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**The overall goal of our study was to further the developement of a safe and effective oxygen-carrying red blood cell (RBC) substitute based on functional hemoglobin encapsulated within lipid vesicles (liposomes). Our studies demonstrated that liposome-encapsulated hemoglobin (LEH) provided an effective means of oxygen delivery in experimental animals. Our results indicated that exposure of experimental animal to liposomes made from conventional lipids was associated with significant immunotoxicity, as measured by impaired host resistance to infectious challenge. Our results thus far suggested that liposomes constructed with "Stealth" lipids were less immunotoxic than were liposomes made with conventional lipids. Both LEH formulations depressed phagocytic function as measured by ingestion of latex beads. Circulation half-life following 50% isovolemic exchange-transfusion in rats with LEH was about 20 hrs; 97% exchange-transfusion demonstrated efficacy in life support. Met-Hb generation accompanying LEH processing (below 10°C) and 1 month storage (-20°C) appeared to be small with only a 3% and less than a 10% increase for encapsulated over precursor, respectively. Oxygen affinity and cooperativity and steady shear viscosity values for LEH suspensions (30% by volume) appeared to be near the normal values expected for whole blood. Incubation in plasma at 37°C resulted in only a small amount of Hb release from LEH; also shear had very little effect on causing Hb leakage from LEH.**

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## INTRODUCTION

Severe blood loss due to traumatic injury and/or surgical procedures as well as the pathological changes following them poses a great threat to human life unless the intravascular volume and oxygen delivery to tissues can be adequately restored. The most widely used blood replacement therapy is the transfusion of whole blood or blood components. Problems associated with these approaches include hemolytic transfusion reactions, allergic reactions, circulatory overload, embolism, citrate toxicity, coagulation disturbances and the transmission of blood borne diseases such as AIDS and hepatitis [1,2]. Whole blood has a short shelf life of 21 days outside the body, which limits its use. The limitations and logistics of distribution of blood of desired type causes additional problems. According to Miller [3], millions of units of blood collected every year are either unused or unaccounted for.

These limitations have formed the basis for efforts to develop an acceptable red cell substitute that could function as a universally transfusable (nonallergenic), oxygen-carrying blood replacement fluid that can be used to provide temporary life support until an adequate supply of whole blood becomes available.. The fact that no product has yet been marketed in the U.S. shows that the problem of finding a suitable red cell substitute is not a simple one. Some specialized uses for artificial red cells are found in the effective oxygen delivery to ischemic tissue; in in vitro and in vivo organ preservation; in the treatment of aplastic anemia and sickle cell anemia; and in veterinary medicine, where blood banking is not practiced. Numerous approaches have been taken toward the development of a blood replacement fluid in the past forty years, resulting in three still-experimental classes of synthetic oxygen-carrying blood substitutes: 1) perfluorochemical emulsions [4-12]; 2) hemoglobin (Hb), either normal [13-20], polymerized or crosslinked intramolecularly, but free in solution [21-24]; and more recently 3) encapsulated Hb, in which Hb solutions are enclosed in a synthetic red cell by any of a number of techniqes. Techniques for Hb encapsulation with liposomes include film hydration followed by sonication [25], membrane extrusion [26,27], french press extrusion [28], microfluidization [29-34] or homogenization [33,35], detergent dialysis [36], reverse-phase evaporation [37], reverse- micelle [38]; and other mechanisms [39]. Most recently, our laboratories have demonstrated that a multiple emuslion approach for encapsulation of Hb can be used to generate an efficient oxygen-carrying red cell substitute [40,41].

Liposome technology provides a mechanism for encapsulation and in vivo delivery of drugs, proteins, etc. which probably would otherwise be degraded, cleared rapidly, or toxic to the host. For example, encapsulation of Hb in phospholipid vesicles (liposomes) may eliminate the toxic effects which accompany the use of perfluorochemical emulsions [7,9,10] and Hb solutions [14-16,20,22,42,43], and may provide reasonable stability of encapsulated Hb at room temperature by inclusion of antioxidants; longer retention times in circulation by adjustment of the phospholipid formulation; normal colloid osmotic pressure with large oxygen carrying capacity, low oxygen affinity, and cooperativity; and oxygen/carbon dioxide mass transport by inclusion of organic phosphates and other materials. However, potential problems still exist in the use of LEH. One of these involves solubilization of phospholipid membrane by human plasma high density lipoproteins [44]. Other aspects of clinical concern with respect to cardiovascular infusion of LEH include tissue/organ toxicity and immunotoxicity [9,10], effect on coagulation system and platelets [45-52], and effect on reticuloendothelial (i.e. the mononuclear phagocyte) host defense system [53-56,80,81,85,86].

