen processing with respect to immunological reactivity (Benacernaf et al., 1954; Fishman et al., 1963; Fishman et al., 1961).

(4) Iron metabolism and bilirubin formation via erythrophagocytosis or Hb uptake and Hb degradation (Vannotti et al., 1957; Barry et al., 1968).

(5) Denatured protein removal and protein metabolism (Hyman et al., 1960; Hyman et al., 1953).

(6) Removal of microaggregates of fibrin and the prevention of intravascular coagulation (Lee et al., 1962).

The tissue macrophages in various tissues differ in appearances because of environmental differences and they are known by different names: Kupffer cells in the liver, reticulum cells in the lymph nodes, spleen, and bone marrow; and alveolar macrophages in the alveoli of the lungs.

In Vivo Fate of Hemoglobin: The in vivo fate of hemoglobin has been extensively reviewed by DeVenuto et al. (1979). The discussion which follows is based on DeVenuto's review. The free Hb present in normal human plasma does not exceed 0.6 mg/dl (Hanks et al., 1960). After introduction into plasma, Hb binds to serum haptoglobin (Muller-Eberhard, 1970). Haptoglobin, an α-globulin, forms a stable complex

with Hb and in normal human plasma is able to bind 50 to 150 mg of Hb/dl (Hershko, 1975). The Hb-haptoglobin complex is removed from the circulation by the macrophages of the RES in the spleen, liver, bone marrow and other organs (Garby et al., 1959). Unbound Hb can be found in the plasma only after the binding capacity of haptoglobin is exceeded.

Free tetrameric Hb dissociates into the dimer and monomeric forms, globin, and free hemes (Bunn et al., 1968; Muller-Eberhard, 1970). The ferrous ion chelated by the heme molecule is readily oxidized to ferric ion (Muller-Eberhard, 1970) by plasma oxidases. Oxidized heme forms complexes with serum hemopexin and serum albumin. Hemopexin is a β-globulin which has a strong affinity for heme and binds it in an equimolar ratio (Heide et al., 1964); in normal man, hemopexin is present at a plasma concentration of 0.1 to 0.5mg/ml (Eyster et al., 1972). The heme bound to hemopexin is quantitatively insignificant compared with the metheme which combines with albumin forming methemealbumin. Since albumin is present in human plasma in high concentrations, 4 to 5 g/dl, considerable quantities of heme, as metheme, can be accomodated. Plasma elimination of the Hb-haptoglobin complex has a $t_{1/2}$ of 20-30 minutes (Freeman, 1964; Garby et al.,1959), with the $t_{1/2}$ for the heme-hemopexin complex being 7 to 8 hours. However, the circulating methemealbumin is cleared from plasma at about the same rate as free albumin. Because of its slow turnover and

relatively high quantities, methemealbumin is the last heme pigment to dissappear following intravascular hemolysis (Sears, 1970; Sears et al.,1966).

Dimeric and monomeric free hemoglobin in excess of the binding capacities of the circulating proteins undergoes renal glomerular filtration and absorption by the proximal tubular cells. Here the iron is cleaved and the globin is probably catabolized in situ (Ericsson, 1965). When a large amount of Hb is present in the circulation, the absorptive capacity of the kidneys is exceeded and free Hb appears in the urine (Bunn et al., 1969; Bunn, 1972; DeVenuto et al., 1977; Muller-Eberhard, 1970; Pimstone, 1972).

DeVenuto and co-workers (1977) have investigated the fate and distribution of hemoglobin after exchange transfusion in rats. At a plasma concentration of 6.7 g/100ml, soon after transfusion, the rate of decrease of plasma Hb was found to be 920mg/ml per hour. The $t_{1/2}$ for disappearence under these conditions was 3.5 hours. At three hours after transfusion, Hb was present in several organs; as a precentage of initial levels, 6% was found in the liver, 6% in the kidney, 10.5% in the bone marrow and 13-14% in the urine.

In Vivo Fate of Liposomes: Whether or not a liposome will be susceptible to plasma-induced injury will depend mainly on the nature of the liposome. Unilamellar vesicles are more easily and extensively damaged than multilamellar ones,

since in the former only one lamella separates the contents from the surroundings. Also, small unilamellar vesicles will often be more vulnerable than larger ones by virtue of their small radius of curvature.

The size, surface area, and the surface charge of the liposomes are three of the major determinants of liposome clearance (Knight, 1981). Thus, SUVs, with diameters 200-500 A persist in the circulation longer than the larger MLVs (1μ) of the same composition, indicating the importance of vesicle size and surface area. Surface charge is also an important determinant of in vivo behavior. SUVs with positive or neutral charge are retained in the circulation for long periods of time, whereas small negative vesicles are rapidly cleared. Gregoriadis and Neeranjan (1974) reported that the rate of removal of the MLVs during the rapid phase was highest for negatively charged liposomes and lowest for positively charged liposomes, with the neutral liposomes in between. During the slow phase the rate of elimination was uniform for all three charges. Juliano and Stamp (1975) obtained similiar results for unilamellar liposomes. They suggested that the rapid clearance of negatively charged vesicles may be due to clumping or coalescing of injected particles. However, even if one ignores the fact of electrostatic repulsion between negative liposomes, liposomes on interacting with plasma components acquire negative charges irrespective of initial surface charge (Juliano et al.,

1978). Juliano and Lin (1980) also found that liposomes with different surface charges bind different arrays of plasma proteins. Possibly the negative charged serum components become associated with the positive and neutral liposomes much more than with negative liposomes, and this factor may alter the tissue distribution and hence blood clearance.

Immune damage to the body by activation of complement may be limited to liposomes bearing glycolipids with sugar residues exposed at their outer surface, although complete clarity on this issue does not exist.

As can be seen several factors mentioned above can influence the liposome's stability as well as other plasma factors such as lipoproteins and opsonins. If the phospholipids used to make liposomes are maintained above the phase transition temperature, then incorporation of cholesterol will decrease liposome permeability (de Gier et al., 1970). When the cholesterol content of egg phosphatidylcholine liposomes is increased, the extent of leakage of entrapped substances decreases both in vitro and in vivo (Papahadjopoulos et al., 1973; and Kirby et al., 1980).

The clearance of liposomes from the circulation can be partially explained by their uptake into various organs. The circulating liposomes are taken up predominately by tissues rich in the reticuloendothelial cells, such as liver, spleen, and to a very small extent by the lungs, bone marrow

and kidneys (Gregoriadis et al., 1972a,b; Jonah et al., 1975; Wisse et al., 1975; Juliano et al., 1978; Bosworth et al., 1982; Abra et al., 1980,1982a).

Despite the preferential uptake by liver and spleen these organs can be saturated (Abra et al., 1981, Ellen et al., 1982). This fact implies that increasing fraction of the injected dose could possibly reside in the circulation if an increased dose of liposomes was given using liposomes as carriers for Hb.

Conclusion: In the development of a blood substitute based on the entrapment of the Hb into lipid vesicles, consideration needs to be given to the areas of Hb function, RBC function, hemoglobin's role in maintaining a physiologic system, Hb removal and how lipid vesicles themselves are cleared. With this background information, criteria to aid in the development and quantification of a blood substitute based on hemoglobin-containing lipid vesicles can be established. These criteria will be discussed in subsequent chapters.

CHAPTER III   THEORETICAL CONSIDERATIONS

I.   The Effect of P_{50} and n on Oxygen Delivery

Two parameters are used to characterize hemoglobin oxy-
gen releasing capabilities: P_{50} (the partial pressure of
oxygen at which 50% of the Hb molecule is saturated with
oxygen) and n (a measure of Hb cooperativity). The term n
is defined in the following empiric relationship.

% Saturation = 100 fo = 100 K $((PO_2)^n /(1 + K (PO_2)^n))$ (1)

where K = $1/(P_{50})^n$ (2)

and fo = fraction saturated

Equation 1, the Hill equation, gives a fairly good fit
for Hb binding of oxygen as a function of PO_2. This equa-
tion can be used to illustrate several important points
about oxygen delivery to tissues. Assume that arterial
blood PO_2 = 100 mm Hg and tissue PO_2 = 20 mm Hg. From Fig-
ure III.1 it can be seen that when n, Hb concentration and
all physiological variables are held constant, an increasing
value of P_{50} increases the amount of oxygen released per
pass (going from lungs to tissue and back). A composite
graph of the effect of simultaneous changes in both P_{50} and
n on oxygen binding to Hb is shown in Figure III.2.
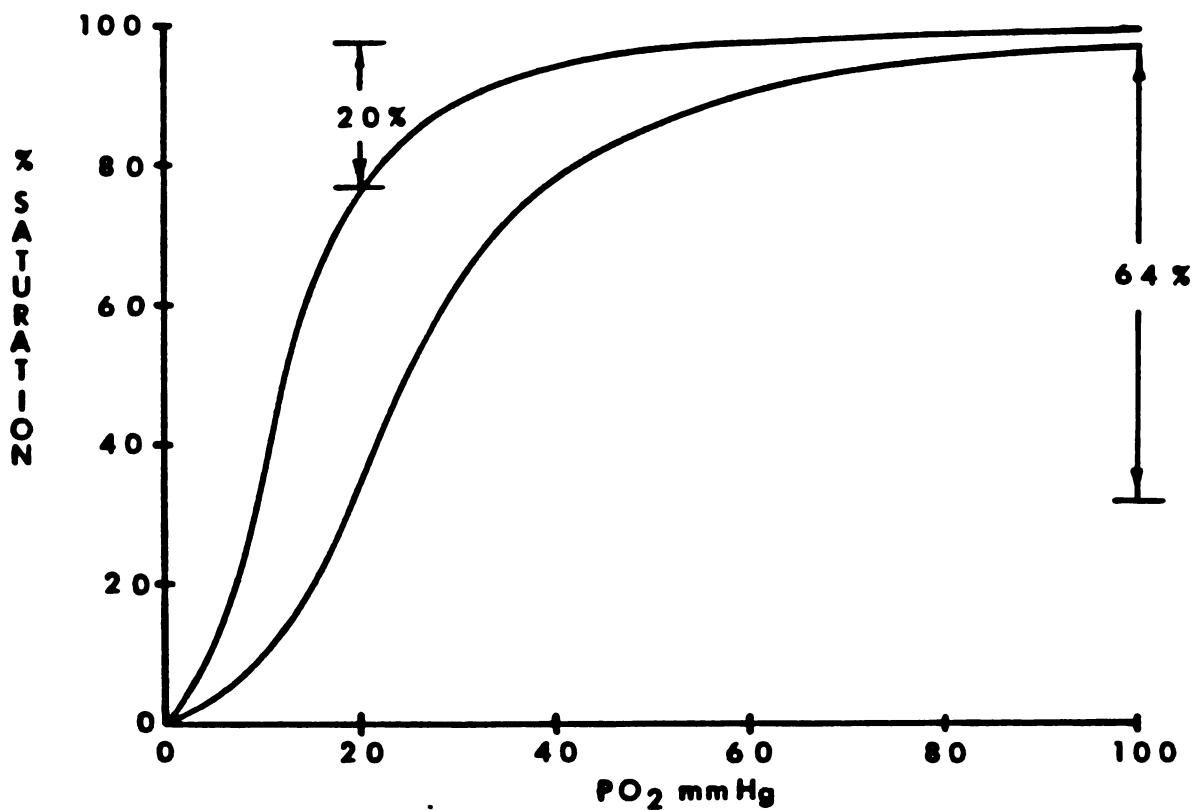
FIGURE III. 1.   Two different oxygen dissociation curves are
                 plotted as a function of PO_2. Curve A has
                 P_{50}=12.5 mmHg and curve B has P_{50} of 26 mmHg.
                 Both curves have the same n value 2.8. Curve
                 A shows a release of 20% of its oxygen and
                 curve B a 64% release for a change in PO_2 from
                 100 to 20 mmHg.
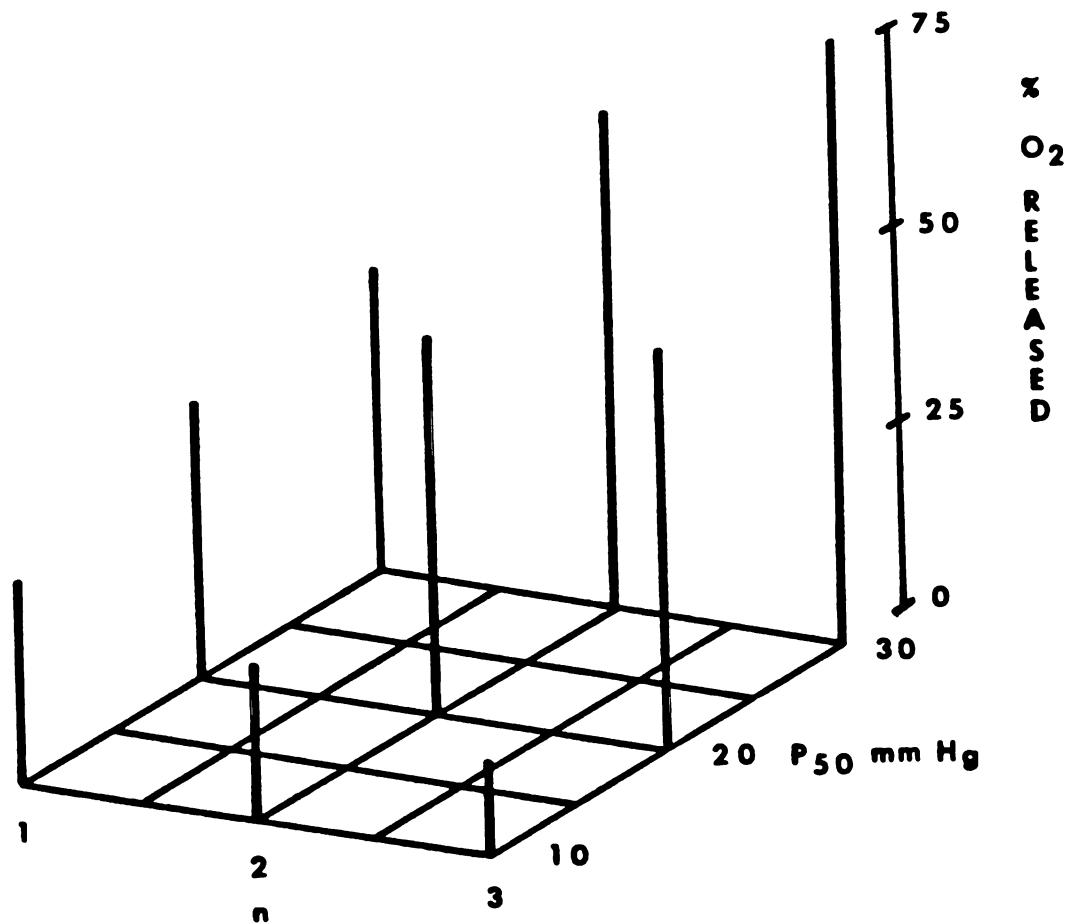
FIGURE III. 2.   The percentage of oxygen (bound to Hb) released
as the oxyHb passes from the lungs (PO_2=100 mmHg)
to the tissues (PO_2=20 mmHg) is plotted as a
function of its P_{50} and n value. Values were
calculated using equations 1 and 2.

When a blood substitute with a low P_{50} is used in vivo the body compensates by lowering the oxygen tension in the tissues to a point where sufficient oxygen can be unloaded. This could provide adequate oxygen for some processes, but not all. Therefore, it is advisable to have a P_{50} and n value comparable to that of whole blood which has a P_{50} = 26 mm Hg and n = 2.8.

When a synthetic resuscitative fluid replaces blood essentially all of the oxygen delivered to the cells comes from the blood substitute. However, when only a fraction of the RBCs are replaced by a blood substitute having a P_{50} and n value different from that of whole blood, the percentage of oxygen delivered by the blood substitute to tissues will not be the same as its percentage in the circulation. Figure III.3 and Figure III.4 show that when a blood substitute such as Hb or NH represents a small fraction of the total blood volume, the percentage of oxygen released is much less than the percentage the blood substitute represents in the circulation. Thus, when only a very small amount of blood substitute is transfused it acts much like a plasma expander even though it can carry oxygen. Figure III.3's data were derived assuming PO_2 starts at 100 mm Hg (arterial pO_2) and falls to 40 mm Hg in the venous system. The arterial and venous blood gas values were chosen because most inferences in clinical work are based on these two blood gas values. Figure III.3 shows that NH deliver on a percentage basis
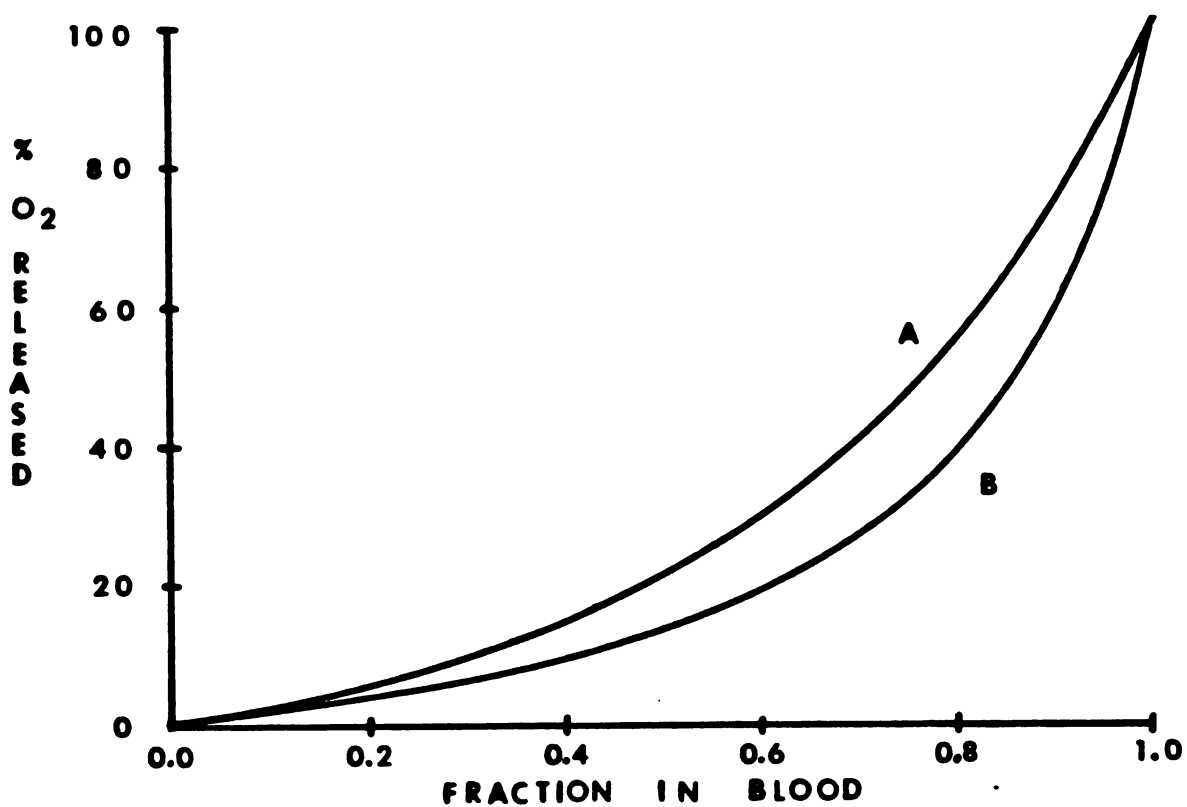
FIGURE III.3.   As the PO_2 falls from 100 mmHg to 40 mmHg, the
percentage of total oxygen released (from just
the blood substitute) is plotted as a function
of its fraction in the 'blood'. Values were
calculated using equations 1 and 2. The Hb
content of each suspension were set equal to
each other. For the NH P_{50}=12.5 and n=2.1;
for the Hb solution P_{50}=26 and n=2.1.
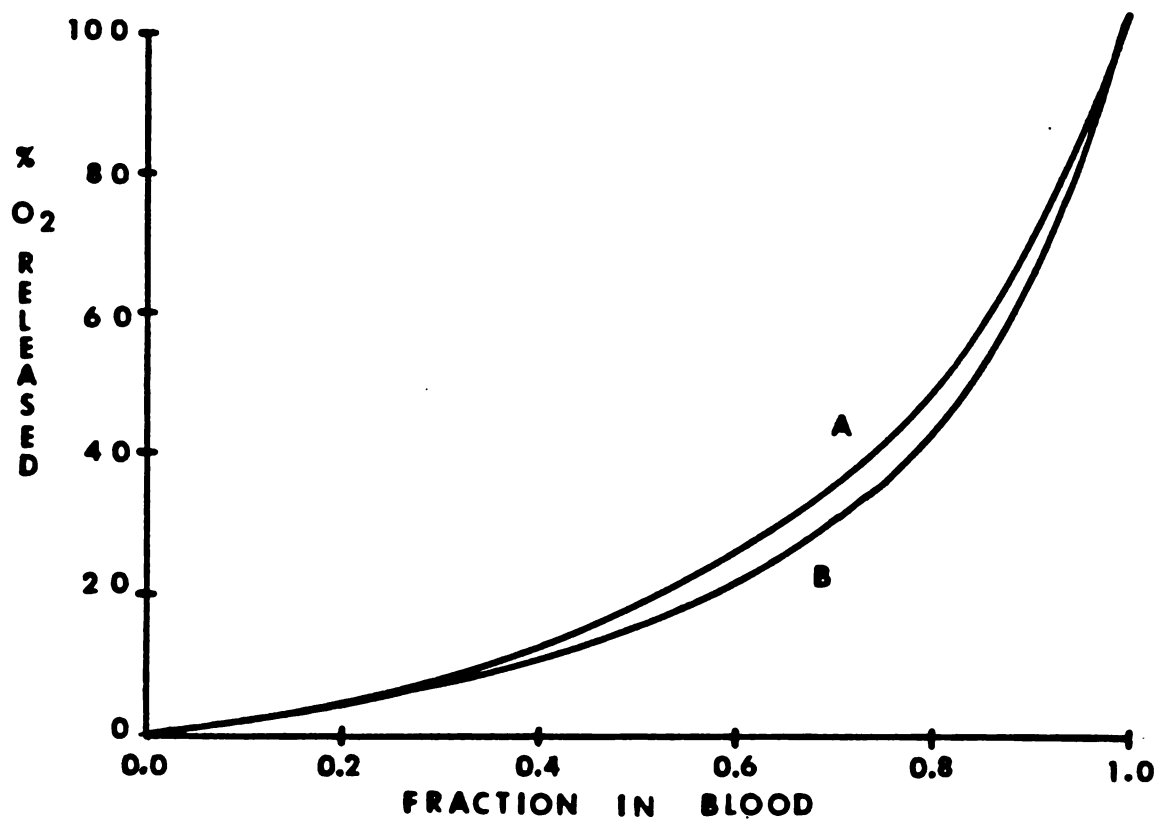
FIGURE III.4.   As the PO_2 falls from 100 mmHg to 20 mmHg, the
percentage of total oxygen released (from just
the blood substitute) is plotted as a function
of its fraction in the 'blood'. The data were
calculated using equations 1 and 2, and the
parameter values listed in Fig. III. 3.

much more oxygen than the Hb even though the concentration
of the Hb (7 g/dl) is greater than the concentration of NH
associated Hb (4 g/dl). However, this gives a false picture
of the relative merits of Hb vs NH. Actually the PO_2 at the
tissue sites, not the venous PO_2, is what determines how
much oxygen will be unloaded from the blood substitute. Fig-
ure III.4 shows the comparison of the abilities of Hb and NH
to release oxygen. Here Hb more closely compares with NH in
the ability to deliver oxygen to the tissues (tissue PO_2=20
mm Hg).