Several reports have suggested that administration of large quantities of lipid-containing materials results in

overloading of the RES [80,81]. Host phagocytes (monocytes, macrophages) are crucial in inactivating and removing bacteria, fungi and viruses, and other foreign material from the body [86]. They also interact extensively with lymphocytes in modulating normal immune responses. Thus, impairment of host phagocytic cells could result in increased susceptibility to pathogenic or opportunistic infections or enhance improper immune modulation resulting in immunosuppression or allergy.

The approaches used in this study, which are based on the further development and biophysical/biochemical characterization of LEH as an oxygen-carrying RBC substitute, incorporate the diverse characteristics required for a practical RBC replacement fluid. They especially address evaluation of efficacy in life support, effect on reticuloendothelial-host defense system, stability and storageability, and feasibility of scaling-up production (using aseptic techniques to obtain pyrogen-free, sterile and virus-free production) in order to support the extensive animal testing required before clinical testing can be seriously considered.

## EXPERIMENTAL DESIGN AND METHODS

### Hemoglobin Solution Preparation

Hb solutions are prepared at 4 °C following aseptic techniques as described elsewhere [27,29,33]. Also, human stroma-free hemoglobin that is sterile and pyrogen-free that was obtained from John Hess at the Letterman Army Institute of Research is also used. All equipment used in the processing is depyrogenated and all water used is sterile and free of pyrogens (as assessed by the USP pyrogen test or the LAL test). To maximize oxygen-carrying capacity high concentrations of Hb solution up to 35 g% (i.e. 35 grams Hb per 100 ml solution) are prepared. This is done using an Amicon 30,000 MW spiral cartridge. The Hb solution concentrate of pH 6.6 to 6.9 is dialyzed against 30 mM phosphate buffered saline (PBS) containing 0.9 wt% NaCl to achieve osmotic equilibrium with the outer aqueous phase used in experiments (see below). The pH of the dialyzed Hb solution is adjusted as necessary using a few ml of 0.1N Trizma base to give a final pH value of 7.4. The dialyzed Hb solution is then centrifuged at 30,000 x g for 30 min to remove any stroma that may precipitate due to the pH adjustment step. To ensure product quality the total Hb, oxy-Hb, reduced-Hb, CO-Hb and met-Hb concentrations are measured spectrophotometrically following the method of Benesch and Benesch [59]. The Hb solution is stored either frozen (-20°C or -80$_o$C) or at 4 °C until use.

### Other Chemicals Added to Hemoglobin Solution

Pyridoxal-5-phosphate (P-5-P) (Sigma Chemical Co.) is added to the hemoglobin solutions to control oxygen affinity of the LEH to a value similar to that of fresh red blood cells. The antioxidant catalase, which acts as a scavenger of free radicals, is added to the Hb solution [60]. In some studies, glutathione and NADH is added to the Hb solution in place of catalase to evaluate their Hb stabilizing effect [58]. Other constituents of the red blood cell hemolysate from which the hemoglobin solution is prepared appear to protect against oxidation of hemoglobin and phospholipid [60]. Since the carbonic anhydrase (Sigma Chem. Co.) in the Hb solution remaining after lysis and dialysis may not be enough to control CO2 mass transfer in vivo, in some studies it was added to the Hb solution to a value similar to that of fresh red blood cells (approx. 2.5 mg/ml).

### Liposome Formulations

The membrane lipids used to encapsulate Hb solution include hydro-genated soy or egg phosphatidylcholine (Soy-PC

or Egg-PC), cholesterol (CHOL), phosphatidylinositol (PI) or the "stealth" phospholipid poly-ethylene glycol distearoyl phosphatidyl ethanolamine (PEG-PE), and α- tocopherol (α-T). Soy-PC, Egg-PC (IV-40) and PEG-PE are a gift from Liposome Technology Inc., Menlo Park, CA. Other lipids will be obtained from Sigma Chemical Co., St. Louis, MO. All materials are used as obtained without further purification. The liposomal membrane is formulated to contain lipid molar ratios Soy-PC or Egg-PC:CHOL:PI or PEG-PE:α-T of 1.0:1.0:0.2:0.02 or 1.0:1.0:0.1:0.02, respectively. The LEH are prepared at a lipid to Hb loading of about 150 μmols per ml of precursor Hb solution (i.e. 20 to 30 g%).

Additions of various components to the lipid phase of the LEH system appear to reduce the oxidative interactions between hemoglobin and membrane lipid. Some of these include the addition of cholesterol to the membrane phase to protect Hb from oxidation [61,62]. Oxidation of Hb to met-Hb may be inhibited by using saturated PC instead of unsaturated PC [27]; also, high cholesterol (Chol) content in the membrane reduces membrane permeability in plasma and prolongs liposome stability in circulation [63]. Because red blood cells have high Chol, they will not lose Chol by mass transfer to liposomes to which they are exposed, and thus will not become osmotically fragile [64]. Negatively charged lipids are used to reduce the aggregation tendency of neutral liposomes [27]. Phosphatidylinositol was chosen as the negatively charged phospholipid in the conventional LEH formulation because it is known to enhance circulation time [65]. It has also been reported that compliment-induced phagocytosis by murine peritoneal macrophages can be suppressed by the inclusion of PI in a phospholipid mix that originally contained only dimyristoyl-PC and Chol [66]. Recent studies have shown that the incorporation of the "stealth" lipid, PEG-PE, significantly increased the circulation time of liposomes in the blood [56,57]. These studies have also shown that "stealth" liposomes have greatly decreased uptake by the reticuloendothelial system compared to many conventional phospholipids. Alpha-tocopherol (α-T) is added in small amounts to the mixture to inhibit lipid peroxidation [60,62,67].

**7.1.4. Preparation of LEH**

Aseptic techniques are followed in the preparation of LEH; all equipment used in the processing is depyrogenated and all water used is bacteria and pyrogen-free. Three methods are used for producing liposome-encapsulated hemoglobin (LEH): film hydration, reverse evaporation and double emulsion. Film hydration following procedures as fully described elsewhere [29, 33] is used to form multilamellar vesicles, which are then reduced in size by a Micro-fluidizerTM M110 (Microfluidics, Newton, Ma). Most recently we have modified this process (prior to Microfluidization) by rotary evaporation (operating under vacuum at room temperature) of as much water as possible from the formed LEH in Hb solution. This results in a somewhat dry Hb/LEH film that deposits on the walls of a round bottom flask. Hb solution is added to rehydrate and resuspend the LEH. Microfluidiza-tion processing at temperatures of about 5°C follows. The LEH so formed are washed at least three times in isotonic PBS and centrifuged at 20,000 x g for 20 mins to remove all unencapsulated Hb solution and any remaining organic solvent (if any). The washed liposomes are then resuspended in isotonic PBS containing 7.5 g% egg albumin.

Reverse-phase evaporation methods [37,68], modified as described below, are used to form LEH. Sonication is not used as it damages the hemoglobin. The approach used herein involves formation of an emulsion of 30 ml aqueous concentrated Hb solution in an organic solvent mixture of 20 ml diethyl ether and 12 ml trichlorotrifluoroethane, which contains in dissolved form the formulated phospholipids/lipids. Organic solvents are slowly evaporated at room temperature

in a rotary evaporator operating under partial vacuum. As the organic solvents are removed, LEH spontaneously form in the excess lipid system. The evaporation procedure is continued until dryness to maximize removal of all organic solvent and water so that the Hb concentration within the LEH is as high as possible. This results in the deposition of anapparently dry Hb/LEH film on the walls of the round bottom flask. Concentrated Hb solution is then added under agitation to rehydrate and resuspend the LEH. Microfluidization, washing and resuspension proced-ures used for LEH are the same as described above for film hydration.

Double emulsion methods include formation of a water-in-oil-in- water type multiple emulsion [69] followed by organic solvent removal and then size reduction in a Microfluidizer to form LEH [70]. First a primary emulsion is formed of Hb solution droplets dispersed in an organic solvent blend containing in dissolved form the formulated lipids/phospholipids. Components and compositions are exactly the same as used above for reverse evaporation. The primary emulsion is then well-dispersed into precursor Hb solution to form the double emulsion. The remaining steps in the process to form LEH that involve organic solvent removal, microfluidization, and washing are similar to those reported above for the reverse evaporation method.

A Wescor Oncometer (Logan, Utah) is used to measure the colloid osmotic pressure of the LEH/PBS-Albumin suspension samples. LEH samples are sterilized as reported previously [29]. Briefly, as a result of addition of hyperosmotic buffered saline solution, the LEH shrink rapidly and passed through a 0.45 micron sterilizing filter using a 400cc, 76mm diameter pressure filtration cell (Nucleopore, Pleasant-ville, CA). Prior to addition of the LEH sample to the filtration cell, it is prefiltered and then filtered under vacuum through 5, 3, and 1 micron CF filters (Nucleopore). Following this step, the LEH sample is loaded into the pressure cell filtered through 0.8, 0.6 and0.45 micron CF filters, using nitrogen (at pressures below 100 psi) as the pressure source.

**Encapsulated Hemoglobin Concentration and Quality**

The encapsulated Hb concentration is determined by dissolving the liposomal membrane with n-Octyl ■-D-glucopyranoside detergent solution [27,70] and then measuring the resulting Hb solution concentration for oxy-, reduced and met-Hb components. A 0.1 ml volume of LEH sample (at a lipocrit of about 30%) is dissolved in 5 ml of concentrated detergent solution (30 mg/ml). The resultant material is stirred for 40-45 mins at room temperature, until the liposome membrane dissolves (or forms micelles), and the solution looses all its turbidity. The solution is then filtered through a 0.1 micron filter. The quality of the encapsulated Hb as a function of storage (4°C) time is determined by measuring the met-Hb concentration in the LEH stored sample. Oxy-, reduced, met, and total Hb concentration for a individual preparations of Hb solution are determined by the method of Benesch et al. [59], modified using the extinction coefficient values provided by Van Assendelft and Zijlstra [72]. Oxygen content in terms of ml O2 per ml of the LEH suspension sample is determined using a Lex-O2-Con (Hospex Fiberoptics, Chestnut Hill, MA) [73].