II. Model for Neohemocyte Formation

Figures III.5 and III.6 depict how NH might ideally be
formed. This model assumes that initially a water in oil
emulsion is formed. This emulsion is stable, has an uniform
dispersed phase droplet size, and has just enough lipid
present to form a monolayer around each dispersed phase dro-
plet (Figure III.5). Removal of the organic phase by eva-
poration produces a lipid bilayer network which "breaks" to
form unilamellar vesicles containing equal amounts of Hb
(Figure III.6).

Actually the dispersed aqueous phase does not have an
uniform droplet size and, if it is assumed that there is
more lipid present than needed to form monolayers around all
aqueous droplets, a different model can be developed (Figure
III.7). The excess lipid forms inverted micelles which are
hydrated with solubilized water present in the organic
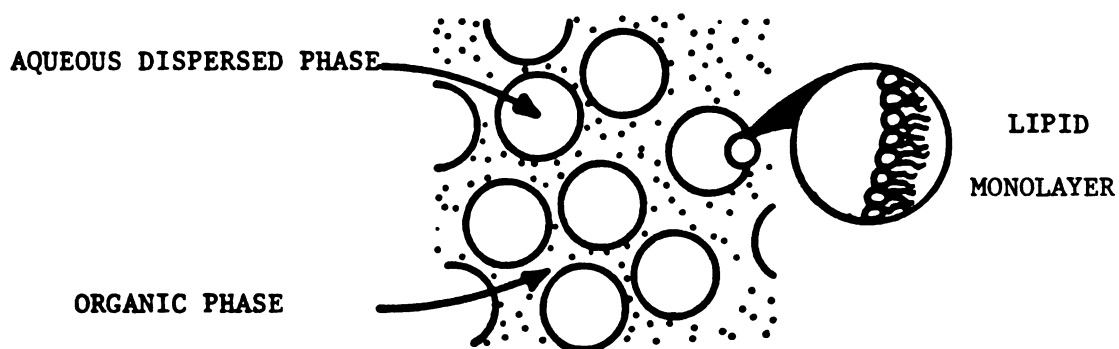
FIGURE III.5. An idealized two dimensional cross-section through a stable water in oil emulsion. The dispersed aqueous phase contains Hb. The lipids are shown adsorbed at the interface between the aqueous droplets and organic phase.
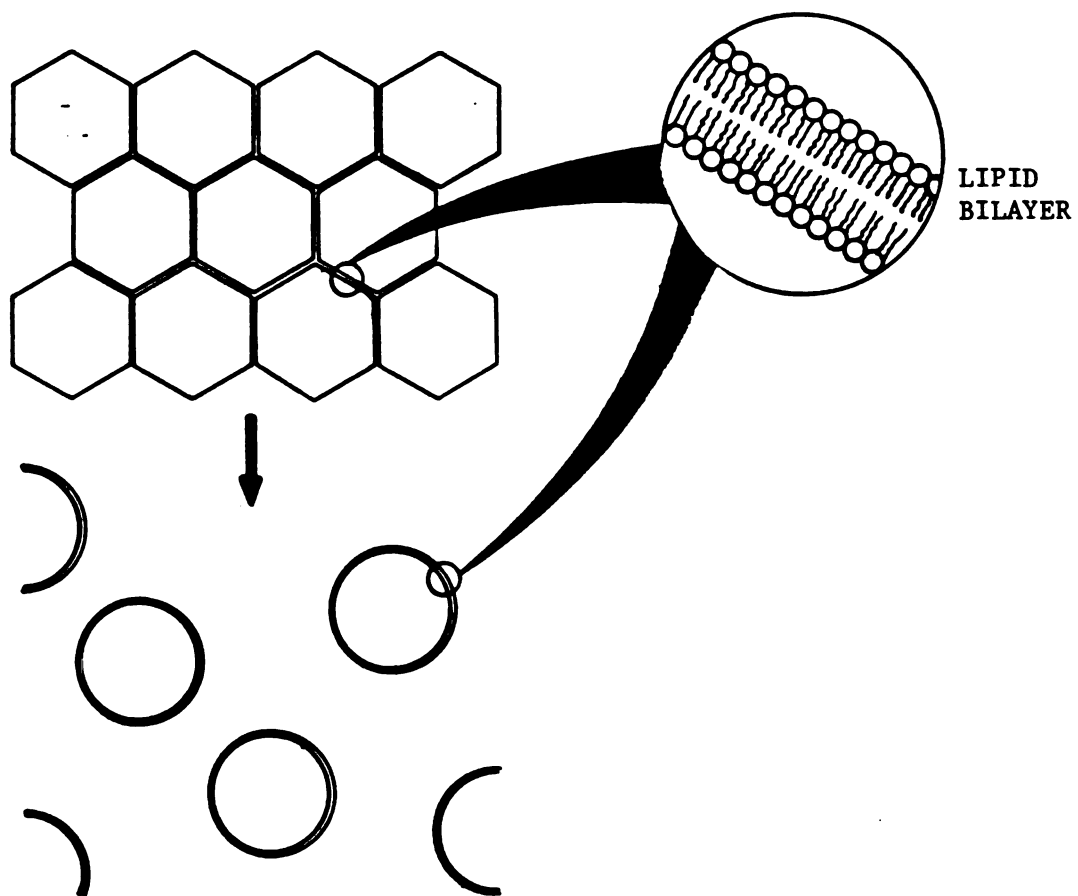
FIGURE III.6. An idealized two dimensional cross-section
through the lipid bilayer network before and
after "breakage".
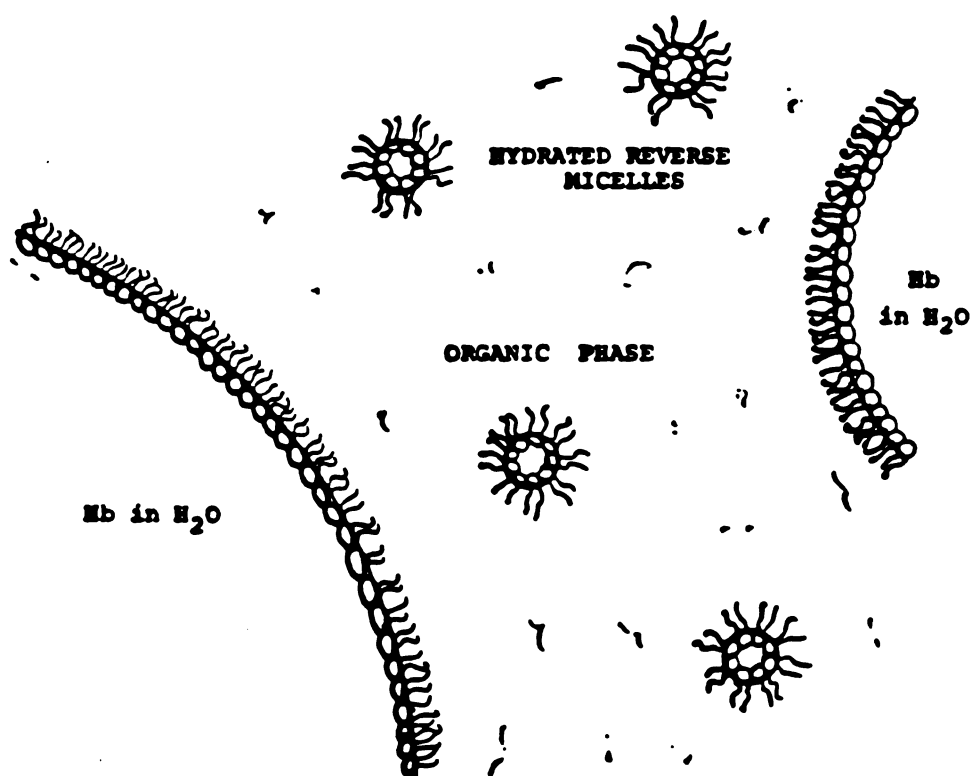
FIGURE III.7. A two dimensional cross-section of a emulsion containing dispersed water droplets of heterogeneous size coated with a monolayer of lipid. The water droplets contain Hb. Also, dispersed in the organic continuous phase are hydrated micelles containing some water, but little if any Hb.

A   B   C
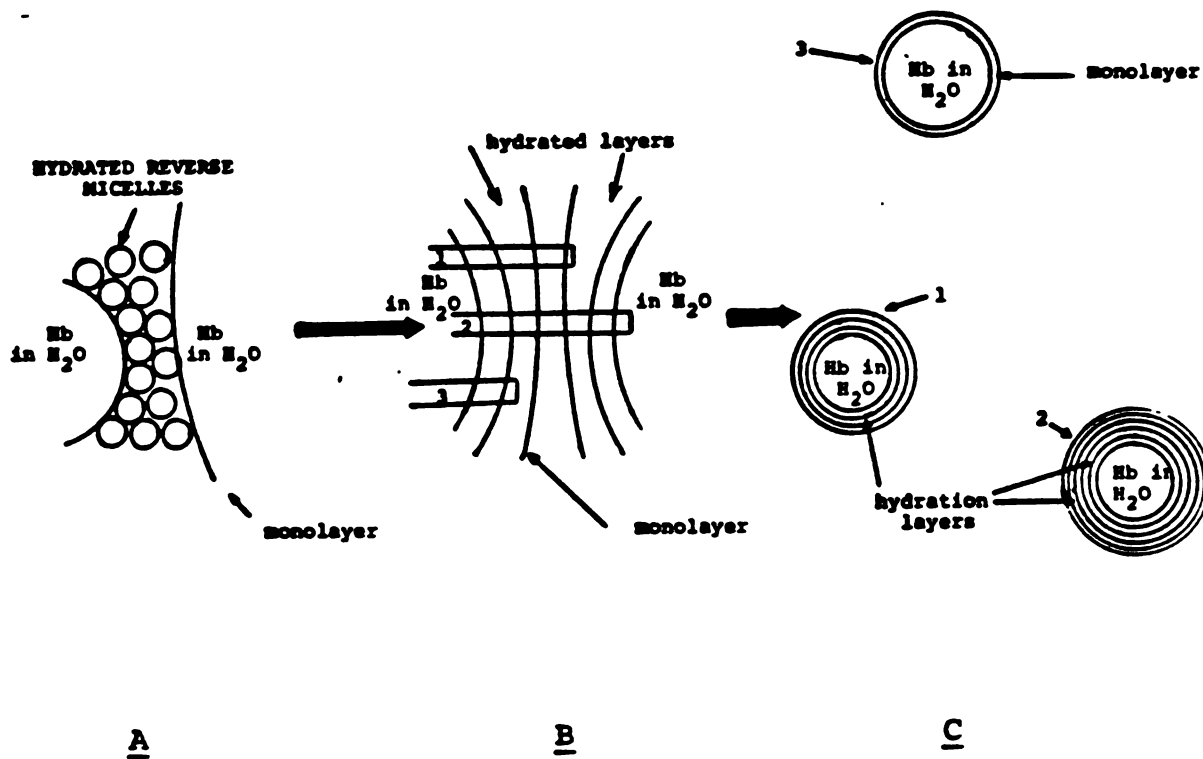
FIGURE III.8.   A is a representation of a compact conglomerate of water droplets containing Hb, each coated with a monolayer of lipid, and hydrated reverse micelles just before complete removal of the organic solvent. B represents an idealized lipid bilayer network prior to "breakage". The numbers 1,2 and 3 refer to hypothetical fracture points in the lipid bilayer network. C illustrate some of the possible lipid vesicles formed after "breakage" at the hypothetical fracture points. The resulting lipid vesicles have different Hb-aqueous and Hb-lipid ratios, and are heterogeneous in size.

phase. Since Hb is insoluble in the organic phase and steric constraints prevent Hb incorporation into these reverse micelles, the reverse micelles contain little if any Hb. As the organic phase is removed the reverse micelles begin to come in close contact with each other and with the monolayer surrounding the aqueous Hb containing droplets. Eventually, as all the organic solvent is removed a hydrated lipid bilayer network is formed around the aqueous Hb containing droplets. Subsequently, this network is sometimes referred to as a "gel", for lack of a better term. Addition of energy (e.g., mechanical agitation) "breaks" the lipid bilayer network forming a variety of vesicles which have regions containing differing amounts of Hb and water (Figure III.8).

III. Neohemocyte Distribution Model

Liposomes accumulate primarily in the liver and spleen but the processes involved can be saturated when a large number of liposomes are administered (Abra et al., 1981). In a total exchange transfusion with NH, one hundred times the doses used by Abra et al. (1981) are administered. The above study indicates that as tissue binding and uptake sites become saturated the fraction of total liposomes remaining in the circulation at any time increases. One would therefore expect that following a massive transfusion of liposomes (neohemocytes) tissue binding and uptake sites would become saturated and the vast majority of liposomes

would remain in the circulation as long as they remained intact. It is important to note that the liposome composition used by Abra and Hunt is essentially the same as that of the NH described in the following chapters.

CHAPTER IV MATERIALS AND METHODS

I. Chemicals

Unless indicated all chemicals were used as received. Egg phosphatidylcholine (PC), sodium dipalmitoyl L-alpha-phosphatidate (PA), cholesterol (CH), DL-alpha-tocopherol (T), phenazine methosulfate (PMS), sodium bisulfite (reagent grade), anhydrous sodium sulfite (reagent grade), sodium chloride, potassium ferricyanide, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 2,3,-diphospho-D-glyceric acid pentasodium salt (DPG), inositol hexaphosphate (IHP), magnesium chloride hexahydrate and DL-lactic acid were purchased from Sigma Chemical Company (St. Louis ,MO). Sodium phosphate monobasic, sodium phosphate dibasic heptahydrate, chloroform, anhydrous diethyl ether, t-butyl hydroperoxide, sodium bicarbonate and potassium chloride were purchased from Mallinckrodt (St. Louis, MO). Hydrochloric acid, sulfuric acid, perchloric acid (72%), potassium cyanide, ferrous sulfate heptahydrate, sodium azide and 1-amino-2-napthyl-4-sulfonic acid (ANSA) were purchased from Fisher (Pittsburg, PA). Triton X-100 was obtained from Amersham/Searle (Arlington Heights, IL). Aquacide 1-A was purchased from Calbiochem-Behring Co. (La Jolla, CA). Ammonium molybdate was received from J.T Baker Company (Phillipsburg,NJ.). 1,1,2-Trichloro-1,2,2-trifluoroethane was obtained from Kodak (Rochester NY).

Bio-Gel A-5M was purchased from Bio Rad Laboratories (Richmond,CA). ^{14}C sucrose, ^{14}C cholesterol and Na ^{125}I was obtained from New England Nuclear (Boston, MA). 1,3,4,6-Tetrachlor-3a,6a-diphenylglycouril (IODO-GEN) was purchased from Pierce Chemical Company (Rockford, IL). Pen/strep antimicrobial reagent was obtained from the Cell Culture Facility at UCSF; it contained 10,000 units of penicillin and 10,000 μg streptomycin per ml of sterilized distilled water.

II.   General Procedures

Buffers:

(1)   Each liter of 300 mosm phosphate buffer solution (PBS-300) contained 22.52 g sodium phosphate dibasic heptahydrate and 4.42 g sodium phosphate monobasic monohydrate. The pH of PBS-300 was 7.5.

(2)   Each liter of 30 mosm phosphate buffer (PBS-30) solution contained 2.33 g sodium phosphate dibasic heptahydrate and 0.18 g sodium phosphate monobasic monohydrate. The pH of PBS-30 was 7.5.

(3)   Each liter of 30 mosm HEPES buffer (HEPES buffer) solution contained 4.77 g HEPES. The pH was adjusted to 7.5 by the addition of sodium hydroxide.

(4)   Each liter of modified lactated Ringer's buffer (Ringer's buffer) contained 5.7 g sodium chloride, 3 g

sodium bicarbonate, 0.3 g potassium chloride and 0.15 g magnesium chloride hexahydrate. Lactic acid was added to the solution until the pH equalled 7.4.

All buffers were prepared at room temperature. Five ml of pen/strep was included per liter; these solutions were filtered through a 0.22 μm millipore filter (type GS) and stored at 4°C until use.

Hemoglobin Solutions Used in Neohemocyte Preparation: Unless otherwise indicated hemoglobin (Hb) was obtained through the Division of Blood Research, LAIR, Presidio of San Francisco; from the University of Minnesota, Department of Surgery, and was prepared under contract from the U.S. Army Medical Research and Development Command. This Hb solution was prepared from outdated red blood cells (RBCs) which had been lysed by hypoosmotic shock.

Hemoglobin Assay: The concentration of Hb was determined using an Eilers' (1967) spectrophotometric assay. Hemoglobin was reacted with Drabkin's reagent to form cyanmethemoglobin (cyanmetHb). The absorbance of cyanmetHb at 540 nm was proportional to the Hb concentration.

Methemoglobin Assay: The percentage of methemoglobin (metHb) in a Hb solution is determined by using a standard spectrophotometric assay (Dubowski et al.,1964; Evelyn et al.,1938). MetHb in a dilute acid solution absorbes at 630

nm; addition of cyanide changes the metHb to cyanmetHb which does not absorb at 630 nm. This change in absorbance is proportional to the amount of metHb present.

Nonenzymatic Regeneration of Oxyhemoglobin from Methemoglobin: The nonenzymatic reduction of metHb to hemoglobin was accomplished using the method of Kajita et al.(1970). Addition of catalytic amounts of PMS in the presence NADH reduced metHb to Hb.

Radioiodinated Lipid Marker: Radioiodinated p-hydroxybenzamidine phosphatidylethanolamine (^{125}I-BPE) was prepared as previously described (Szoka et al., 1982). It was used as a lipid membrane marker to follow the in vivo fate of NH, described subsequently.

Radioiodination of Hemoglobin: The radioiodination of Hb and subsequent purification was accomplished by following the method of Markwell et al. (1978). The exposed tyrosine residues of Hb were iodinated in a rapid and gentle fashion by running the radioiodination reaction in vessels coated with a thin film of IODO-GEN. The thin film of IODO-GEN provided a catalytic surface for the iodoination reaction to occur on.

Phosphorus Assay: A method based on a phosphorus assay developed by Bartlett (1959) was used.