**LEH Suspension Rheology and LEH Stability Under Shear**

Steady shear viscosity of the suspension samples is measured in a uniform shear field with a Wells-Brookfield Syncro-Lectric Microvis-cometer (Model LVT) equipped with a 0.80o cone (Model CP-40, Soughton, MA). Shear rates from 45 to 450 $s^{-1}$ at 37°C are evaluated. Also, a temperature controlled Weissenberg Rheogoniometer Model 18 (Sangamo Weston Controls Ltd., Sussex, England) equipped with a cone (0.5°)- and-plate (CP) platen system is also used for

viscometric character- ization of the LEH suspensions as well as for measurement of shear- induced leakage of Hb for shear rates to 3000 $s^{-1}$, which corresponds toshear stress levels to 100 dynes/cm2). The rheological properties of LEH suspension samples are characterized as a function of storage age and lipocrit. The cone-and-plate geometry is very useful as it provides a good approximation of viscometric flow with constant shear rate throughout the flow field [74].

The fragility of the LEH, as determined by leakage of Hb, is evaluated by shearing the LEH samples for 10 mins in the viscometer as a function of shear rate. Following mild centrifugation (150 x g) the concentration of total Hb in the supernatant of the sheared sample is compared with that of the unsheared sample. The benzidine method [75] is used for determining plasma Hb concentration in the mg/dl range which are found as a result of leakage of Hb from liposomes. This method has been demonstrated to be accurate at concentrations as low as 1 mg/dl.

Steady-state viscosity and complex viscosity measurements are made to determine the viscoelastic properties of the suspensions, following methodology previously employed for whole blood [76]. This characterization is important to ensure that the LEH suspension samples have rheological properties comparable to those of whole blood.

**LEH Size and Ultrastructure**

Thin-section electron micrographs are used to assess the lamellarity and level of the encapsulated Hb concentration of the prepared liposomes at various processing stages. Long term stability of the LEH under various conditions of storage are also evaluated. Negative-stain and thin-section electron micrographs are used to provide data on the size distribution of the LEH. A Philips 201c or a Zeiss EM900 transmission electron microscope are used in these studies.

**Oxy-Hemoglobin Equilibrium Dissociation Curve**

The Oxy-hemoglobin equilibrium dissociation and association curves for the various LEH samples, measured as a function of oxygen partial pressure, will be measured using a Hemox-Analyzer (TCS Medical Products Co., Huntingdon Valley, PA). Oxygen affinity (P50) and cooperativity (Hill exponent n) are determined from the generated curves.

**Interaction of LEH With Blood Components**

The influence of blood components on permeability [44] on Hb leakage from LEH is evaluated. The inherent stability of different LEH formulations, with respect to Hb release, in whole blood and plasma is measured by methods as described above. In a typical experiment, 0.1 ml of LEH suspension sample (30%-50% lipocrit) is added to a suitable volume of incubation medium such as 0.5 ml plasma or 0.5 ml PBS/7.5 g% albumin control. At several time intervals during exposure to experimental medium at 37°C (i.e. 1, 2, 4, 8 and 24 hrs), duplicate 10µl samples of the incubation mixture are withdrawn, diluted into PBS and assayed for released Hb by the benzidine method [75].

**Wet and Dry Storage Stability**

Wet storage stability will be evaluated for LEH formulations that have been determined to be effective and safe. Samples of LEH suspended in isotonic/isooncotic PBS-albumin(7.5 g%) solution stored at 4°C and -20°C are tested for oxygen-carrying capacity, liposome-encapsulated Hb concentration and functionality (i.e. % oxy-, reduced, and met-), LEH size and Hb leakage (i.e., extravesicular Hb), using methods as described above.

**Experimental Model**

**Experimental Animal**

The experimental animal used in the efficacy and blood clearance studies is the male Sprague-Dawley rat, to be

purchased from Harlan/Sprague- Dawley, Indianapolis, IN. Sprague-Dawley rats have been used success-fully at IITRI in an extensive series of efficacy studies with LEH and other blood substitutes, and are the outbred rat strain of choice for preclinical toxicity studies. Rats are obtained at 6 to 7 weeks of age, and held in quarantine for a minimum of one week after receipt. All studies are conducted using rats with a minimum body weight of 225 grams (8 weeks old).