Method:

(1) Five mM sodium phosphate monobasic was used as a phos-
     phorus calibration standard. Each liter of the aqueous
     ammonium molybdate solution contained 4.4 g ammonium
     molybdate and 14 ml concentrated, reagent grade sul-
     furic acid. Each liter of the ANSA solution contained
     10 g of sodium bisulfate, 2 g sodium sulfite, and 0.168
     g ANSA.

(2) The phosphorus standards were made by using 5, 10, 20,
     30, 40, and 50 μl of the 5 mM phosphorus calibration
     standard. Duplicate aliquots were placed into each of
     two 100 mm X 12 mm test tubes. Phosphate free buffer
     was used as blanks.

(3) Aliquots of unknown samples were added to 100 mm X 12
     mm test tubes.

(4) Concentrated perchloric acid (0.2 ml) was added to all
     tubes.

(5) Each tube was maintained at $180^{\circ}C$ for 2 hours and then
     cooled.

(6) To each sample 1.2 ml of the ammonium molybdate solu-
     tion and 1.2 ml of the ANSA solution were added.

(7) Samples were subsequently heated for 10 minutes in
     boiling water.

(8) Following cooling the absorbance at 700nm of each
     cooled sample was recorded.

A typical calibration curve is presented in Figure IV.1.

By knowing the percentage of phospholipid present in a lipid sample the lipid concentration of the sample can be determined.

Removal of Peroxides from Diethyl Ether: Peroxides were removed by shaking diethyl ether for at least 8 hours with an equal volume of 5 g/dl ferrous sulfate in 0.01 M sulfuric acid. Ether was separated from the sulfuric acid solution in a separatory funnel. The aqueous solution was discarded. The remaining diethyl ether was washed three times with equal volumes of distilled water, and used within 48 hours.

Gel Filtration Chromatography: Gel chromatography (Bio-Gel, A-5m) was used to separate NH from unencapsulated Hb and to change the composition of the suspending medium. Packed columns were stored in 0.02 g/dl sodium azide, and were conditioned with 5 to 6 column volumes of eluting buffer prior to use. Used gels were cleaned of denatured protein and other contaminates by adding 0.2 to 0.4 ml of 10 g/dl aqueous triton X-100 to the column and then eluting with 5 to 6 column volumes of distilled water containing 0.02 g/dl sodium azide.

Preparation of Hb/DPG Mixtures for use in Preparation of Neohemocytes: If the percentage of methemoglobin was greater than 4 g/dl, then the Hb solution was treated with NADH and
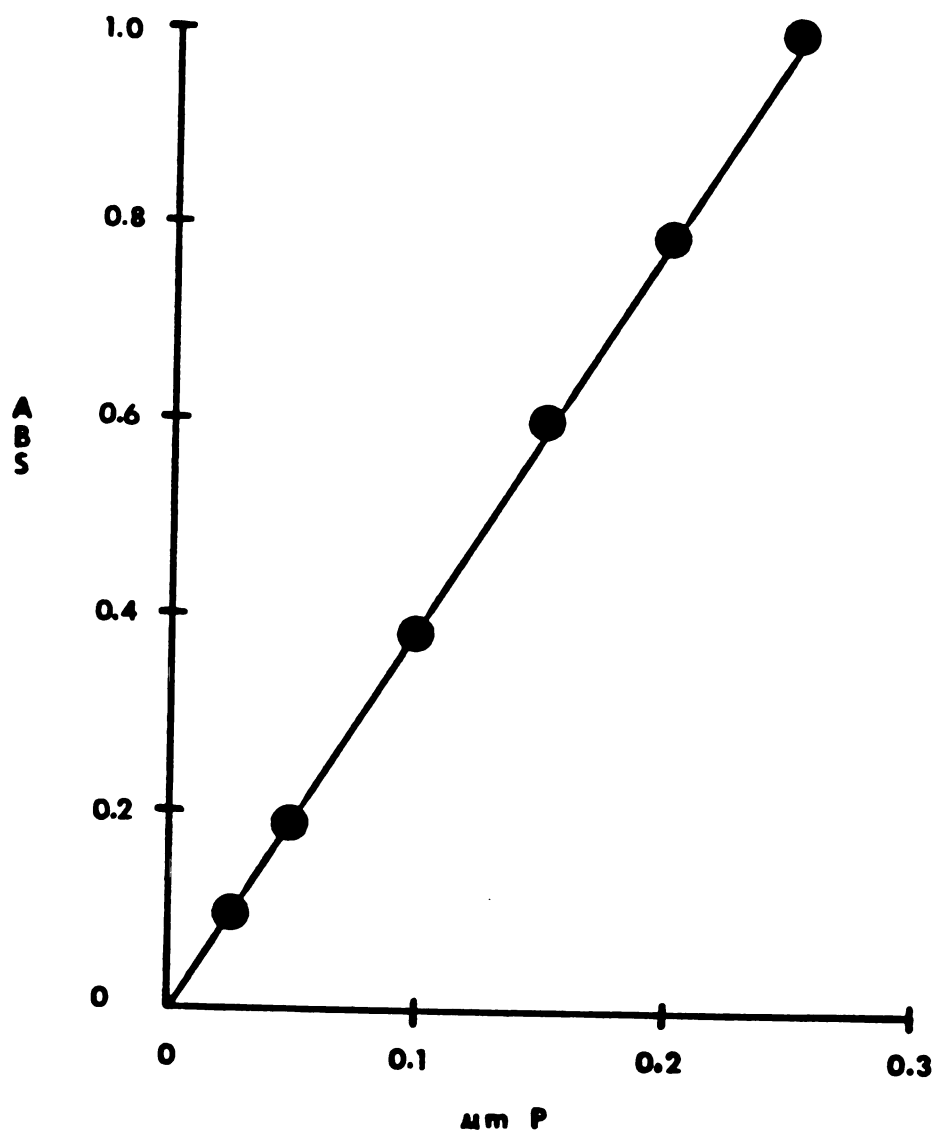
FIGURE IV.1.   A typical calibration curve for the phosphorus
                assay. Absorbance (ABS) was measured at 700 nm
                and plotted vs phosphorus concentration.

PMS to reduce the metHb levels. This treatment was accomplished during the concentration step as follows: The Hb, NADH, and PMS were placed in a dialysis bag, covered with aquacide 1-A, and left at room temperature for 3 to 12 hours. The aquacide 1-A, being very hygroscopic, removed water, concentrating the Hb. The time required to complete the concentration was more than sufficient to complete the reduction. After concentration, the Hb solution, still in the dialysis bag, was equilibrated overnight with pH 7.4 PBS-30. The majority of unreacted NADH and PMS, their reaction products, and any other low molecular weight solutes were removed during this dialysis. The concentrations of both metHb and Hb were again determined. The DPG was then added (1.5 moles DGP: 1 mole Hb) and the encapsulation process begun.

Dialysis: Dialyses were carried out at $4^{\circ}C$ for 24 to 48 hours. Dialysis tubing (12,000 MW cut off) was purchased from VWR Scientific (San Diego, CA). The tubing was boiled in distilled water for one hour and then rinsed with distilled water before use. After clamping and tying one end of the dialysis tubing, the experimental suspension was added, and then the other end was clamped and tied to give a dialysis bag. The bag was placed in a glass beaker with a stirring bar and at least 100 volumes of buffer were added. The buffer was changed twice during each 24 hour period.

Hemoglobin Encapsulation: The Hb encapsulation procedure evolved from the method of Szoka et al. (1978), which described formation of a water-in-organic emulsion and subsequent evaporation and processing to form the liposomes. For Hb encapsulation a mixture of peroxide free diethyl ether and 1,1,2-trichloro-1,2,2-trifluoroethane (the mixture had a density equalling 1) was added to the Hb solution until the ratio of the volumes of the organic and Hb phase was approximately equal to 2.4:1. Either diethyl ether or 1,1,2-trichloro-1,2,2-trifluoroethane was then added until the density of the organic phase equalled the density of the aqueous phase. The densities were assumed to be matched when shaking the mixture 10 seconds gave an emulsion which did not phase separate after standing for 5 minutes. Matching phase densities improved emulsion stability, and proved to be an essential requirement. The organic phase contained PC, PA, CH and T in the molar ratio 4:1:5:.02, respectively. There were 30 μmol lipid/ml Hb solution. The mixture was mechanically shaken for 30 minutes to form an emulsion. A teflon coated stirring bar was placed in the mixture to aid in the breakage of the to be formed gel. The nonaqueous solvent was then removed in two stages. The majority of the organic phase was removed by evaporation at 200-400 mm Hg, in a Buchi Rotovapor R Evaporator. By keeping the pressure above 200 mmHg frothing was minimized. When the mixture reached a gel-like consistency, the vacuum was released. The second stage of evaporation was started at atmospheric

pressure. The pressure was decreased slowly until the gel broke (at approximately 20 mmHg) forming a crude mixture of NH suspended in a solution of Hb. The suspension was next shaken, to break up the remaining gel, and then evaporated starting at atmospheric pressure. The pressure was then progressively lowered until the remaining gel was disrupted.

Neohemocyte Purification and Physiological Adjustment: The N/Hb suspension was centrifuged at 12,000 g and 4°C for 20 minutes. The pellet containing denatured protein was discarded. The supernatant, which contained the NH and unencapsulated Hb, was decanted into dialysis bags. These dialysis bags were then placed into a beaker containing PBS-30. Sodium chloride ,2.7M, was then infused at a rate of 8.4 ml/hr until the buffer reached 300 mosm.

Free Hb was separated from NH on a Biogel A-5M column. The NH were recovered in the void volume; Hb was retained on the column. The ratio of Hb to NH retention volumes was 3 to 1. The NH were next concentrated by centrifugation at 6000 g and 4° for 20 minutes. The supernatant containing 20 percent of the NH was discarded leaving a NH pellet which was somewhat more rich in hemoglobin. This pellet was resuspended in a 0.9 g/dl sodium chloride solution which contained 3 g/dl bovine albumin. The resulting NH suspension had an apparent Hct of 25.

III. Procedures Used to Analyze Neohemocytes

Measurement of Oxygen Dissociation Curve: Oxygen dissociation curves were obtained using the technique of biotonometry (Neville, 1974). The assay required placing 6 ml of buffered yeast (pH about 7.4-7.5) into a 10 ml test tube and warming the mixture to $37^{\circ}C$ in a temperature controlled block. The air space remaining in the tube was flushed with a 95% oxygen, 5% carbon dioxide mixture for 5 seconds, the tube was capped and then shaken for 10 seconds to oxygenate the suspension. A 0.5 to 1 ml Hb solution or NH suspension was introduced, the head space was again flushed, the tube capped and shaken. An aliquot of this mixture was immediately introduced into a capillary pH electrode $(37^{\circ}C)$ and the pH recorded. The remaining solution was transferred to a test tube $(37^{\circ}C)$ which contained an oxygen electrode. The response of the oxygen electrode was recorded on a strip ch.ɹt recorder; pH measurements were made when the observed PO_2 reached 100 mm Hg and again when it reached 0 mmHg. The oxygen dissociation curves were analyzed to determine the P_{50}, n and apparent Hb content (Neville, 1974).

Effect of DPG/Hb Molar Ratio on Neohemocytes: A standard Hb solution which had been dialized against PBS-30, was divided into four 20 ml aliquots, each having one of the following DPG/Hb molar ratios: 0.5 : 1, 1:1 , 1.5:1, or 2:1. Each of these four solutions were used to prepare NH. Values of

P_{50}, n and apparent Hb were then determined for each preparation.

Determination of the Effect of Initial Osmolarity and Hb Concentration on the Neohemocyte Characteristics: Hemoglobin solutions were prepared at the following concentrations and osmolarities by equilibration with a pH 7.5 phosphate buffer having the appropriate osmolarity.

g/dl Hb

All Hb solutions had 1.5 moles of DPG per mole of Hb and contained ^{125}I-Hb as a marker. Oxygen dissociation curves were obtained for each NH preparation from which the n, P_{50} and apparent Hb concentration were determined. The percentage of starting Hb encapsulated equals:

((Vol. NH)(conc. Hb in NH)/(Vol. starting Hb)(conc. starting Hb))100

Negative Stain Electron Micrographs (EMs): Grids for negative stained electron microscopy were prepared as follows:

(1)   A thin film of soap (Ivory) was coated onto a glass slide support.

(2)   The slide was dipped into a 0.5% Formavar/chloroform solution.

(3)   A 200 mesh grid was placed at the bottom of a clean funnel containing distilled water. The soap film was floated off the slide and onto the surface of the grid by slow removal of water.

Immediately prior to use, the grids were ionized by subjecting them to a glow discharge. Approximately 50μl (1 drop) of the NH suspension was placed on the grid. The lipid concentration of the NH suspension was adjusted to 2 μmole/ml of Hb solution. After one minute, excess suspension was removed by touching the edge of the grid with filter paper. Because Hb is electron dense the sample was not stained.   The grid was allowed to dry for 10 to 15 minutes.

A Seimans 1A electron microscope run at 80 kV was used to take the EMs. The size of the NH was determined using a calibration grid.

Freeze Fracture Electron Micrographs: Samples were prepared at a lipid concentration of 10 to 30 μmole/ml. Lipid concentration was adjusted by the addition of 0.15M ammonium acetate buffer. Glycerol was added to yield a 33 % (v/v) glycerol-liposome mixture. After the drop of glycerol-liposome solution had sat for 30 seconds on the support, the disks were plunged into a liquid freon chamber surrounded by liquid nitrogen, thus rapidly freezing the sample. These frozen samples were stored in liquid nitrogen until freeze-fracture replicas were made on a Balzar 360M freeze-fracture apparatus. Replicas were carbon-platinum casts of the frozen liposome's surface that had been exposed by a horizontal fracture. Replicas were either cleaned by suspending them on a wire screen above refluxing a chloroform in acetone solution for 1 hour, allowed to dry and then soaked overnight in 10 percent sodium hypochlorite, or simply soaked overnight in 10 percent hypochlorite. A final 30 second acetone rinse removed any remaining lipid. Replicas were transferred to a flamed 200 mesh grid and evaluated on a Sieman's 1A electron microscope at 80 kV. Magnification was determined using a carbon calibration grid.

Neohemocyte Effect on Standard Measures of Blood Clotting:
The prothrombin time (PT) was measured using the method
described by Quick (1963a). The activated partial thrombo-
plastin time (aPTT) was determined using the method of
Proctor et al., (1961); Quick et al., (1963b) and Gonlin et
al., (1965). The method used to determine the partial
thromboplastin time (PTT) was the same as used in the deter-
mination of the aPTT except kaolin was not added.

Viscosity Measurements: Viscosity measurements were made on
Hb solutions, NH suspensions, whole blood and distilled
water using a rotating torque meter at 37°C (Wells-
Brookfield Microviscometer; Stoughton, MA) following the
manufactures recommended procedure.

Neohemocyte Lipid Extraction: The lipid present in a NH
suspension was removed by extraction into chloroform follow-
ing the procedure of Bligh and Dyer (1959). This allowed the
phospholipid fraction to be analyzed separately.

Bleaching of Neohemocyte Samples in Preparation for Liquid
Scintillation Counting: A sample of up to 0.2 ml of NH was
added to a scintillation vial along with 0.2 ml t-butyl
hydroperoxide and 0.1 ml potassium hydroxide (0.1N). The
scintillation vial was capped, shaken for one minute and
then placed into a heating block at 50° C for 12 hours.
Water, 0.7 ml, and 3.5 ml Aquasol (PCS) were added to each

vial; it was shaken and then analyzed.

Determination of the Lipid/Aqueous Ratio as a Function of Density When a 7 g/dl Hb Solution is Encapsulated:

Hemoglobin was dialyzed against a pH 7.5 HEPES buffer containing $1X10^{-5}M$ EDTA. After dialysis 60 μl of ^{14}C sucrose was added. Neohemocytes were made, extruded and separated from the free Hb. An aliquot of NH suspension was transferred to an Hct tube and centrifuged (IEC MB centrifuge, Micro Hematocrit). That fraction of the Hct that contained the pelleted NH was divided into three equal portions by first scoring the Hct tube with a file and then breaking it into fractions. The fractions were designated as high, medium and low density. Each NH fraction was placed into a liquid scintillation vial and counted. The cpm in each fraction were used to determine the amount of water present in each fraction. A phosphorus assay was done on each fraction to determine the fraction's lipid content.

Determination of Neohemocyte Hemoglobin Concentration as a Function of Neohemocyte Density:

Neohemocytes were prepared from 10 g/dl and 20 g/dl Hb solutions which contained ^{125}I-Hb (5mC/mmole) and had been dialyzed against PBS-30. An aliquot of NH suspension was transferred to a Hct tube, centrifuged and divided into three equal fractions as described above. The amount of Hb in each sample was determined by measuring the relative amounts of ^{125}I-Hb.

Neohemocytes and Their Hydronium Ion Permeability: Neohemo-
cytes were prepared using a 15 g/dl Hb solution which had
been dialyzed against 150 mosm, pH 7.5 phosphate buffer.
These NH were divided into three fractions. One was placed
into a dialysis bag and dialyzed against 1 liter of 300
mosm, pH 8 phosphate buffer for 24 hours. Buffer was
changed once during this period. Similar treatment was
given to the second and third fractions, except the buffer's
were pH 6 and pH 7.5 respectively. The pH 7.5 sample served
as control. Each fraction was removed from its dialysis bag
and centrifuged at 6000 g for 20 min at 4°C. The supernant
was discarded and the remaining NH were resuspended in the
same buffer used for dialysis, and then oxygen dissociation
curves were obtained.

In Vivo Neohemocyte Distribution: Samples of 7 g/dl Hb were
dialyzed against PBS-30 or PBS-300. DPG was added to the
resulting Hb solution such that the DPG-Hb ratio was
1.5:1. ^{125}I-BPE was included in the lipid mixture.
Neohemocytes were made, extruded, and separated from unen-
capsulated Hb. The 30 mosm NH preparation was divided into
two batches. One batch was placed into a dialysis bag and
equilibrated with PBS-30 at 4°C. Dextrose (2.7M) was then
infused at the rate of 8.4 ml/hr until the solution reached
300 mosm. Samples were taken from each fraction and a Bligh
and Dyer lipid extraction carried out in order to separate
phospholipids from the DPG and phosphate ions which remained

in the aqueous phase. After extraction, phosphate content of the extracted lipids was measured; this value was used to calculate lipid concentrations.

For each NH preparation, the equivalent of 2 μm lipid were injected into a tail vein of six mice. For each dosage, 3 mice were sacrificed at 2 and 5 hours. At the time of sacrifice, the liver, lung and spleen were removed, weighed and placed into a gamma counting vial. A blood sample was also taken. Total blood volume was assumed to be 7.3% of the total mouse's weight. The amount of marker in each of these samples was determined. Knowing the total dose given and the fraction of dose present in the liver, spleen, lungs and blood the percentage of NH present in each organ at 2 and 5 hours was calculated.

Exchange Transfusions in Rats: Male Sprague-Dawley rats weighing 140-160 g were used in all the exchange transfusion studies. The exchange was performed as described by De Venuto et al. (1977). In this procedure the rats were lightly anesthetized with ether and the jugular vein was cannulated with a catheter. The transfusion was done by removing one ml of blood and immediately infusing one ml of Hb solution or NH suspension. This process was repeated until the desired level of exchange was obtained.

Bacterial Growth in Neohemocyte Suspensions: Blood/agar cultures were plated with 50 μl of a 1:1000 dilution of NH

suspension and placed into an incubator (Model #6300, National Appliance Company, Portland, OR) The cultures were kept at 37°C in an atmosphere containing 5 percent carbon dioxide. After 24 hours, cultures were removed and the number of colonies counted.

CHAPTER V RESULTS

I. Neohemocyte Preparation

Method of Emulsification: Three methods of emulsification
(sonication, homogenization and mechanical shaking) were
investigated to see which produced the most satisfactory
emulsion. Sonication caused excessive Hb denaturation.
Homogenization produced excessive and undesired frothing
along with some Hb denaturation. Mechanical shaking in com-
bination with matching phase densities (see below) produced
the most satisfactory emulsion. There was minimal Hb dena-
turation, no frothing and there appeared to be adequate
potential for scale up.

Effect of Organic Phase Composition on Emulsion Formation:
Ordinary diethyl ether initiated polymerization and dena-
turation of Hb unless peroxides were removed. A mixture of
peroxide free diethyl ether and a Hb solution (4:1, v/v)
could be emulsified by mechanical shaking without Hb dena-
turation. However, the emulsion consistently separated into
two phases within 10 seconds after stopping the shaking. Of
the variables normally available for adjustment to stabilize
the emulsion, only the densities of the two phases and the
ionic strength of the aqueous phase could be adjusted.
Matching the densities of the organic and aqueous phases by
mixing carbon tetrachloride into the ether did not work

because of denatured Hb formation. A mixture of peroxide free diethyl ether and a freon, 1,1,2-trichloro-1,2,2- trifluoroethane, did work. The density of this mixture was matched to that of the Hb phase and, following shaking, the emulsion that formed did not separate within thirty minutes.