Following the quarantine period, rats are entered into efficacy studies (exchange transfusion studies) in groups of three (one control and two experimentals). Studies with other blood substitutes have indicated that performing simultaneous exchanges with three rats provides the most efficient use of technical personnel; furthermore, through the use of paired statistics, this approach generates immediately comparable efficacy data for control versus experimental groups. Rats are matched for age and body weight in all studies. Prior to cannulation, rats are housed in trios and allowed free access to Purina Lab Chow 5001 (Ralston-Purina, St. Louis,MO) and drinking water.

Where indicated, special toxicity studies are conducted using CD-1 mice and rats, both obtained from Charles River Breeding Laboratories, Portage, MI. CD-1 (mice and rats) is the strains most commonly used at IITRI for host resistance and immune function assays; a large background database for both strains is available in our laboratories. **Surgical Technique**

Efficacy and blood clearance studies are conducted using a simultaneous isovolemic exchange protocol which has been developed and used in our laboratories for studies with several blood substitutes. One day prior to the exchange, rats are anesthesized with ketamine and surgically implanted with an intraatrial silastic cannula via the jugular vein. Immediately prior to the exchange transfusion, rats are reanesthesized and a second cannula implanted into the femoral vein. Isovolemic exchange transfusions will be initiated immediately after implantation of the second cannula.

**Exchange Technique**

All exchange transfusions are conducted in rats maintained under ketamine anesthesia, and involve simultaneous isovolemic replacement of blood with LEH or control solution (albumin). To conduct the exchange, the atrial and femoral cannulas from one rat are attached to the inflow and outflow sides of a single cassette in a Manostat peristaltic pump. The exchange rate is set at 0.33 ml per minute, and blood samples are taken for determination of hematocrit at 10 minute intervals. Preliminary studies in our laboratory have demonstrated that this exchange protocol has no adverse effects on animals in the range of hematocrits between baseline (approximately 40 - 45%) and 10%; rats exchanged with dextran or albumin solutions from baseline to a 10% hematocrit show essentially no mortality or acute toxicity other than a temporary lethargy. Overnight recovery appears to be complete, and no adverse effects have been observed over a 7 to 14 day observation period.

**Efficacy (Exchange) Studies**

To demonstrate efficacy, survival curves are developed as a function of hematocrit for animals transfused with LEH and with albumin (control) solutions. Using this approach, efficacy is demonstrated by (a) improved survival at hematocrits throughout the "critical range" (i.e., 5 to 10% hct) where mortality is commonly observed, culminating in (b) survival of rats transfused with LEH at a hematocrit (i.e., < 5% hct) where 100% mortality is observedin rats exchanged with a control solution.

In order to address these endpoints, experimental rats receive isovolemic exchange transfusions with LEH, and

controls are transfused isovolemically with albumin solution. Exchanges continue until 100% mortality is encountered in both groups; mortality is related to hematocrit at the time of death. The results of these studies are used to construct a survival curve which relates mortality to hematocrit in the experimental and control groups.

**Immunotoxicology Studies**

Several reports have suggested that administration of large quantities of lipid-containing materials results in overloading of the RES [80,81]. These studies have demonstrated decreased phagocytic activity in animals treated with liposomes. Normal RES clearance pathways are crucial to the maintenance of proper immune responsiveness; thus, RES overload and resulting alterations in phagocytic cell number and/or activity may increase host susceptibility to infectious agents [85]. On this basis, it was deemed important to evaluate the safety of LEH through studies to determine the actual effects of the LEH on host clearance and defense mechanisms.

In vivo: Mice receive an i.v. infectious chanllenge with a 10-30% lethal dose of L. monocytogenes on Day 0. On Day -3 or Day +1, groups of 18-20 mice receive 7.5 g% albumin control solution or 0.2 to 0.5 ml of LEH1 or LA* at a 5*EH2 suspensions prepared to 25%63y5c5ences, 5nc., Warr5ngt6n, or %0% lipocrit concentrations. Mice are observed for survival for 10 days after infectious challenge. Percent mortality and mean survival time for each dose group is calculated.

In vitro: LEH1 and LEH2 formulated to 30% lipocrits are tested for effect on phagocytosis of rat alveolar macrophages (AM). AM are obtained by tracheobronchial lavage with warm, sterile saline [94]. LEH formulations are incubated with $1x10^6$ viable AM/ml for 4 hrs prior to addition of latex beads (3.06 µm diameter: Polysciences, Inc., Warrington, PA) at a 50:1 bead to cell ratio. After 1 hr, trypan blue dye is added and cells are cytocentrifuged onto glass slides. Cells are fixed in formalin vapor and unengulfed beads are dissolved in methylene chloride. One hundred viable cells per slide are microscopically counted and the number of beads ingested per cell are determined.

**Bacteria**: L. monocytogenes (American Type Tissue Collection strain 13932 serotype 4b) is used. Stock cultures are obtained from 24 hr petri plate cultures on Brain Heart Infusion Agar incubated at 35°C. Bacteria are resuspended in sterile nutrient broth containing 15% v/v of glycerol as a cryoprotective agent. Frozen stock cultures are thawed and resuspended in sterile 0.85% saline. A volume of 0.2 ml (approximately $2.66x10^5$ colony forming units per mouse) is injected i.v. into the tail vein using a 1 ml syringe with a 25g 5/8" needle. Following infection, mice are observed at least once daily for a 10-day period.

Percent mortality and mean survival time are determined in the Listeria challenge study. Significance between exposure groups are assessed using life table analysis (BMDP Statistical Software, Inc., Los Angeles, CA). Differences in phagocytic index (% of cells ingesting at least one bead) and number of beads per cell are tested by ANOVA and Dunnett's test. Probabilities of $P \leq 0.05$ are accepted as significant.

**RESULTS AND DISCUSSION**

An effective and safe red blood cell substitute is being developed based on film hydration or double emulsion/evaporation followed by rehydration and high pressure homogenization techniques to form liposome-encapsulated hemoglobin (LEH). Formulations are made up of hydrogenated phospha-tidylcholine (PC, soy or egg), cholesterol, phosphatidylinositol (PI), and α-tocopherol in a molar ratio of 1:1:0.1:0.02, respectively. Most recently formulations have

been made up with the "stealth" lipid, polyethyleneglycol distearoylphosphatidyl-ethanolamine (PEG-PE) in place of PI. Only the most relevant results obtained during the grant period are covered in this report. The results described below are with conventional LEH formulations that contain PI (LEH1), unless specified as "stealth" liposomes that contain PEG-PE (LEH2).

Results obtained suggest that Microfluidizer™ processing at temperatures below 9°C (preferably at 4°C), have little or no effect on met-Hb generation. Oxygen content, which is primarily a measure of oxy-Hb concentration, was determined using a Lex-O2-Con. Neither an oxygen content reduction in precursor Hb solution or in LEH encapsulated Hb solution was found as either material was passed up to 15 times through the Microfluidizer™ (see Figure 1). However, oxygen content was found to be reduced when processing temperatures were above 10°C. This most likely represents generation of met-Hb. Encapsulated met-Hb concentrations of from about 2 to 5% of the total Hb concentra-tion were obtained when all processing steps in the formation of the LEH product were taken in account. Storage of the LEH for up to one month at -20°C resulted in percent met-Hb concentrations to levels of about 9%. Although catalase had been added to the Hb solution encap-sulated in the LEH, results have been reported recently that suggest that the addition of glutathione and NADH results in reduction of met-Hb LEH concentration during storage at 4°C [58]. Future studies will examine this approach to Hb stabilization.

We have evaluated three general methods for producing LEH. These include film hydration (LEH 3 is unmodified, LEH 4 is modified), reverse evaporation (LEH 1 is unmodified, LEH 2 is modified) and double emulsion (LEH 5a uses Soy-PC, LEH 5b uses Egg-PC, and LEH 5c is Soy-PC based and suspended in human plasma instead of 7.5% albumin/PBS). All of the LEH samples prepared have been followed by a homogenization and liposome size reduction step using a MicrofluidizerTM M110. Note that LEH 1, LEH 2, LEH 3 and LEH 4 are made with Soy-PC. Figure 2 shows the resulting encapsulated Hb concentrations for typical batches produced following the various methods. LEH made by double emulsion processing resulted in the highest percentage of precursor Hb solution concentration encapsulated, i.e. about 80% for LEH 5b. However, results obtained using modified film hydration processing methods also gave high results. As shown in Figure 1, the results for LEH 5b correspond to an oxygen content for an LEH suspension sample (50% by volume LEH) of 15 volume % oxygen. For double emulsion processing, Soy-PC or Egg PC appeared to give similar results for the percentage of precursor Hb solution concentration encapsulated, i.e. either were equally good. LEH processing resulted in encapsulation efficiencies of about 50%. For example, Hb and lipid (i.e. total lipid including cholesterol) in the "Stealth" LEH formulations were loaded at about 3.1 μmoles per 150 μmoles, respectively and resulted in LEH containing 0.9 μmoles encapsulated Hb per 82 μmoles of total lipid. These results were determined from a total phosphorous analysis in conjuction with Bligh-Dyer extraction on 1 ml samples of "Stealth" LEH at a lipocrit of 30% containing an encapsulated Hb solution concentration of 20 g%. The phosporous analysis was performed at Liposome Technology Inc.'s (Menlo Park, CA) quality control laboratory. The remainder of this report refers to results obtained for LEH produced using the modified LEH methods.