Effect of Buffer Composition on Neohemocyte Formation: After a 9 g/dl Hb solution was equilibrated with a pH 7.5, 30 mosm HEPES buffer an equal molar amount of DPG was added. Neohemocytes formed from this Hb solution had a P_{50} of 15 mm Hg. When the same protocol was followed except that pH 7.5, 30 mosm phosphate buffer was used rather than HEPES buffer, the resulting NH had a P_{50} of 21.5 mm Hg.

Role of Organic Phosphate Needed in Neohemocyte Formation: As the DPG to Hb molar ratio in individually prepared NH increased, the P_{50} also increased and approached an asymptotic value of approximately 23 mm Hg, as shown in Figure V.1. However, when the molar ratio of DPG to Hb increased from 1:1 to 10:1, Hb entrapment decreased 30 percent. When IHP was used in place of DPG at a 1:1 molar ratio, the resulting NH had a higher P_{50}, 25 mm Hg, but lower n value, 1.04.

Effect of Initial Hemoglobin Concentration and Osmolarity on Neohemocyte Properties: As the starting Hb concentration was increased, regardless of the osmolarity, the percer ge that
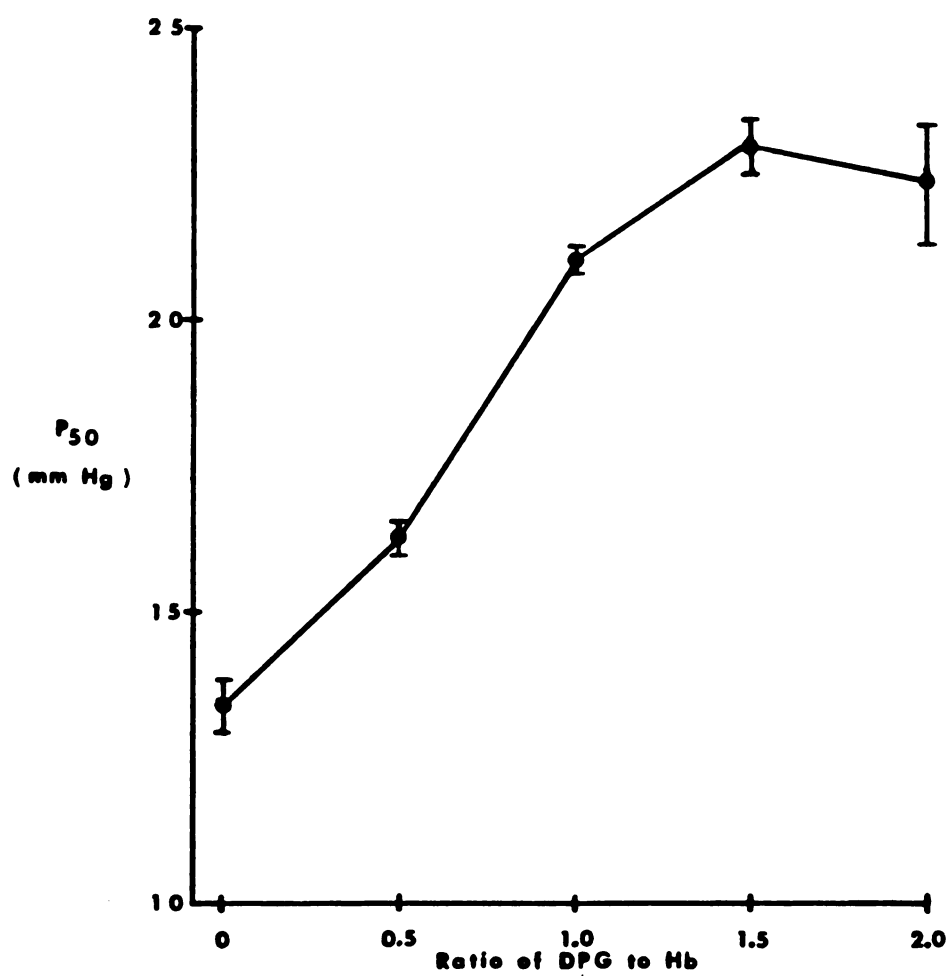
FIGURE V.1. The mean P_{50} of the neohemocytes was plotted vs the starting DPG–Hb molar ratio. The vertical bars show $\pm$ one standard deviation for n=3.

was entrapped decreased (Figure V.2). However, at 30 mosm, the amount of entrapped Hb increased as the starting Hb concentration increased (Figure V.3), even though the percentage of entrapment decreased.

Loss During Neohemocyte Preparation: The amount of lipid lost at each major stage of the NH preparation procedure is shown in Table V.1. Only approximately 50 percent of the starting lipid is retained in the final transfusable suspension.

Neohemocyte Hemoglobin and Methemoglobin Assay: An attempt was made to assay the Hb content of NH by first treating the NH with a 10 percent (w/v) Triton X-100 solution. After lysis, or-more-likely destruction of the bilayer membrane, the freed Hb was assayed spectrophotometrically. Results were unreliable due to substantial light scattering.

Assay for metHb in NH was attempted by converting the metHb to cyanmethemoglobin as described by Masakatsu et al. (1982). The conversion was to be accomplished by adding potassium cyanide to NH. The technique failed because the NH were impermeable to the cyanide ion.

II. Properties of Neohemocytes

Neohemocyte Characteristics from Electron Micrographs: Negative stain EMs revealed that NH were heterogeneous in both
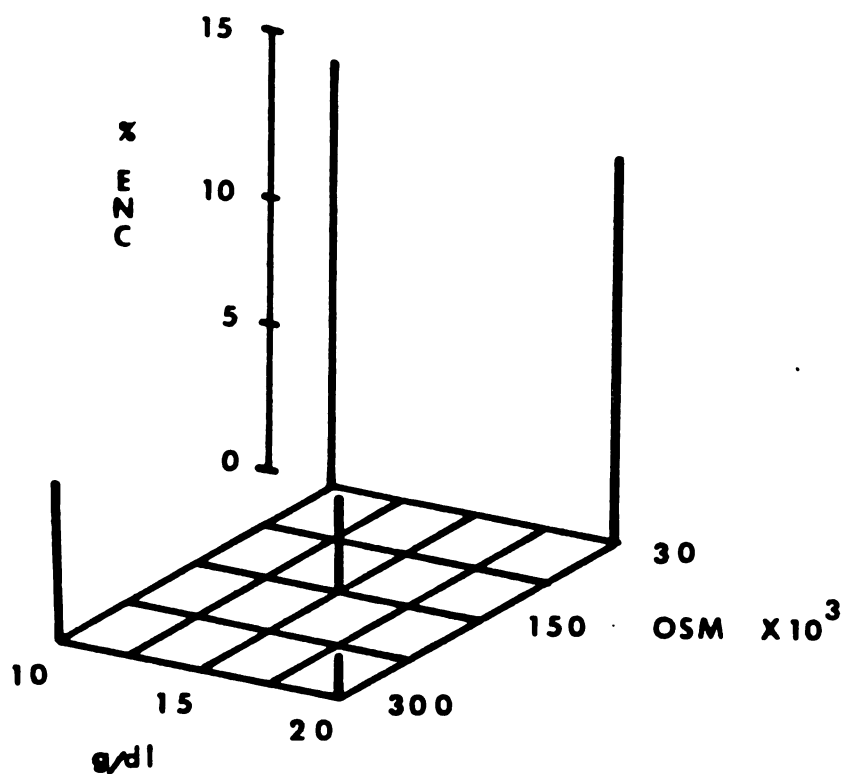
FIGURE V.2.   The percentage of starting Hb encapsulated in neohemocytes
              is plotted as a function of the starting Hb concentration
              and osmolarity. In each case the starting amount of lipid
              used was 3 mmol/dl of Hb solution.
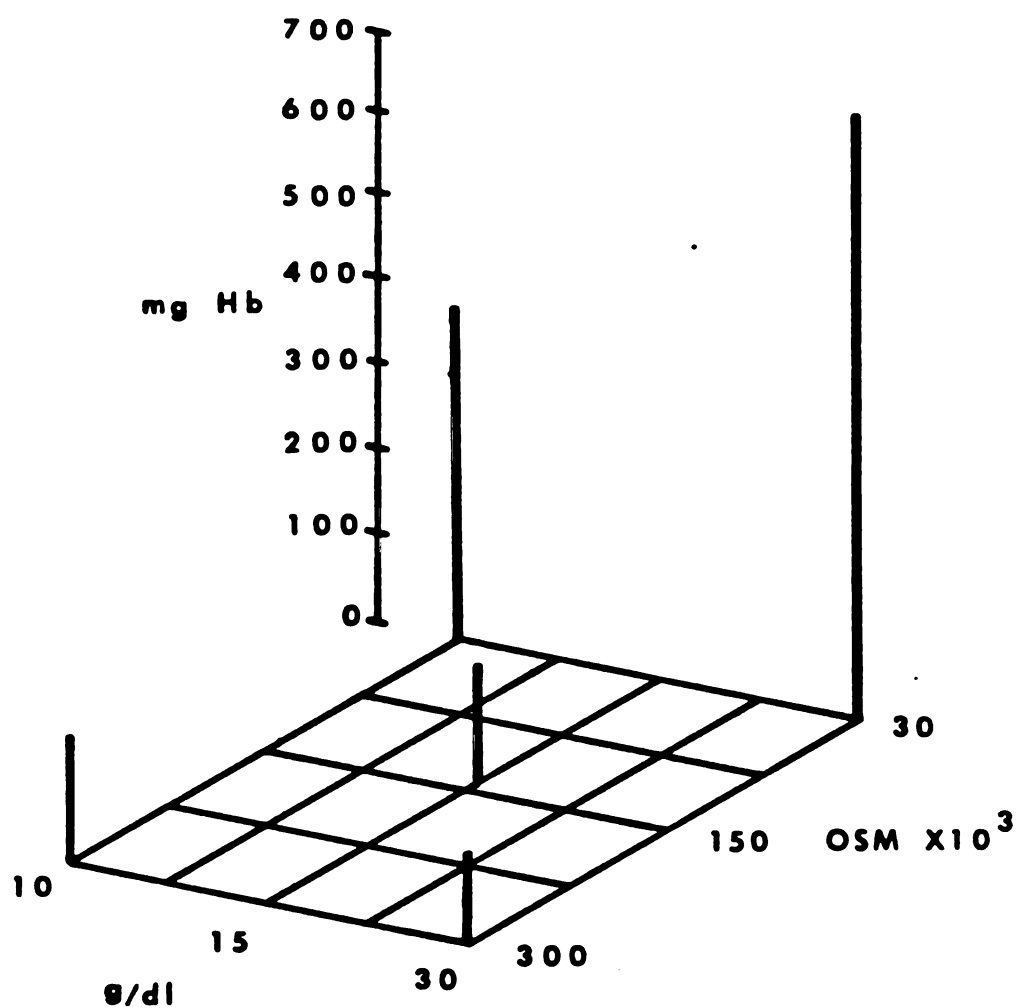
FIGURE V.3.   The mg of starting Hb which was encapsulated in the
              neohemocytes is plotted as a function of starting
              Hb concentration (g/dl) and osmolarity (mosm).

NEOHEMOCYTE PREPARATION STAGE	PERCENTAGE LOST AT EACH STAGE	CUMMULATIVE PERCENT LOST
EMULSION	0	0
AFTER CENTRIFUGATION	13.8	13.8
AFTER EXTRUSION	18.6	32.4
AFTER GEL CHROMATOGRAPHY	1.0	33.4
AFTER RESUSPENSION	16.0	49.4

TABLE V.1. Sequential loss of lipid. The lipid loss was based on using ^{14}C-CH as a lipid marker.

size and Hb content, with some apparently exhibiting an internal chambered structure (Figure V.4). No apparent correlation between size and Hb content was observed. Freeze fracture EMs also showed that NH were heterogenous in size (Figures V.5 and V.6) with some apparently having an internal chambered structure (Figure V.6).

Effect on In Vitro Clotting Times: The PT, PTT, and aPTT were increased above control values when either NH, liposomes or Hb were added to the test system (Table V.2). The only exception was that Hb did not effect aPTT. The increase in clotting time was greatest when the tests were done in the presence of NH.

Neohemocyte Viscosity: A NH suspension having an apparent Hct of 25 was less viscous than rat blood but more viscous than distilled water (Figure V.7).

Heterogenity of Lipid-Aqueous Ratio: As the density of the NH fraction increased the lipid-aqueous ratio decreased (Figure V.8). The preparation of the NH suspension differed from the normal NH preparation in that 50 μM lipid/ml of Hb solution was used instead of 30 μM lipid/ml of Hb solution, HEPES buffer was used instead of phosphate buffer and the final centrifugation was done at 12,000 g instead of 6000 g.

Determination of Neoheocyte Hemoglobin Concentration as a Function of Neohemocyte Density: When NH were made starting
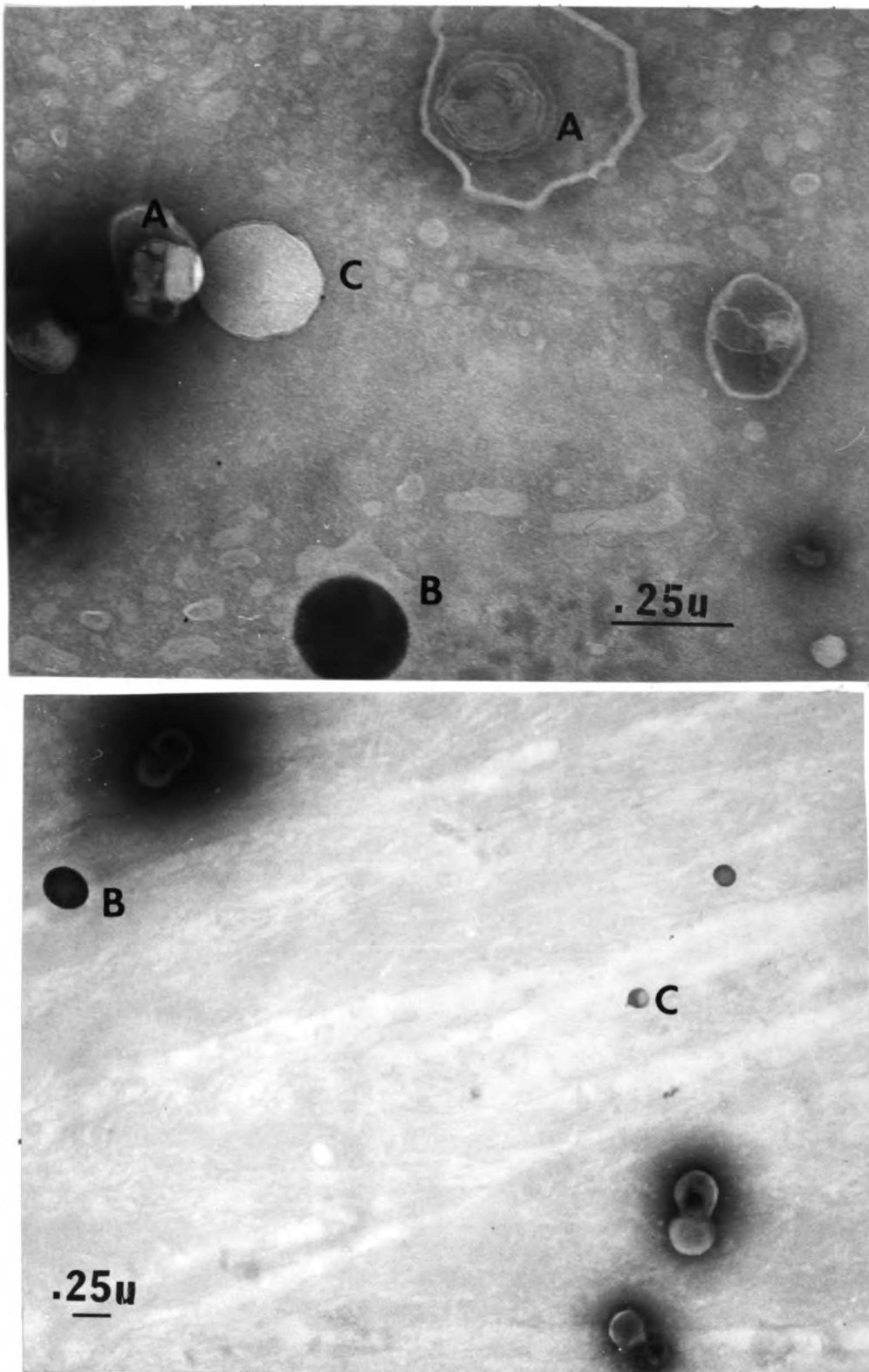
FIGURE V.4.   Negative stain electron micrographs of a typical
               neohemocyte suspension. A: shows evidence of
               internal structure. B: hemoglobin-rich vesicles.
               C: hemoglobin-poor vesicles.
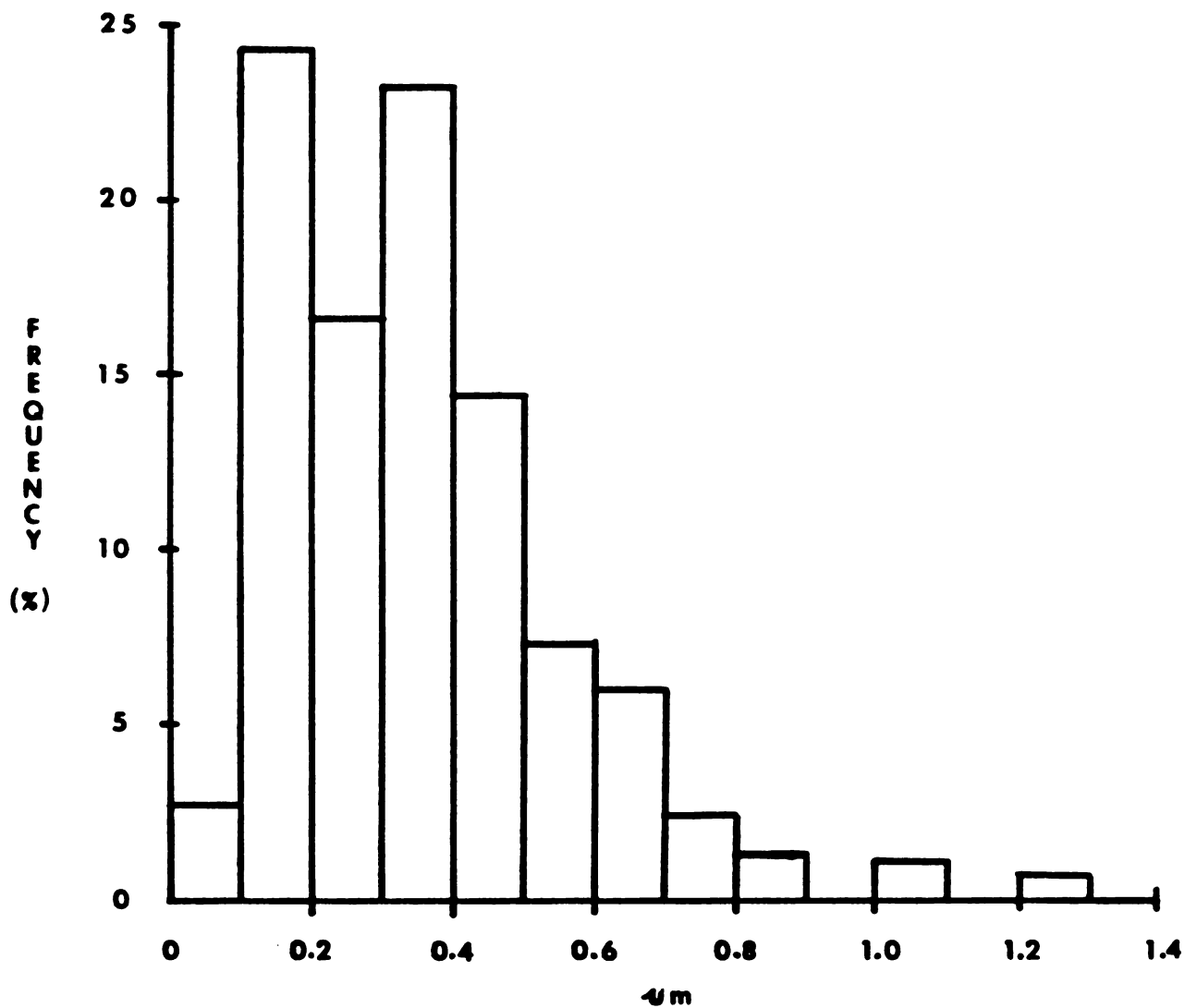
FIGURE V.5.   The frequency distribution of a typical neohemocyte
preparation is plotted vs the observed diameter
measured on freeze fracture micrographs. A total
of 554 replicas were measured. The number average
diameter was 0.37 μm.
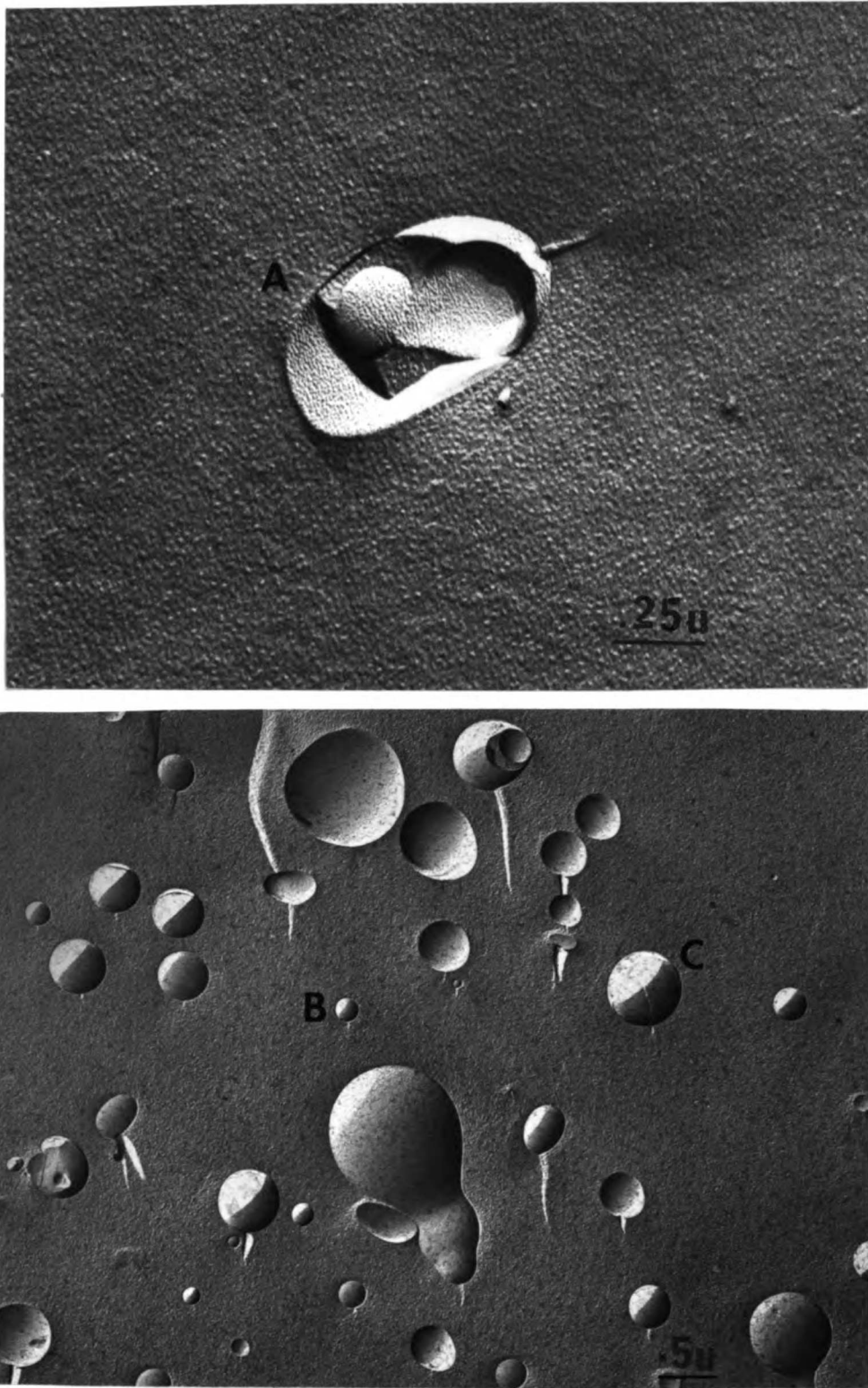
FIGURE V.6.   Freeze fracture electron micrographs of a typical
              neohemocyte suspension. A shows evidence of internal
              structure. B is a neohemocyte with a diameter of
              0.2 μm. C is a larger neohemocyte with a diameter
              of 0.7 μm.

SAMPLE	PT	PTT	aPTT
Buffer Control	$11.6^{\pm}0.3$	$64.0^{\pm}8.5$	$26.4^{\pm}1.1$
7 g/dl Hb	$12.7^{\pm}0.2$	$98.6^{\pm}12.3$	$24.4^{\pm}2.2$
Liposomes	$14.9^{\pm}0.1$	$82.8^{\pm}2.0$	$35.1^{\pm}1.0$
Neohemocytes	$15.6^{\pm}0.3$	$108.1^{\pm}7.9$	$30.5^{\pm}0.6$

Table V.2.   Effect of Hb, liposomes and neohemocytes on three
             in vitro measures of clotting time. Both liposomes
             and neohemocytes were prepared in the same manner
             except that the liposomes did not contain Hb. Values
             shown are means $\pm$ one SD.
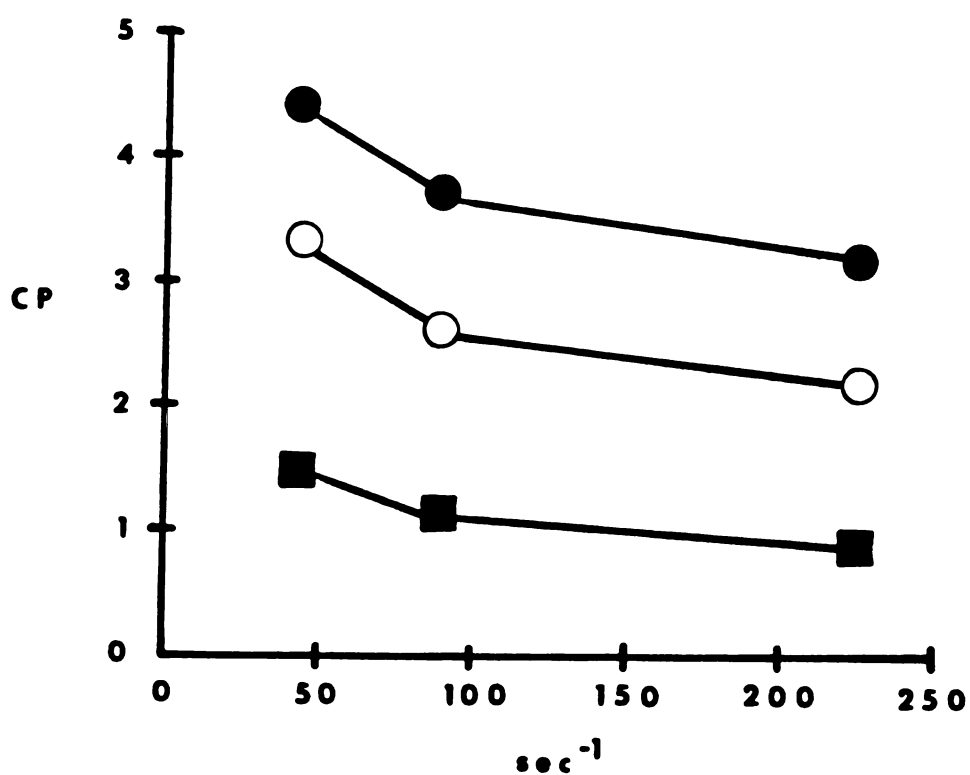
FIGURE V.7.　　Mean viscosity (CP) in centipoise, for three preparations at 37^6 is plotted vs shear rate (sec^{-1}). Data were obtained using a Wells-Brookfield Microviscometer. (●) fresh, whole rat blood; (O) neohemocytes with an apparent hematocrit of 0.4 suspended in a saline buffer containing 5 g/dl of human serum albumin; (■) distilled water control. Vertical bars are $^{+}_{-}$one SD for n=3.
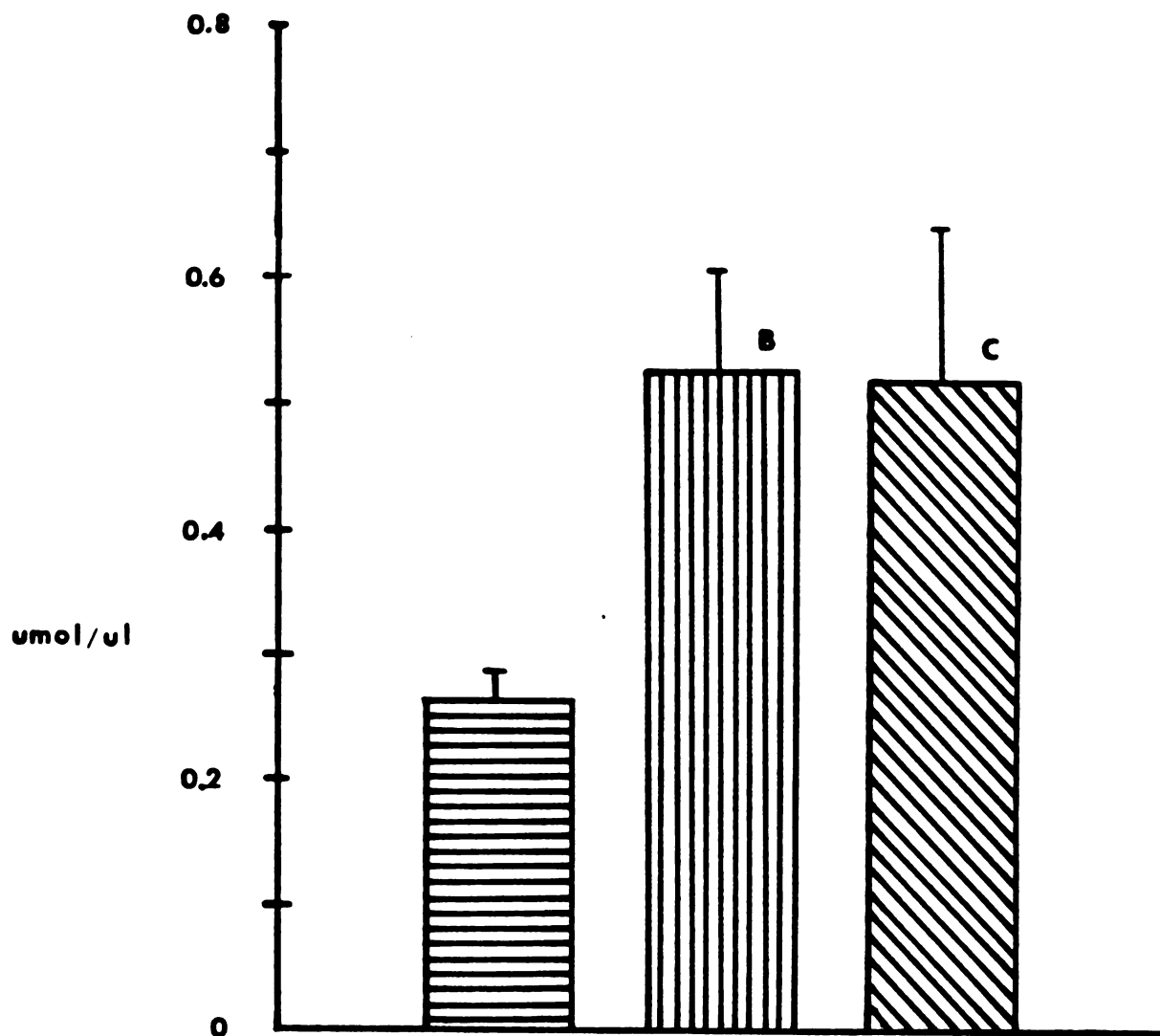
FIGURE V.8.   The mean lipid-aqueous ratios of different density
              fractions of a typical neohemocyte preparation
              are indicated. The preparation was centrifuged.
              The lower third, A, had the highest density, the
              middle third, B, was of intermediate density, and the
              upper third, C, had the lowest density. Vertical
              bars show one SD for n=3.

with 10 g/dl or 20 g/dl Hb, the density of the NH decreased as the percentage of incorporated Hb decreased (Figures V.9 and V.10). In the preparation of this NH suspension approximately 20 percent of the original NH were discarded (those with the lowest density).

Permeability of Neohemocyte Membranes to Hydronium Ion: The internal pH of aliquots of a NH suspension were equilibrated to either pH 6, 7.5 or 8 by prolonged equilibration in buffers having the target pH. The P_{50} of these modified NH were then determined at pH 7.5 by biotonometry. Had the internal and external pH rapidly equilibrated, the P_{50} values would have been identical. As shown in Figure V.11, this was not the case when the biotonometry reading was begun one minute after addition to the pH 7.5 media.

III. In Vivo Results

In Vivo Distribution of Neohemocytes: Following intravenous doses of 2 μmole/mouse, all three NH preparations distributed preferentially to the liver (Figure V.12). The organ distribution pattern of the 300 mosm preparation was essentially identical, except for higher spleen levels, to that previously observed for identical composition vesicles not containing Hb (Abra and Hunt, 1981). The 30 mosm preparation was only modestly different, producing higher liver levels and as a consequence, lower blood, spleen and lung
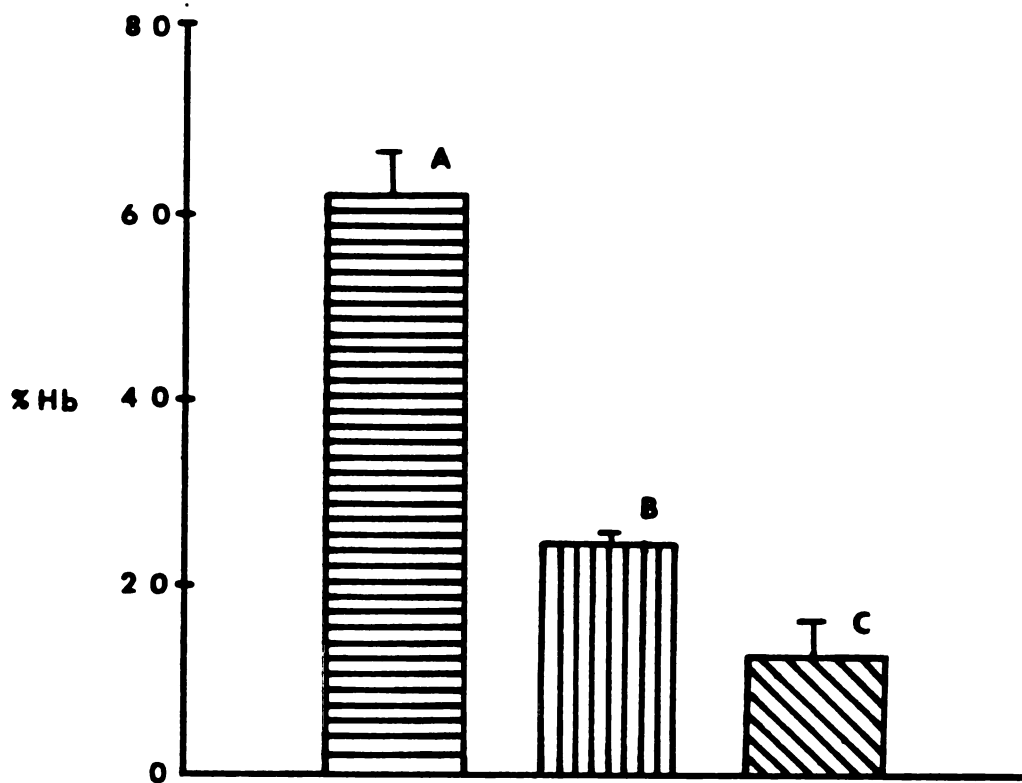
Figure V.9. The percentage of the encapsulated Hb associated with the high (A), medium (B) and low (C) density fractions of the centrifuged neohemocytes is shown. Each of the fractions were of equal volume. The neohemocytes were prepared from a 10 g/dl Hb solution. The vertical bars represent one SD for N=3.
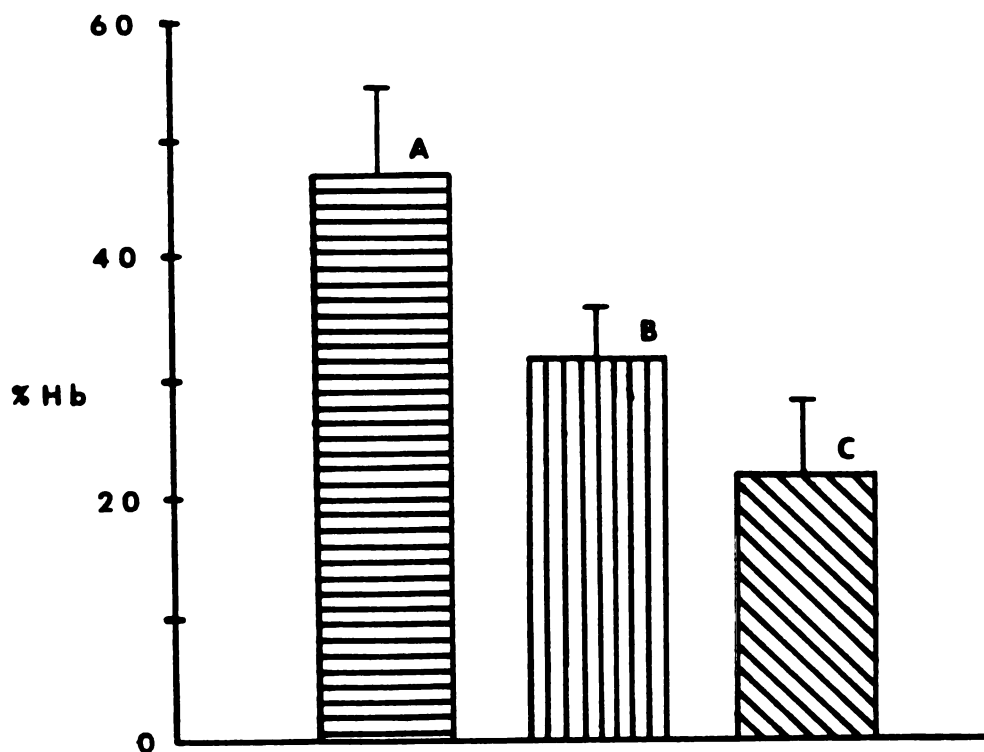
FIGURE V.10. The percentage of the encapsulated Hb associated with
the high (A), medium (B) and low (C) density fractions
of centrifuged neohemocytes is shown. Each of the fractions
were of equal volume. The neohemocytes were prepared
from a 20 g/dl Hb solution. The vertical bars represent
one SD for N=3.
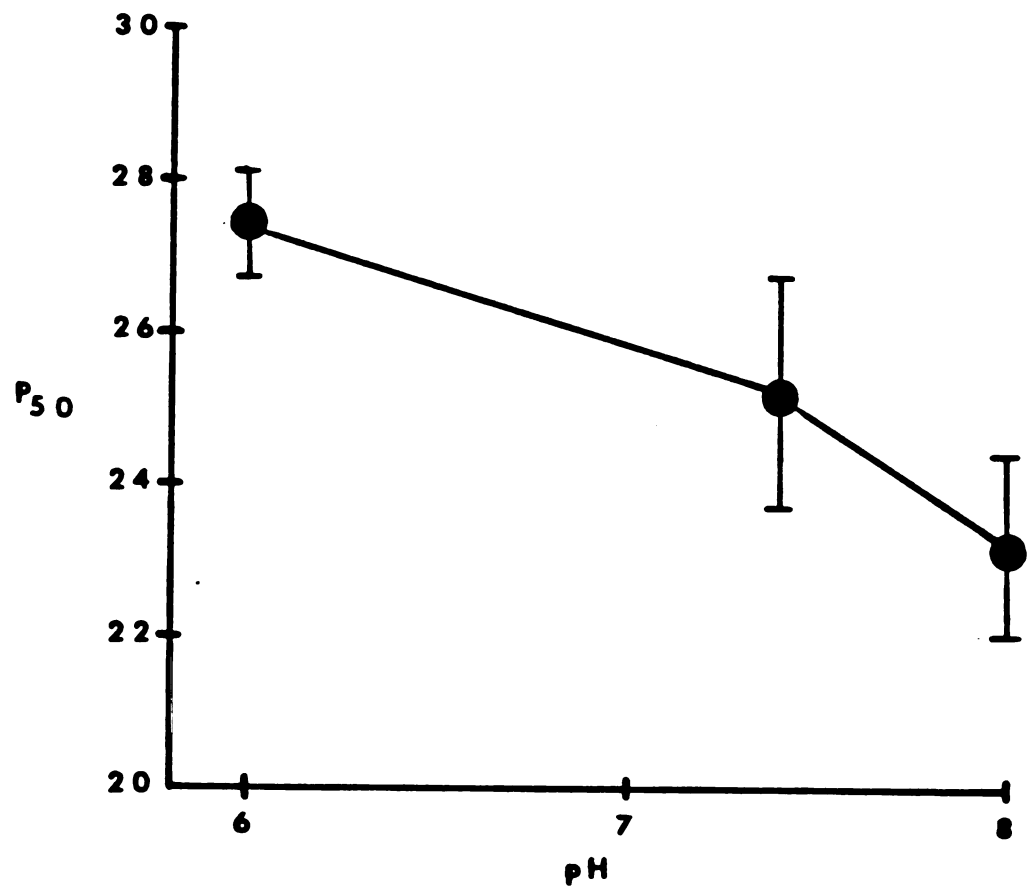
FIGURE V.11. P_{50} values of different neohemocyte preparations is plotted vs the pH of the preparation. After equilibration at pH 6, 7.5 or 8, neohemocytes were placed in pH 7.5 buffer for one minute before their P_{50} was determined by biotonometry. Verticle bars are $\pm$ one SD for n=3.