A thin-section electron micrograph for LEH passed 10 times through the MicrofluidizerTM is shown in Figure 3 at a magnification of 25,000. The particle size range of the 3-micron-filtered sample appearsto be from as small as 50 nm to a little greater than 1 micron. However, the average particle size is about 300 to 400 nm. The oxygen saturation curve

obtained for LEH and the precursor Hb solution using a Hemox Analyzer is shown in Figure 4. The P50 for LEH and Hb solution samples (both containing P-5-P) are 22 and 19 mm Hg, respectively. Oxygen cooperativity values for LEH are within the normal range of values expected for whole blood.

Steady shear viscosity results were obtained (see Figure 5) for LEH suspension samples (in PBS containing 7.5 g% albumin at isooncotic levels). The viscosity results for both conventional and "stealth" LEH suspension samples (both prepared at 50% lipocrit) were higher than that obtained human and rat whole blood (both at 45-46% hematocrit), however viscosity results obtained for LEH suspension samples of 30% lipocrit were slightly lower than that measured for the whole blood samples. All viscosity measurements were made at 37°C. Note that the "stealth" LEH had higher viscosity than the conventional for shear rates below 100 s-1. The effect of shear rate on leakage of encapsulated Hb was obtained for freshly prepared LEH samples in either 7.5 g% egg albumin/PBS or human plasma, both at 30% by volume LEH (see Figure 6). Although leakage was highest for the LEH sample suspended in plasma, only 0.5% leakage of the encapsulated Hb was measured at the highest shear rate value tested of about 500 s-1. Egg-PC based-LEH suspended in 7.5%albumin/PBS (LEH 5b) showed only 0.25% leakage at the highest shear rate compared to 0.4% for the Soy-PC based-LEH suspension sample. The effect of storage of conventional LEH at 37°C on leakage of encapsulated Hb obtained for freshly prepared LEH samples in either 7.5 g% egg albumin/PBS or human plasma (both at 30% by volume LEH) is shown in Figure 7.

Circulation half-life after 50% isovolemic exchange-transfusions in rats typically were approximately 15 to 20 hrs for the conventional and "stealth" LEH samples tested. Results shown in Figure 8 show circulation half-life to be about 20 hrs for both types of LEH samples. These times are desirably long and compare very favorably to results reported in another study for a similar phospholipid/lipid formulation [29]. Also, high cholesterol content in the membrane, as is the case for both LEH1 and LEH2 formulations, prolongs liposome stability in circulation [95]. Other recent studies have also found that the "stealth" lipid, PEG-PE, significantly increased the blood circulation time of liposomes formulated with it [56,57,96-99]. Also those studies have shown "stealth" liposomes to have greatly decreased uptake by the reticuloendothelial system and thus enhanced circulation time compared to many other conventional phospholipids formulations. A possible explanation for this behavior is that the PEG polymer sterically stabilizes the LEH [99,100] like that previously reported for ionic surfactant coating of colloidal particles [101]. This results in "limited accessibility" [97] of the liposome surface to adsorption by plasma proteins [102] such as immunoglobulins and high density lipoproteins, which could cause vesicular breakdown [44] and opsonization followed by RES uptake [66].

Recently it was found that replacement of conventional lipids in LEH with "Stealth" lipids may provide a mechanism for <u>in vivo</u> oxygen delivery with less adverse impact on host resistance and immunity [19]. It was also found that both conventional and "Stealth" LEH caused significant reduction of phagocytic activity [19]. Yet, as shown in this study both types of LEH have approximately the same clearance rates. Therefore, it appears that prediction of impact on host resistance should not be based solely on <u>in vitro</u> tests of the effect of test material on phagocytosis.

As shown in Figure 9, nearly total (97%) isovolemic exchange transfusion demonstrates efficacy of the LEH suspension samples, since administration of LEH supported life in rats whose hematocrit had been reduced to levels below 5% which are incompatible with survival when performing exchange transfusion with isotonic/isooncotic PBS containing 7.5 g% albumin. These data concerning efficacy of LEH are consistent with those found in other recent studies for terminal