BLOOD

12

8

$\overline{x}$

4

C

A

B

0

0   2   4   6

hr

LIVER

120

80

$\overline{x}$

A

B

C

40

0

0   2   4   6

hr

SPLEEN

15

C

10

$\overline{x}$

B

A

5

0

0   2   4   6

hr

LUNG

1.5

1.0

$\overline{x}$

C

0.5

B

A

0

0   2   4   6

hr
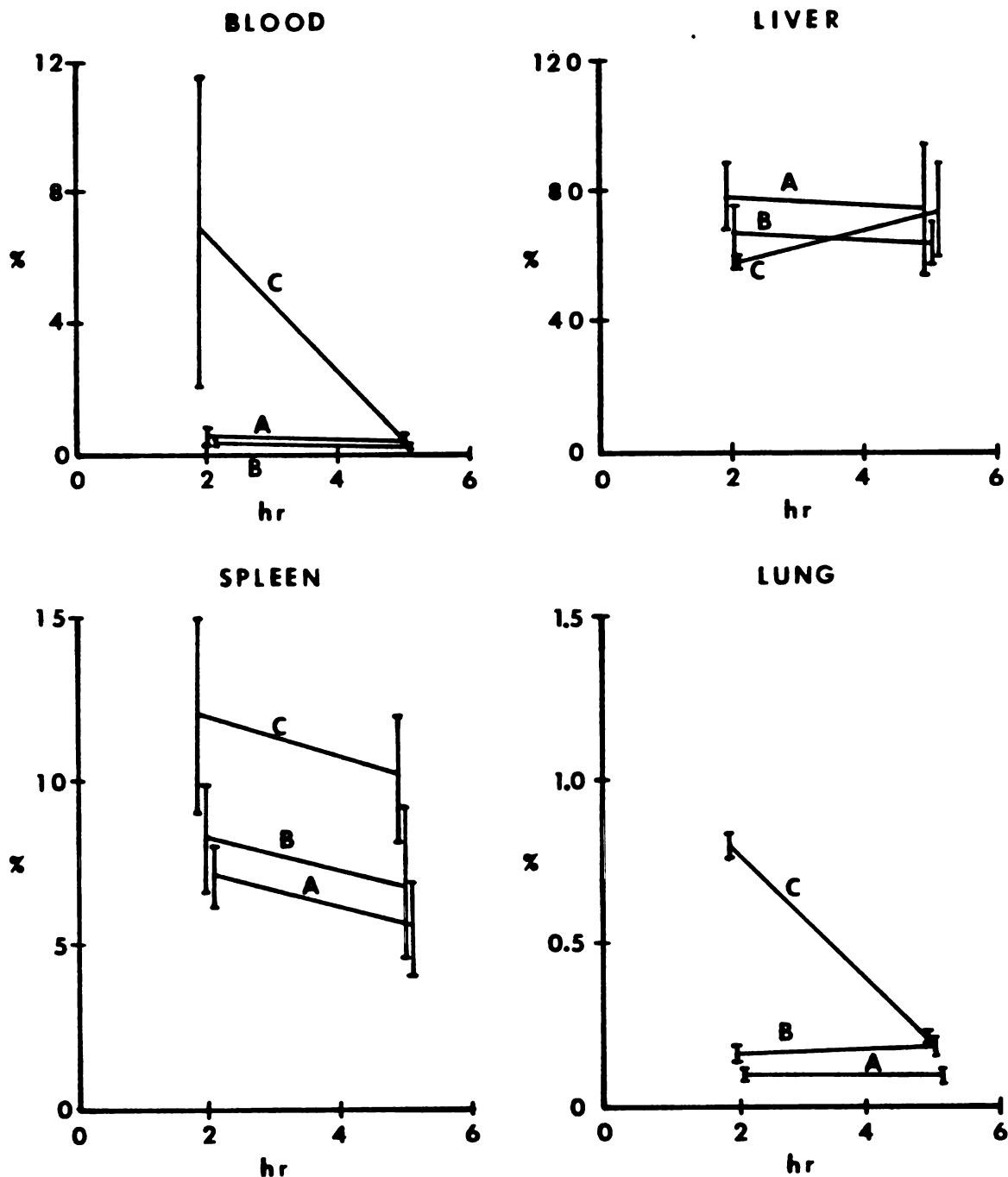
FIGURE V.12.   The percentage of neohemocytes associated with blood, liver, spleen and lung of mice is plotted vs time after an intravenous bolus injection. A dose of neohemocyte suspension containing 2 μm lipid was given. A, B and C were neohemocyte suspensions made at 30 mosm, 300 mosm and 30-300 mosm, respectively.

levels. The disposition pattern of the 30-300 mosm neohemocytes, however, was distinctly different. Blood and lung levels were substantially higher at two hours, but not significantly so at five hours. Liver and spleen levels were dramatically depressed at two hours. Liver, but not spleen levels were essentially the same as for the other two preparations. Carcass levels were not measured.

Bacterial Growth in Neohemocyte Suspensions: All starting buffers and Hb solutions were sterilized by filtration prior to use. Sterility was checked at major stages in the NH preparation procedure on several occasions and routinely after completion of the procedure. When NH were transferred to a sterile container after NH formation, but before centrifugation to remove particulate material and before separation of free from encapsulated Hb, the suspension showed no evidence of bacterial growth. However, after completion of the preparation, there were typically about 100,000 bacteria per ml, primarily E. coli and pseudomonas. Attempts to decrease the bacterial contamination met with minimal success.

Exchange Transfusion with Neohemocytes and Hemoglobin:

After a transfusion of NH (Tables V.4, V.5, and V.6) or Hb (Tables V.3 and V.6), samples taken immediately had n values that were lower than that of the rat blood or transfusion agent. The P_{50} value of a sample taken

SAMPLE	SURVIVAL TIME (hr)	n	P_{50} (mm Hg)	Hb (g/dl)	RBC Hct
RAT #1					
PRETRANSFUSION		2.58	39.5	9.11	37.1
POSTTRANSFUSION		1.98	16.6	2.88	3.3
DEATH	4.5	1.64	30.0	2.49	7.0
RAT #2					
PRETRANSFUSION		2.68	39.4	8.59	32.6
POSTTRANSFUSION		1.97	16.3	3.18	3.5
DEATH	3.4	1.71	20.7	2.14	5.3
RAT #3					
PRETRANSFUSION		2.59	42.3	9.27	39.9
POSTTRANSFUSION		1.85	17.4	3.41	3.7
DEATH	2.3	1.64	18.9	2.39	3.6
RAT #4					
PRETRANSFUSION		2.73	37.1	7.64	33.5
POSTTRANSFUSION		2.35	12.6	5.52	2.9
DEATH	6.0	1.61	15.0	2.71	3.8

TABLE V.3.   Results of control transfusions. Rats #1, #2 and #3 were transfused with a 0.9 g/dl sodium chloride solution containing 3.2 g/dl Hb and 1.8 g/dl bovine albumin. Rat #4 was transfused with 0.9 d/dl sodium chloride solution containing 7.2 g/dl Hb. Samples were taken before the transfusion (pretransfusion), immediately after completion of the transfusion (posttransfusion) and at the time of death.

SAMPLE	SURVIVAL TIME (hr)	n	P_{50} (mm hg)	TOTAL Hb (g/dl)	RBC Hct	NH Hct	Hb in NH (g/dl)
RAT #1							
PRETRANSFUSION		2.63	39.6	8.71	34.5	0.0	0.00
POSTTRANSFUSION		1.95	29.0	4.47	4.0	17.0	3.46
DEATH	6	1.81	32.4	2.13	5.5	10.4	0.74
RAT #2							
PRETRANSFUSION		2.70	34.5	7.19	34.9	0.0	0.00
POSTTRANSFUSION		1.98	27.4	4.23	4.5	18.9	3.30
TERMINATE	6	1.81	27.0	2.47	5.0	10.8	1.44
RAT #3							
PRETRANSFUSION		2.43	32.4	9.11	36.0	0.0	0.00
POSTTRANSFUSION		2.01	25.8	4.52	4.8	19.9	3.31
TERMINATE	6	2.06	28.0	2.86	5.6	10.6	1.44

TABLE V.4.   Results of transfusions with NH. For rat #1, the NH were suspended in a Ringer's buffer which contained 3 g/dl human albumin. For rats #2 and #3, the NH were suspended in 0.9 g/dl sodium chloride solution which contain 3 g/dl bovine albumin. Samples were taken before the transfusion (pretransfusion), immediately after the transfusion (posttransfusion) and at the time the rat died (death) or was sacrificed (terminate).

SAMPLE	SURVIVAL TIME (hr)	n	P_{50} (mm Hg)	TOTAL Hb (g/dl)	RBC Hct	NH Hct	Hb in NH (g/dl)
RAT #4							
PRETRANSFUSION		2.67	36.7	10.01	35.9	0.0	0.00
POSTTRANSFUSION		2.07	28.1	4.14	3.2	18.4	3.25
DEATH	21	1.90	32.4	1.98	4.8	9.8	0.64
RAT #5							
PRETRANSFUSION		2.40	44.8	10.80	43.6	0.0	0.00
POSTTRANSFUSION		1.89	30.9	4.47	1.5	24.3	4.10
DEATH	22	1.68	32.2	1.45	2.0	9.1	0.95
RAT #6							
PRETRANSFUSION		2.70	37.7	8.06	35.2	0.0	0.00
POSTTRANSFUSION		2.09	26.7	4.33	3.6	20.9	3.51
SURVIVED	RECOVERED						
NH SUSPENSION	-	2.0	26.0	3.80	-	-	3.80

TABLE V.5. Results of transfusions with NH. For all rats the NH were suspended in 0.9 g/dl sodium chloride solution which contained 3 g/dl bovine albumin. Samples were taken before the transfusion (pretransfusion), immediately after the transfusion (posttransfusion) and at the time the rat died (death).

SAMPLE	n	P_{50} (mm Hg)	Hb (g/dl)	Hct
3.2 g/dl Hb	2.20	13.6	3.16	-
7.2 g/dl Hb	2.23	12.0	7.20	-
WH SUSPENSION	2.00	26.0	3.80	25.0

TABLE V.6. Properties of solutions and suspensions used in transfusions. These data were for the preparations used in the rat exchange transfusions, in Tables IV.3, IV.4 and IV.5. The 3.2 g/dl Hb solution contained 0.9 g/dl sodium chloride and.1.8 g/dl bovine albumin. The 7.2 g/dl Hb solution contained 0.9 g/dl sodium chloride solution containing 3 g/dl bovine albumin.

immediately after the tranfusion was higher than that obtained for the transfusion agent and lower than that obtained for rat blood. The n and P_{50} values continued to change after the transfusion was completed. After transfusion with NH, the P_{50} values of samples removed from a rat were higher, and remained higher, than those obtained after Hb was transfused. The survival times for rats transfused with NH were longer than those obtained with Hb alone (Figure V.13). Rats transfused with a Hb solution died while their Hb concentration was still above 2 g/dl. Rats transfused with a NH suspension lived even after their Hb concentration fell below 2 g/dl.

After a NH transfusion, the apparent Hct initially decreased rapidly and then more slowly (Figure V.14). The oxygen carrying ability of the NH decreased more rapidly than did the apparent Hct (Figure V.14).

Rats transfused with a 7 g/dl Hb solution continue to urinate a dark red urine at the rate of about 0.9 ml/hr. Rats transfused with a NH suspension continued to urinate at a rate of about 0.5 ml/hr. The urine was initially pale red changing to clear yellow within a hour. Untreated control rats urinated at the rate of about 0.2 ml/hr.

In Vivo Toxicity Caused by a Neohemocyte Transfusion: Rats transfused with a 7.2 g/dl Hb solution, NH suspended in lactated Ringer's solution containing 3 g/dl human albumin or
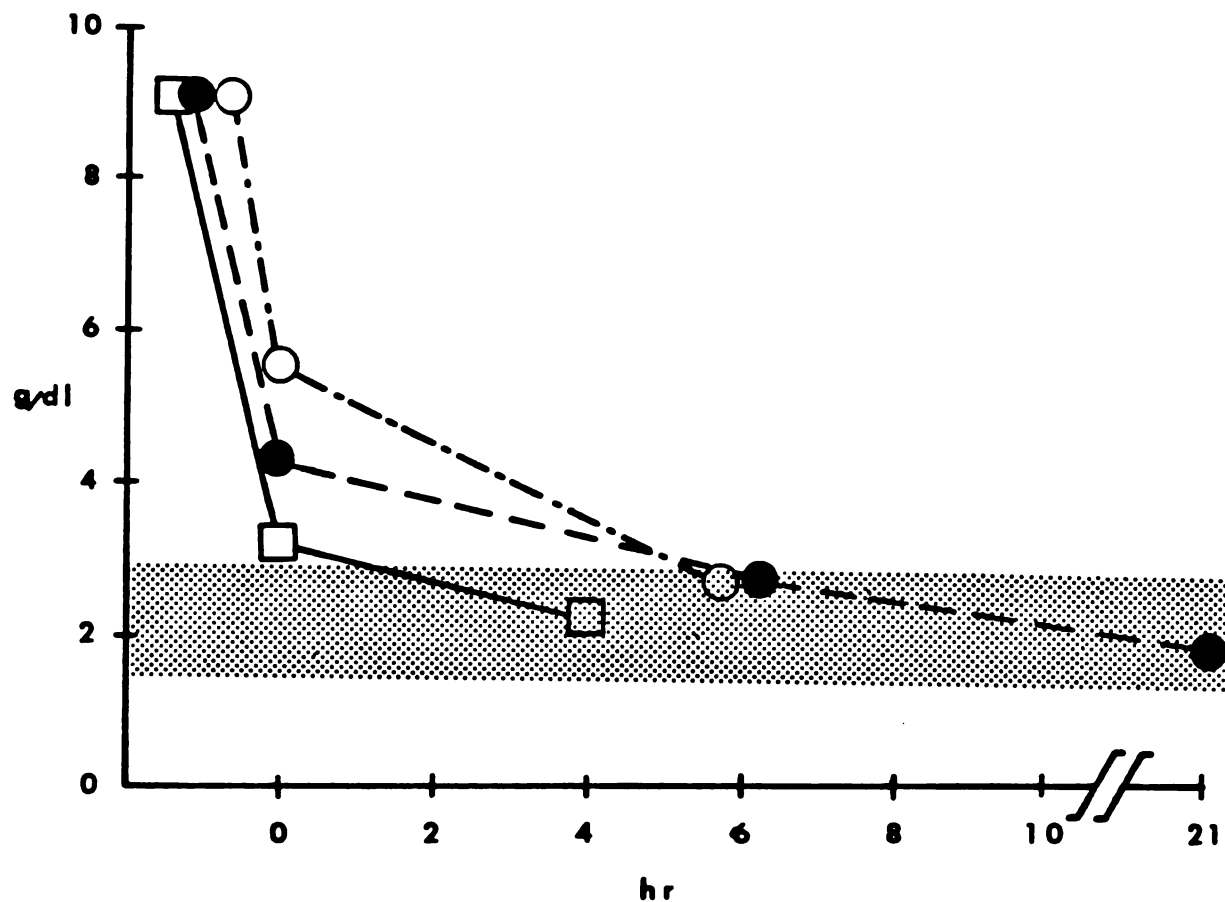
FIGURE V.13.   The Hb content of 'blood', calculated from
                biotonometry measures, for transfused rats is
                plotted vs time. Zero time is when the trans-
                fusion was completed; earlier points are pre-
                transfusion values. The shaded region gives the
                lower limit for Hb concentrations known to support
                life (death usually occurs shortly after values
                drop into or below this range). (O): rats
                transfused with a 7.2 g/dl Hb solution; (●) rats
                transfused with the NH suspension; (□)- controls:
                transfused with a 3.6 g/dl Hb solution. In each
                case the final values were at the time of death.
                Additional data from this study are given in Tables
                IV.3. IV.4 and IV.5.
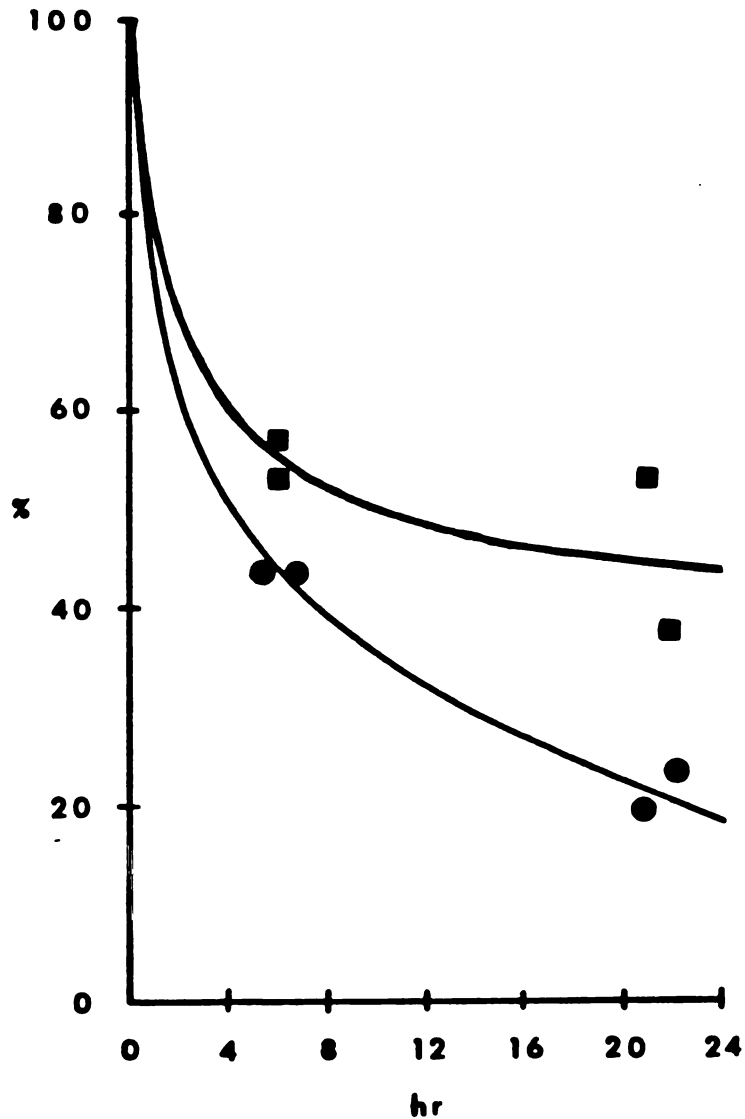
FIGURE V.14.   The percentage change in the apparent Hct (■)
and oxygen content (●) of 'blood' is plotted vs
time post-transfusion. The 100% values are
based on determinations made immediately after
transfusion. Apparent Hct refers to NH and does
not include RBCs. Values at about 6 hour were
from rats that were killed. The final values were
from rats that died. Each pair of data points is
for one rat.

with NH suspended in 0.9 g/dl sodium chloride solution containing 3 g/dl of bovine albumin were examined for pathological tissue changes. The liver, kidney, spleen, lung, heart and bladder were examined with the unaided eye. Examination under a light microscope was done on tissue samples taken from the liver, kidney, spleen, lung and heart. Tissue samples were stained and mounted on slides.

Transfusion with a 7.2 g/dl Hb solution caused no noticeable changes in the liver, lungs or heart. The kidney tubules contained protein casts and there was mild degeneration and protein leakage in the glomeruli.

Rats transfused with NH suspended in lactated Ringer's buffer containing 3 g/dl human albumin showed pathologic changes in all tissues studied. Examination by eye revealed hemorrhagic spots on the lung and liver, and a bladder containing a white precipitate. Histological examination showed necrosis in all tissues examined. The severity of tissue damage was greatest in the kidney followed by the liver, lung and heart in decreasing order of severity. Epithelial cells were missing from the kidney tubules and there was evidence of protein leakage in Bowman's capsule. The liver had diffuse cellular damage throughout, secondary to protein infiltration.

Rats transfused with NH suspended in 0.9 g/dl sodium chloride containing 3 g/dl bovine albumin showed some pathological changes in the tissues examined. There were

hemorrhagic spots in the lungs and liver, but no white particulate material in the bladder. Histological examination showed that the kidney tubules contained protein casts with some protein leakage from the glomeruli, and exhibited substantially less damage than seen in kidneys from rats transfused with Hb solution or NH suspended in lactated Ringer's buffer containing 3 g/dl human albumin. Similarly, the liver, lungs, spleen and heart had less damage than that seen in rats transfused with NH combined with human albumin.

CHAPTER VI DISCUSSION

I. Lipids

Cholesterol (CH) is used to stabilize the liposomes via hydrophobic and possibly H-bonding interactions directly beneath the phospholipid head groups. This interaction reduces the flexibility of the acyl chains and thus stiffens the membranes. This stiffening is accompanied by a reduction of surface area per molecule and a decrease in permeability. Additionally, CH broadens or eliminates the gel to liquid crystalline phase transition (Ladbrooke et al., 1968). This phase transition is not desirable since there is an increase in permeability at such a transition. Therefore, the incorporation of CH into bilayers of phospholipid vesicles renders them much less susceptible to the destructive action of plasma protein (Kirby et al., 1980). Egg phosphatidyl choline (PC) used in our experiments was a mixture of neutral phosphatidyl cholines and thus has no phase transition.

Phosphatidic acid (PA) is negatively charged at physiological pH (7.4). The negative charge causes charge repulsion in the lipid bilayer, thus, increasing the distance between bilayers of the liposome (Gulik-Krzywicki et al., 1967). The increased distance between bilayers allows for the possibility of greater Hb entrapment. Possible disadvantages of this charge repulsion within the bilayer are

increased permeability and decreased stability. This change, however, is compensated for by the inclusion of CH.

All liposomes undergo autoxidation that is accelerated at elevated temperature, and catalyzed by light, metal ions and some solutes. As a result there is often an abrupt change in the liposomes' permeability. Incorporation of alpha-tocopherol (T) into liposomes prolongs the characteristic induction phase of autoxidation. The induction period is defined as the time separating preparation of the liposomes and the confirmed leakage of trapped solute or rapid rise of oxidation products. Addition of CH enhances the effect of T. A benefit of incorporation of T into liposomes is improved stability in plasma. However, the addition of increasing amount of T to liposomes results in increasing evidence of oxidation at early times. For this reason only 0.2 parts in 10 on a molar basis was used (Hunt et al., 1981).

II Qualitative Indicators of Neohemocyte Preparation Quality

Several qualitative indicators can be used to evaluate a preparation of the NH. If the peroxides are not sufficiently removed from the diethyl ether, mixing diethyl ether with Hb resulted in its polymerization. If MetHb accounts for more than 5 percent of the total Hb, then during the removal of organic solvent from the emulsion excessive amounts of denatured Hb/lipid-protein are formed. This

problem may be the result of MetHb being less stable and more subject to denaturation. The observation of denatured Hb/lipid-protein aggregates indicates a poor preparation whatever the cause. After the mixture of NH and unencapsulated Hb is centrifuged, the presence of a large amount (greater than 5 percent by volume) of precipitate indicates excessive denaturation. When the supernatant obtained after this centrifugation is difficult to extrude, an unacceptable amount of denaturation should be suspected. If there was a reddish tinge present throughout the column after separation of NH from unencapsulated Hb the preparation should be considered suspect. This reddish tinge may be due to lipid-Hb aggregates bound to the Bio-Gel. If the rat's respiration became erratic during the transfusion, then the rat's chance of survival over the next few hours is small, and the preparation should, again, be considered suspect. The orgin of these various problems remains to be identified.

III. Neohemocyte Formation

The procedure described produces NH that are heterogenous in size, and that have a range of Hb content and encapsulated aqueous fractions. These characteristics suggest that the NH also have complex internal structures, a hypothesis that is supported by electron microscopy. In chapter III, a model (Figures III.7 and III.8) for NH formation was given. This model provides one explanation for the heterogeneous size, differing Hb content and complex struc-

ture.

As Hb concentration increases, the percent of initial Hb encapsulated decreases. A reasonable explanation of this consistant observation is related to the higher viscosity characteristic of the more concentrated Hb solutions. As the relative viscosity of the aqueous phase increases, dispersed droplet size increases resulting in decreased emulsion stabiltity. As a consequence, fewer NH are formed, and thus, the total amount of initial Hb entrapped decreases.

When either the concentration of DPG or the osmolarity of the buffer increases, the amount of encapsulated Hb decreases. The increased DPG concentration and buffer osmolarity results in increased ionic strength. The increased ionic strength should affect hydration of the lipid monolayer surrounding the dispersed aqueous droplets; it may also alter the ability of Hb to interact with this monolayer. If these changes result in a less ordered monolayer, then emulsion stability would decrease and an increasing fraction of the lipids would exist as hydrated reverse micelles. These hydrated reverse micelles would pack together upon removal of the organic phase. When the resulting bilayer network is disrupted a variety of vesicles would form which contain little Hb. In fact a dramatic reduction of NH Hb content results when either DPG concentration or osmolarity is increased.

IV. Neohemocyte Characteristics

The Permeability of Neohemocytes: In the hydronium ion permeability experiment, the P_{50} was chosen as an indicator of internal pH because increasing hydronium ion activity decreases the P_{50}. If internal equilibration of pH occurred in one minute or less all three NH preparations should have given the same P_{50} value. Since the P_{50}s are not the same, equilibration must require more than one minute. Also, failure of the metHb assay was due to the inability of cyanide to enter the NH. Both of these observations indicate that NH are poorly permeable to ions. Nichols et al. (1980a and b) and Gutknecht et al. (1981a and b) have reported hydronium ion permeabilities ranging from 10^{-4} to 10^{-9} cm/sec. This wide variation in hydronium ion permeability depends on the lipid composition and on how the vesicles were formed. Thus, the available information in the literature allows for hydronium ion permeabilities consistent with the data obtained.

Although bicarbonate or chloride permeability was not determined, the results indicate that these should be evaluated. Low chloride permeability might be expected since NH contain PA, a negatively charged phospholipid at pH 7.5 (Papahadjopoulus et al., 1967). Increasing NH permeability to bicarbonate and chloride without decreasing lipid vesicle stability, along with coencapsulation of carbonic anhydrase would further enhance NH utility. An increased

permeability and coencapsulation of carbonic anhydrase would increase the NH's ability to transport carbon dioxide from tissues to the lungs.

Effect of DPG-Hb Molar Ratio on Neohemocyte Performance: As the DPG-Hb molar ratio increases, P_{50} increases and slowly approaches an asymptotic value. This response is to be expected since one molecule of DPG interacts within the central cavity of each Hb molecule. Additional DPG molecules can associate with the outside of Hb, but would have no effect on oxygen affinity to Hb. Since increasing amounts of DPG leads to a decrease in the amount of Hb entrapped in NH, a molar ratio of 1.5:1 (DPG-Hb) is used. This provides P_{50} values close to the maximum obtainable while not effecting Hb encapsulation.

Viscosity of Neohemocytes: In order for a blood substitute to reach the microvasculature it must have acceptable flow properties. The main variable governing flow is viscosity. Viscosity data for rat blood, NH and distilled water have been presented. The NH behave similar to RBCs in that at low shear rates viscosity is higher than at high shear rates. This behavior is probably due to increased NH-NH interaction at the lower shear rates.

The viscosity of NH is lower than that of rat blood, and, as expected, is a function of the Hct. It is the level and nature of NH, rather than the free protein or suspending

buffer, which primarily determines the viscosity. Interactions between the NH and components of rat blood might raise, lower or not effect the viscosity. In order to avoid a life threatening situation, caused by an elevated viscosity, the NH suspension's apparent Hct has been chosen to be significantly less than that of rat blood. This is a compromise because in doing so the Hb content in the circulation after an exchange transfusion will be less.

Neohemocyte Effects on Blood Clotting: The measures for clotting time (PT, PTT and aPTT) were all prolonged in the presence of NH, liposomes or Hb. The prolongation of the clotting time was greatest for NH and least for Hb. Since the PT and PTT were prolonged this could indicate deficiencies in fibrinogen, prothrombin, or factors V and X (Guyton, 1981).

During a total exchange transfusion about 80 percent of the clotting factors are removed. In circumstances where there are no foreign substances present which could alter clotting times this remaining 20 percent is completely adequate in maintaining normal coagulation times (personal communication with F. DeVenuto, LAIR). When a foreign substance, such as NH, is transfused and does affect clotting times it may be necessary to supplement this remaining 20 percent of clotting factors. Additional clotting factors could be included in the NH suspending medium.

V. In Vivo Distribution

The distribution of lipid vesicles has been extensively studied (e.g. Abra and Hunt, 1981). Important variables affecting disposition are vesicle composition, lipid dose, total surface area and effective mean diameter. Comparisons between different disposition studies are difficult at best. In fact, Abra and Hunt (1981) emphasize that there is a tendency for variation between individual experiments even at identical lipid doses, possibly resulting from several uncontrolled and yet unidentified variables. The nature of the vesicle marker may be particularly important. Even though Abra (1982b) points out that no apparently different distribution results are obtained using ^{14}C inulin as opposed to ^{125}I-BPE, at least at early times (e.g. 0-5 hours post dose), the choice of the marker still remains a potential variable. The large MLVs of Abra and Hunt have the same composition and similar size as the 300-NH, and therefore would be expected to have similar in vivo distribution properties, which they do. These similarities in disposition at similiar doses suggest that the different preparation procedure used for NH and the presence of encapsulated Hb do not result in substantially different vesicles.

However, there are differences in disposition among the three NH preparations, and these differences must be the result of the different osmotic treatments used. Any speculation about the reasons for different disposition proper-

ties requires a few assumptions. One assumption is that each type of NH is equally stable in vivo. If these preparations have slightly different stabilities, then the level of the marker present in any tissue will not reflect just intact NH. The distribution pattern of the 30-NH and 300-NH are statistically different ($\alpha=0.1$), but without repetitive evaluations of preparations prepared identically, yet at different times, and without knowledge of in vivo stabilities, one can not be sure how different these two types of NH really are.

The disposition characteristics of the 30-300-NH preparation are quite different from the other two (30-NH and 300-NH) at 2 hours, but not necessarily so after 5 hours. Assuming identical in vivo stability, this difference may be due to alterations in nonspecific binding brought on by shape modifications (e.g. the 30-300-NH have a shape different than the 300-NH because of osmotic shrinkage), which in turn are the result of the different osmotic treatments. The degree to which protein binding to these vesicles is different in vivo is unknown. If there are differences, however, they could account for all or part of the differences in disposition.

VI. Transfusion Results

The data obtained from transfusions of either Hb or NH, show that the post transfusion values of n are lower than the n's for rat blood, NH suspensions or Hb solutions.

Also, the post transfusion P_{50} values were higher for the NH suspension than for the Hb solution but lower than that of rat blood. Further, the n and P_{50} values continue to change with time, which may be explained by noting that these were not complete transfusions. Some RBCs remain, and as time passes their relative amount increases in relation to the transfused substance. Mixtures of two oxygen carriers having different Hill numbers and P_{50}s will always have a (apparent) lower Hill number than either of the components, and a P_{50} somewhere in between the P_{50}s of the different oxygen carriers. The actual composite n and P_{50} values will depend on the relative amounts of the individual components. Because the relative amounts of functional RBC hemoglobin and transfused hemoglobin change with time, the overall n and P_{50} values must also change. To demonstrate that the above explanation can account for the experimentally observed P_{50} and n values, the data obtained from the control 3.6 g% Hb transfusions can be compared with the calculated P_{50} and n values using known n and P_{50} values of Hb and rat RBCs along with their known relative amounts. Figure VI.1 is a plot of the composite oxygen dissociation curves obtained from such a calculation and allows easy determination of P_{50} values. Figure VI.2 shows the data in Figure VI.1 replotted in order to calculate the Hill number directly from the slope. Data from Figures VI.1 and VI.2 are summarized and compared to the experimentally determined P_{50} and n values in Table VI.1. Although the calculated and
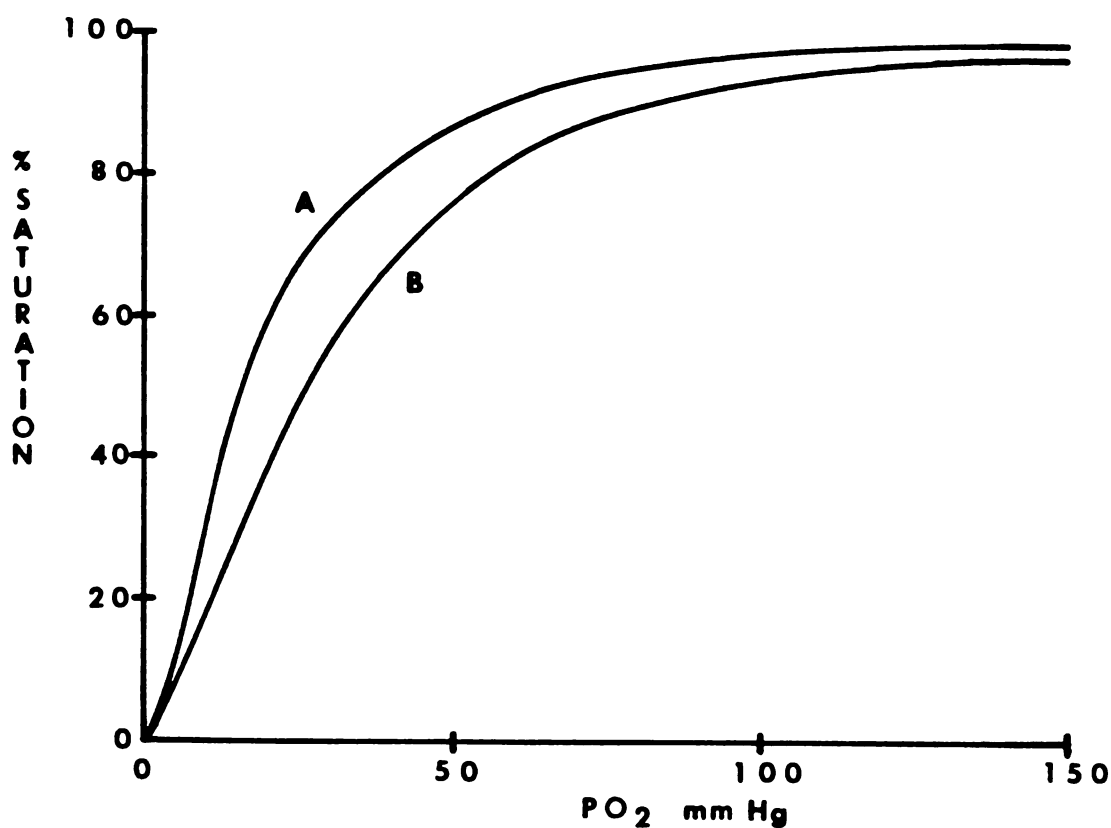
FIGURE VI.1. Calculated oxygen dissociation curves were determined
for rats transfused with a Hb solution. The calculated
curves were derived by using the weighted average of the
oxygen dissociation curves for rat blood and Hb solution.
The weighting factors were equal to the relative fraction
of the rat blood and Hb solution present immediately
after the transfusion and at the time of death. Curve
A is the calculated curve obtained for rats immediately
after the transfusion. Curve B is the curve obtained
for rats at the time of death.
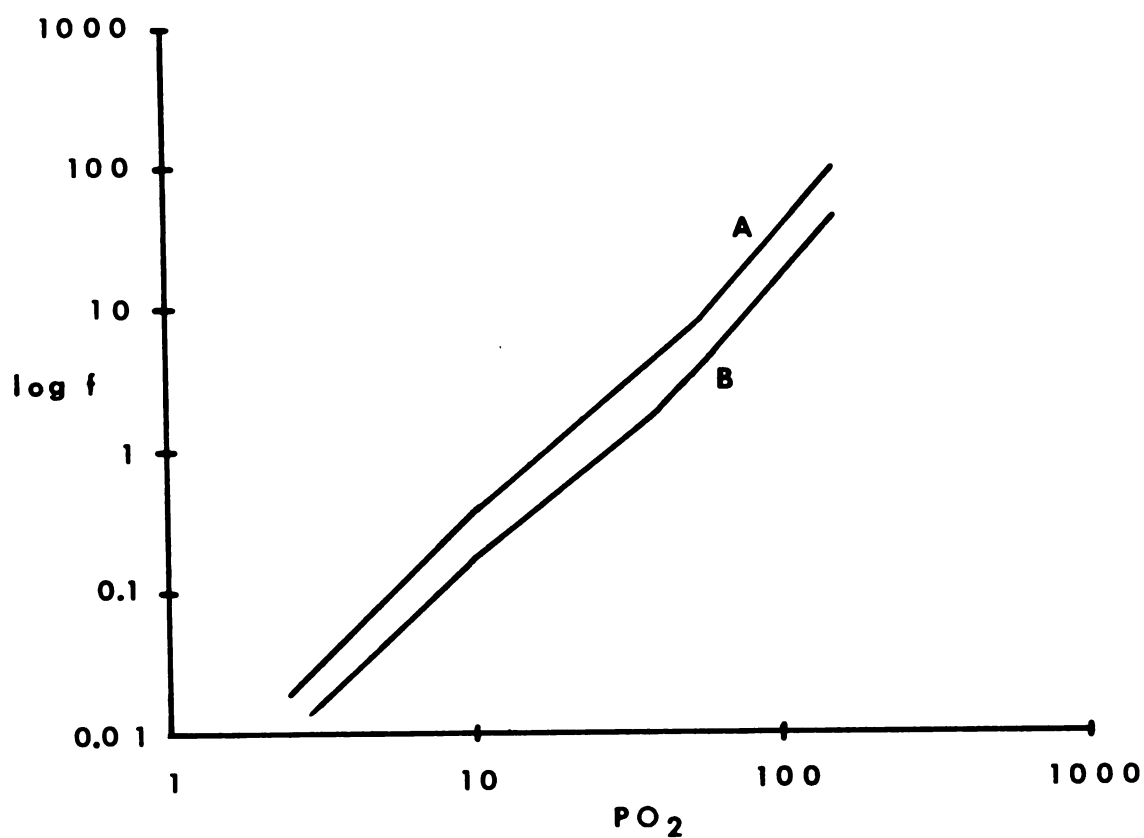
FIGURE VI.2. The calculated oxygen dissociation curves in Figure VI.1
were transformed into lines whose midpoint slope is
approximately equal to the Hill number (n).
f = fo/(1-fo), where fo is the fraction of Hb saturated
with oxygen. The line obtained for rats immediately
after the transfusion is A. The line obtained for rats
at the time of death is B.

SAMPLE	CALCULATED		EXPERIMENTAL	
	P_{50} (mm Hg)	n	P_{50} (mm Hg)	n
POSTTRANSFUSION	17.3	1.70	16.7	1.9
AT TIME OF DEATH	27.6	1.66	23.3	1.6

TABLE VI.1. Calculated and experimental oxygen binding parameters. For a rat transfused with a Hb solution, the P_{50} and Hill number were determined immediately after the transfusion and at the time of death. The calculated values were obtained from Figure VI.1 and Figure VI.2.

observed values are not exactly the same they are close enough to show that mixing of these two oxygen carriers (rat blood and 3.6 g/dl Hb) does not result in an interaction that changes P_{50} or n values.

The Hb transfused rats died while their Hb concentration was still above 2 g/dl. Rats transfused with a NH suspension remained alive even when their Hb concentration dropped below 2 g/dl. The data is too limited to test if this lower value is real or not. If it is, then one explanation may be that the rats transfused with NH survived with lower Hb concentrations because the NH contained DPG. Because DPG raises the P_{50} of the Hb, it should allow more oxygen to be released as the NH pass through capillaries.

The P_{50} observed after NH transfusions was definitely higher than the P_{50} observed after the control Hb transfusions and remained higher over the course of the study. This must indicate that DPG remained encapsulated even when the NH were present in an in vivo environment.

The survival times for the animals transfused with NH were longer than those obtained for animals transfused with Hb. This was true even though, in one case, the free Hb concentration was about twice that for the NH transfusion. One rat totally recovered from a transfusion using a NH suspension. A reasonable conclusion is that the NH must have kept its entrapped Hb in circulation longer than when the same amount of Hb was transfused unencapsulated. This

retention was not due to blockage of kidney excretion (the main route of elimination for Hb) since urine flow continued at above normal rates after NH transfusion. Also, histological studies revealed only mild effects on the kidney.

A plot of apparent Hct as a function of time (Figure V.14) shows that it initially decreases rapidly and then decreases more slowly. This apparent plateau indicates that the NH saturated the majority of uptake and elimination processes.

A plot of the oxygen carrying ability of the NH as a function of time after transfusion (Figure V.14) shows that these values decrease more than does the apparent Hct. One likely explanation is that encapsulated Hb is being oxidized to methemoglobin. The NH do not contain any reducing agents (as RBCs do) and therefore, are more subsceptible to oxidation. Examination of blood samples removed after transfusion reveal an increasingly brownish color consistant with increasing methemoglobin levels.

VII. In Vivo Toxicity

Observations on the liver, heart, lungs, spleen, and kidney obtained from rats transfused with NH revealed mild hyperproteinemia in the immediate extracelluar space surrounding the vasculature. An explanation for hyperproteinemia is that upon administration of the NH there is some tissue binding to the blood vessel walls. Once bound the NH could be lysed (e.g. due to interaction with lipoproteins).

This would result in a high local protein concentrations in the stagnant layer adjacent to the blood vessel wall. The resulting large concentration gradient would drive the protein into the extracellular space.

Rats transfused with a NH suspension containing human albumin had a white precipitate present in the bladder. Rats transfused with a NH suspension containing bovine albumin did not have this white precipitate. The white precipitate can be explained by realizing that human albumin binds heme whereas bovine albumin does not (Bunn, 1968). When NH are administered there is some lysis and release of Hb into the circulation. Haptoglobin's carrying capacity for Hb is rapidly saturated leaving free Hb in solution. The concentration of this free Hb would be low since only a small percentage of the NH are lysed. At this lower Hb concentration, dissociation to dimers and monomers is more favored. These dimer and monomeric units are more susceptible to heme oxidation than is the tetramer because the heme moieties are less protected. Once oxidized the heme groups more easily dissociate from the globular chains. The heme units then bind to human albumin resulting in the formation of metalbumin. The remaining globular protein is unstable in solution and precipitates out as a white solid. When bovine albumin is present the heme does not as readily dissociate from the globular protein. This may stabilize the globular protein preventing it from precipitating.

VIII.<u>CONCLUSION</u>

By encapsulation of Hb and DPG into lipid vesicles (NH), the vascular retention time and P_{50} were increased over that of free Hb. As a result of the increased retention time and elevated P_{50}, survival times of rats transfused with NH were longer than those obtained with rats transfused with Hb. The NH were relatively nontoxic causing mild hyperproteinemia and diuresis. The NH effected the kidney less than Hb. Future work should focus on the prevention of MetHb formation, and on the development of a sterile and stable product.

REFERENCES

Abra, R.M., Hunt, C.A., "Liposome Disposition In Vivo IV: The Interaction of Sequential Doses of Liposomes having Different Diameters," Res. Commun. Chem. Pathol. Pharmacol., 36 (1): 17-31 (1982a).

Abra, R.M., Schreier, H. and Szoka, F.C., "The Use of a New Radioactive-Iodine Labelled Lipid Marker to Follow In Vivo Disposition of Liposomes: Comparison with an Encapsulated Aqueous Space Marker," Res. Commun. Chem. Pathol. Pharmacol., 37 (2): 199-213 (1982b).

Abra, R.M., Hunt, C.A., "Liposome Distribution In Vivo III. Dose and Vesicle-Size Effects, "Biochim. Biophys. Acta, 666 : 493-503 (1981).

Abra, R.M., Bosworth, M.E., and Hunt, C.A., "Liposome Disposition In Vivo 29 (2): 349-360 (1980).

Amberson, W.R., "Blood Substitutes," Biol. Rev., 12 : 48-86 (1937).

Amiconi, G., Antonini, E., Brunori, M., Wyman, J. and Zolla, L., "Interaction of Hemoglobin with Salts Effects on the Functional Properties of Human Hemoglobin," J.Mol.Biol., 152 : 111-129 (1981).

Baldwin, J.E. and Gill, B., "Approaches to the Preparation of Oxygen Carriers for Use as Blood Substitutes," Med. Lab. Sci., 39 , 45-51 (1982).

Barry, W.E. and Tallarida, R.R.BF, "Kinetics of Release Iron from Reticuloendothelial System," J. Reticuloendothel. Soc., 5 : 412-421 (1968).

Bartlett, G.R., "Phosphorus Assay in Column Chromatography," J.Biol.Chem., 234 : 466-468 (1959).

Benarcerraf, B., (1958), Quantitative Aspects of Phagocytosis. In "Liver Function", (Ed. Brauer, R.W.), 205-234, Waverly Press, Baltimore. sp. 1 Benarcerraf, B., Halpern, B.N., Biozzi, G., and Others, "Quantitative Study of the Graulopectic Activity of the Reticuloendothelial System: III. Effect of Cortisone and Nitrogen Mustard on the Regenerative Capacity of the R.E.S. after Saturation with Carbon," Brit. J. Exp. Path., 35 : 97-106 (1954).

Benacerraf, B., Biozzi, G. and Halpern, B.N., (1957), Physiology of Phagocytosis of Particles by the Reticuloendothelial System. In "Physiopathology of Reticuloendothelial System", (Ed. Halpern BN), Charles C. Thomas

Publisher, Springfield, Ill.

Benesch, R.E., Benesch, R., Renthal, R.D. and Maeda, N., "Affinity Labeling of the Polyphosphate Binding Site of Hemoglobin," Biochemistry, 11 : 3576-3582 (1972).

Benesch, R.E., Benesch, R., and Yu, C.I., "The Oxygenation of Hemoglobin in the Presence of 2,3-Diphosphoglycerate. Effect of Temperature, pH, Ionic Strength, and Hemoglobin Concentration," Biochemistry, 8 (6): 2567-2571 (1969).

Biozzi, G. and Stiffel, C., (1965) The Physiology of the Reticuloendothelial Cells of the Liver and Spleen. In "Progress in Liver Disease", (Eds. Popper, H. and Schaffner, F.), Grune and Stratton Inc. New York.

Biozzi, G,. Bernacerraf, B., and Halpern, B.N. "Quantitative Study of the Granulopectic Activity of the Reticuloendothelial System:
II. A. Study of the Kinetics of the Granulopectic Activity of the R.E.S. in Relation to the Dose of Carbon Injected: Relationship between the Weight of the Organs and their Activity," Brit. J. Exp. Path., 34 : 441-457 (1953).

Bligh, E.G. and Dyer, W.G., "A Rapid Method of Total Lipid Extraction and Purification," Can. J. Biochem., Physiol., 37 (8): 911-917 (195^).

Bosworth, M.E. and Hunt, C.A., "Liposome Disposition In Vivo II: Dose Dependency," J. Pharm. Sci., 71 (1): 100-104 (19^2).

Boyden, S., "Cellular Recognition of Foreign Matter," International Reviews of Experimental Pathology, 2 : 311-356 (1963).

Bunn, F., "Erythrocyte Destruction and Hemoglobin Catabolism," Semin. Hematol., 9 : 3-17 (1972).

Bunn, H.F., Esham, W.T., and Bull, R.W., "The Renal Handling of Hemoglobin-I, Glomerular Filteration," J. Exp. Med., 129 : 909-924 (196^).

Bunn, H.F., and Jandi, J.H., "Exchange of Heme among Hemoglobins and between Hemoglobins and Albumin," J. Bio. Chem., 243 : 465-475 (1968).

Chang, T.M.S., "Semipermeable Microcapsules," Science, 146 : 524-525 (1964).

Crosby, W.H., "Proposal for the Adoption of an International Method and Standard Solution for Hemoglobinometry, Specifications for Preparation of a Standard Solution and

Notification of Availability of a Reference Standard Solution," Blood, 26 : 104-107 (1965).

De Geir, J., Haest, C.W.M., Mandersloot, J.G., and Van Deenen, "Valinomycin-Induced Permeation of $^{86}Rb^+$ of Liposomes with Varying Composition Through the Bilayers," Biochim. Biophys. Acta, 211 : 373-375 (1970).

DeVenuto, F., Friedman, H.I., Neville, J.R. and Peck, C.C., "Appraisal of Hemoglobin Solution as a Blood Substitute," Sur. Gynecol. Obstet., 149 : 417-436 (1979).

DeVenuto, F., Moores, W.Y., Zeyna, A.I., and Others, "Total and Partial Blood Exchange in the Rat with Hemoglobin Prepared by Crystallization," Transfusion, 17 : 555-562 (1977).

Djordjevich, L. and Miller, I.F., "Synthetic Erythrocytes from Lipid Encapsulated Hemoglobin," Ex. Hemat., 8 (5), 584-592 (1980).

Dobson, E.L., Jones, H.B.,"The Behavior of Intravenously Injected Particulate Material," Acta. Med. Scand.(suppl)., 273 1-71 (1952).

Dubowski, K.M.,(1964) Hemoglobin, its Precursors and Metabolites, (Eds. Sunderman, F.W., and Sunderman, F.W.Jr.), J.B. Lippincott Co., Philadelphia.

Duling, B.R., Berne, R.M., "Longitudinal Gradients in Periarteriolar Oxygen Tension: A Possible Mechanism for the Participation of Oxygen in Local Regulation of Blood Flow," Circ. Res., 27 : 669-678 (1970).

Eilers, R.J. "Notification of Final Adoption of an International Method and Standard Solution for Hemoglobinometry: Specifications for Preparation of Standard Solution," Am. J. Clin. Pathol. 47 : 212-214 (1967).

Ellens, H., Mayhew, E., and Rustum, Y.M., "Reversible Depression of the Reticuloendothelial System by Liposomes," Biochim. Biophys. Acta, 714 : 479-485 (1982).

Endrich, B., Greenburg, A.G., Intaglietta, M. and Peskin, G.W. "Fluorocarbon Emulsions as Blood Replacement Fluid: Influence on the Omental Microcirculation," J. Sur. Res., J. Surg. Res., 26 : 185-198 (1979).

Ericsson, J.L.E., "Transport and Digestion of Hemoglobin in the Proximal Tubule-I. Light Microscopy and Cytochemistry of Acid Phosphatase," Lab. Invest., 14 : 1-15 (1965).

Evelyn, K.A. and Malloy, H.T., "Microdetermination of

Oxyhemoglobin, Methemoglobin, and Sulfhemoglobin in a Single Sample of Blood," J. Biol. Chem., <u>126</u> : 655-662 (1938).

Eyster, M.E., Edgington, T.S., Liem, H.H., and others, "Plasma Hemopexin Levels Following Aortic Valve Replacement; a Valuable Screening Test for Assessing the Severity of Cardiac Hemolysis," J. Lab. Clin. Med., <u>80</u> : 112-116 (1972).

Fishman, M., Hammerstrum, R.A., and Bond, V.P., " <u>In Vitro</u> Transfer of Macrophage RNA to Lymph Node Cells," Nature, <u>198</u> : 549-551 (1963).

Fishman, M., "Antibody Formation <u>In Vitro</u> ," J. Exp. Med., <u>114</u> : 837-855 (1961).

Freeman, T., "Haptoglobin Metabolism in Relation to Red Cell Destruction," Protides Biol. Fluids, <u>12</u> : 344 (1964).

Garby, L. and DeVerdier, C.H., "Affinity of Human Hemoglobin A to 2,3-Diphosphoglycerate. Effect of Hemoglobin Concentration and of pH , "Scand. J. Clin. Lab. Med., <u>27</u> : 345-350 (1971).

Garby, L, and Noyes, W.D.,"Studies on Hemoglobin Metabolism-J, The Kinetic Properties of the Plasma Hemoglobin Pool in Normal Man," J. Clin. Invest., <u>38</u> : 1479-1483 (1959).

Geyer, R.P., "Potential Uses of Artificial Blood Substitutes," Fed. Proc., <u>34</u> : (6) 1525-1528 (1975).

Goodford, P.J., "The Hemoglobin Molecule: Is it a Useful Model for a Drug Receptor?," Trends in Pharmacological Sciences, <u>1</u> : (2) 309-312 (1980).

Gould, S.A., Rosen, A.L., Sehgal, L., Sehgal, H., Rice, C.L. and Moss, G.S., "Hemoglobin Solutions as Red Cell Substitutes," Trans. Am. Soc. Artif. Intern. Organs, <u>26</u> : 350-353 (1980).

Goulian, M. and Beck, W.,"The Partial Thromboplastin Time Test," Amer. J. Clin. Path., <u>44</u> : 97-103 (1965).

Gregorviadis, G.,"The Carrier Potential of Liposomes in Biology and Medicine," N. Eng. J. Med., <u>295</u> : 704-710 (1976).

Gregoriadis, G., Neerunjun, D.E., "Control of the Rate of Hepatic Uptake and Catabolism of Liposome-Entrapped Proteins Injected into Rats. Possible Therapeutic Applications," Eur. F. Biochem., <u>47</u> : 179-185 (1974).

Gregoriadis, G., and Ryman, B.E., "Fate of Protein-Containing Liposomes Injected into Rats: An Approach to the

Treatment of Storage Diseases," Eur. J. Biochem., 24 : 485-491 (1972a).

Gregoriadis, G. and Ryman, B.E., "Lysosomal Localization of β-Fructofuranosidase-containing Liposomes Injected into Rats," Biochem. J., 129 : 123-133 (1972b).

Gulik-Krzywicki, T., Rivas, E., and Luzzati, V., "Structure et Polymorphisme des Lipids: Etude pas Diffraction des Rayon X du Systeme forme de Lipides de Mitochondries de Coeur de Boeuf et d'Eau," J. Mol. Biol., 27 : 303-322 (1967).

Gutknecht, J. and Walter, A., "Hydroxyl ion Permeability of Lipid Bilayer Membranes," Biochem. Biophys. Acta, 645 : 161-162 (1981a).

Gutknecht, J. and Walter, A., "Transport of Protons and Hydrochloric Acid through Lipid Bilayer Membranes," Biochem. Biophys. Acta 641 : 183-188 (1981b).

Guyton, A.C., (1981) Textbook of Medical Physiology, 6th edition, W.B. Saunders Companay , Philadelphia.

Hanks, G.E., Cassel, M., Ray, R.N., et al, "Further Modification of the Benzidine Method for Measurement of Hemoglobin in Plasma," J. Lab. Clin. Med., 56 : 486-498 (1960).

Hauser, C.J., Kaufman, C., Frantz, R., Shipping, C., Schwartz, S. and Shoemaker, W.C., "Use of Crystalline Hemoglobin as Replacement of RBC Mass," Arch. Surg., 117 782-786 (1982).

Heide, K., Haupt, H., Storiko, K. and Others, "On the Heme-Binding Capacity of Hemopexin," Clin. Chim. Acta, 10 : 460-469 (1964).

Hershko, C., "The Fate of Circulation Hemoglobin," Br. J. Haematol., 29 : 199-204 (1^75).

Hunt, C.A., Tsang, S., "α-Tocopherol Retards Autoxidation and Prolongs the Shelf-Life of Liposomes," International J. Pharmaceutics, 8 : 101-110 (1981).

Hyman, C. and Paldino, R.L. "Possible Role of the Reticuloendothelial System in the Protein Transport," Ann. N.Y. Acad. Sci., 88 : 232-239 (1960). Hyman, C., "Role of the Reticuloendothelial System in Protein Turnover," Intern. Physiolcong., 19 : 486-487 (1953).

Jonah, M.M., Cerny, E.A. and Rahman, Y.E., "Tissue Distribution on EDTA Encapsulated within Liposomes of Vary Surface Properties," Biochim. Biophys. Acta, 401 : 336-348 (1975).

Juliano, R.L., and Lin, G., (1980) "The Interaction of Plasma Proteins with Liposomes: Protein Binding Effects on Clotting Complement system," In Immunobiology (Eds. Tom, B. and Six, H.), Elsevier, New York.

Juliano, R.L., Stamp, D.,and McCallough, N., "Pharmacokinetic of Liposome-Encapsulated Antitumor Drugs and Implications for Therapy," Ann. N.Y. Acad. Sci., 308 : 411-425 (1978). Juliano, R.L., stamp, D., "The Effect of Particle Size and Charge on the Clearance Rates of Liposomes and Liposome Encapsulated Drugs, "Biochem. Biophys. commun., 63 (3): 651-65ª (1975).

Kajita, A., Noguchi, K., and Shukuya, R., "A Simple Non-Enzymatic Method to Regenerate Oxyhemoglobin from Methemoglobin," Biochem. Biophys. Res. Commnun., 39 (6) 1199-1204 (1970).

Kirby, C., Clarke, J. and Gregoriadis, G., "Effect of the Cholesterol Content of Small Unilamellar Liposomes on Their Stability In Vivo and In Vitro , Biochem. J., 186 : 591-598 (1980).

Knight C.G.,(1981) Liposomes from Physical Structure to Therapeutic Applications. Research Monographys in Cell and Tissues Physiology. (Eds. Dingle J. T. and Gordon, J.L.), Elsevier/North-Holland Biomedical Press.

Ladbrook, B.D., Williams, R.M. and Chapman, D., "Studies on Lecithin-Cholesterol-Water Interactions by Differential Scanning Calorimetry and X-Ray Diffraction," Biochim. Biophysica. Acta, 150 : 333-340 (1968).

Lee, L., "Reticuloendothelial Clearance of Circulating Fibrin in the Pathogenesis of the Generalized Schwartzman Reaction," J. Exp. Med., 115 : 1065-1082 (1962).

Muller-Eberhard, U., "Hemopexin", N. Eng. J. Med., 283 : 1090-1094 (1970).

Markwell, M.A.K., Fox, F.C., "Surface Specific Iodination of Membrane Proteins of Viruses and Eucaryotic Cells Using 1,3,4,6-Tetrachloro-3α, 6α-Diphenylglycoluril," Biochemistry, 17 (22): 4807-4817 (1978).

Nemoto, E.M., "Pathogenesis of Cerebral Ischemia: Anoxia, Crit. Care Med., 6 : 203-214 (1978).

Neville, R.J., "Hemoglobin Oxygen Affinity Measurement Using Biotonometry," J. Applied Physiol., 37 (6): 967-971 (1974).

Nichols, J.W. and Deamer, D.W., "Net Proton-Hydroxyl

Permeability of Large Unilamellar Liposomes Measured by an Acid-Base Titration Technique," Proc. Natl. Acad. Sci. USA, 77 : (4) 2039-2042 (1980a).

Nichols, J.W., Hill, M.W., Bangham, A.D. and Deamer, D.W., "Measurement of Net Proton-Hydroxyl Permeability of Large Unilamellar Liposomes with the Fluorescent pH Probe, 9-Aminoacridine," Biochim. Biophys. Acta, 596 : 393-403 (1980b).

Papahadjopoulos, D., Cowden, M. and Kimelberg, H., "Role of Cholesterol in Membranes Effects on Phospholipid- Protein Interactions, Membrane Permeability and Enzymatic Activity," Biochim. Biophys. Acta, 330 : 8-26 (1973).

Perutz, M.F., "Hemoglobin Structure and Respiratory Transport," Sci. Am., 92-125 (Dec. 1978).

Pimstone, N.R., "Renal Degradation of Hemoglobin," Semin. Hematol., 9 : 31-42 (1972).

Proctor, R.P., and Rapaport, S.I. "The Partial Thromboplastin Time with Kaolin," Amer. J. Clin. Path., 36 : 212-219 (1961).

Quick, J.A., "Control of Anticoagulant Therapy," Arch. Internal Med., 111 : 234-239 (1963a).

Quick, A.J. and Geppert, M.,"Screening for Bleeding States-The Partial Thromboplastin Test," Amer. J. Path., 40 465-472 (1963b).

Saba, T.M.,"Physiology and Physiopathology of the Reticuloendothelial System," Arch. Intern. Med., 126 : 1031-1052 (1970).

Sakata, M., Yoshida, A., and Huga, M., "Methemoglobin in Blood as Determined by Double-Wavelength Spectrophotometry," Clin. Chem., 28 (3): 508-511 (1982).

Sears, D.A., "Disposal of Plasma Heme in Normal Man and Patients with Intravascular Hemolysis," J. Clin. Invest., 49 : 5-14 (1970).

Sears, D.A., Anderson, P.R., Foy, A.L., and others, "Urinary Iron Excretion and Renal Metabolism of Hemoglobin in Hemolytic Diseases," Blood, 28 : 708-725 (1966).

Sellards, A.W. and Minot, O.R., "Injection of Hemoglobin in Man and its Relation to Blood Destruction, with Special Reference to the Anemias," J. Med. Res., 34 : 469-494 (1916).

Stryer, L.,(1975) Biochemistry, W.H. Freeman and Company, San Francisco.

Sunder-Plassmann, L., Dieterle, R. Seifert, J., Jesch, F. and Messmer, K., "Stroma Free Hemoglobin Solution as a Blood Replacement. Actual State and Problem," Eur. J. Intern. Care. Med., 1 : 37-42 (1975).

Szoka, F. and Mayhew, E.M., "Synthesis and Characterization of an Iodinatable Phospholipid," Chem.and Phys. Lipids, in Preparation, (1982).

Szoka, F., Papahadjopoulos, D., "Procedure for Preparation of Liposomes with Large Internal Aqueous Space and High Capture by Reverse-Phase Evaporation," Proc. Natl. Acad. Sci. U.S.A. 75 : 4194-4198 (1978).

Udkow, M.P., Lacelle, P.L. and Weed, R.I., "The Effects of pH, Oxygen Tension and 2,3-Diphopshoglycerate Concentration upon the Binding of Adenosine Triphosphate to Concentrated Hemoglobin A Solution," Nouv. Rev. Fr. Hematol., 13 (6) 817-834 (1973).

Vannotti, A., (1957) The Role of the Reticuloendothelial System in Iron Metabolism. In "Physiopathology of the Reticuloendothelial System," (Ed. Halpern B.N.), Charles Thomas Publisher, Springfield, Ill.

Wisse, E. and Gregoriadis, G., "The Uptake of Liposomes by the Rat Liver RES," Res. J. Reticuloendothel. Soc., 18 : 10a (1975).