hematocrit obtained for the control-exchanged animal and the LEH-exchanged animal [30,35,37]. The effect of nearly total (97%) isovolemic exchange transfusion of blood with LEH on rat arterial blood pressure is reported in Figure 10 as a function hematocrit dilution. Note that exchange transfusion using the Hb control solution was not lethal even at 0% hematocrit. The corresponding arterial blood pressure was not much reduced from the value at the start of exchange at 45% hematocrit. Rat internal organs were harvested at terminal hematocrit following exchange with the albumin/PBS control and the LEH suspension sample. There appeared to be no apparent difference in organ weights between rats exchanged with control compared to rats exchanged with LEH. It has been shown above that transfusion with LEH can maintain survival in experimental animals whose hematocrits have been reduced to levels which cannot otherwise support life. However, the following results address the question, will the quantity of LEH required for life support overload the reticuloendothelial system, thereby compromising host immunity? Based on the experimental design as described in methods below results as shown in Table 1 were obtained. Mice dosed with LEH1 solutions on Day -3 retained essentially normal immune response against Listeria infection. However, mice dosed with LEH2 solutions had increased mortality. Mice challenged on Day 1 with LEH1 rapidly succumbed to Listeria infection. LEH2 dosed mice dosed also died more rapidly than controls, but mortality rates were significantly decreased as compared to mice exposed to LEH1. These data suggest that LEH1 clearance by the RES is extremely rapid so that mice dosed on Day -3 had already overcome RES overload while challenge on Day 1 caused extreme immunosuppression resulting in overwhelming Listeria infection and death. LEH2 also impaired immune defenses, but to a lesser degree, indicating perhaps a more prolonged clearance pattern with less impairment of the phagocytic capabilities of the RES. These results demonstrate that changes in lipid composition can modulate the immunotoxicity of LEH; the differential effects of LEH1 and LEH2 appear to result from differences in both half-life and intrinsic immunotoxic activity. Replacement of conventional lipids in LEH with Stealth lipids may provide a mechanism for in vivo oxygen delivery with less adverse impact on host resistance and immunity.

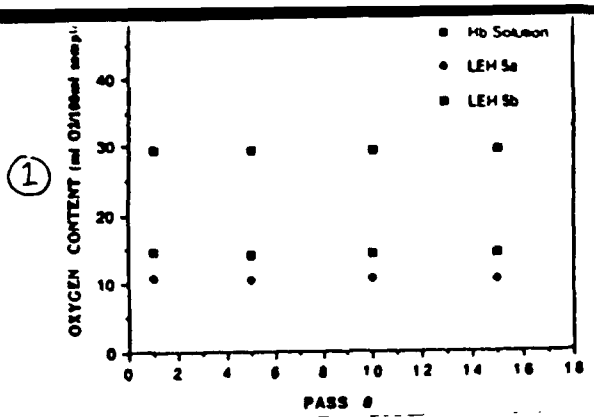
## CONCLUSIONS

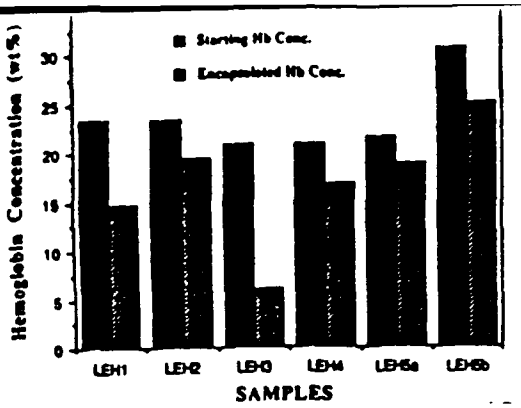
Further development of double emulsion and film hydration methods resulted in improvements in processing of LEH. Rehydration methods produced high encapsulation efficiencies and high encapsulated Hb concentrations that were limited only by the maximum viscosity and lipid loading. A successful method to filter significant quantities of LEH suspensions at pressure drops below 50 psi through cellulose ester filters down to a cut off filter diameter of 0.45 was developed. Met-Hb generation accompanying LEH processing (below 10°C) and during 1 month storage (-20°C) was small with only a 3% and less than a 10% increase for encapsulated over precursor, respectively. Oxygen affinity and cooperativity and steady shear viscosity values for LEH suspensions (30% by volume) appear to be near the normal values expected for whole blood. Incubation in plasma at 37°C resulted in only a small amount of Hb release from LEH; also shear had very little effect on causing Hb leakage from LEH.

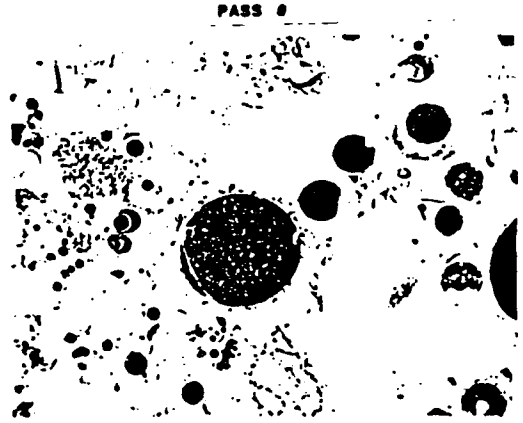
Our in-vivo studies demonstrated that liposome-encapsulated hemoglobin (LEH) provided an effective means of oxygen delivery in experimental animals. Our results indicated that exposure of experimental animal to liposomes made from conventional lipids was associated with significant immunotoxicity, as measured by impaired host resistance to infectious challenge. Our results thus far suggest that liposomes constructed with "Stealth" lipids are less immunotoxic than were liposomes made with conventional lipids. Both LEH formulations depressed phagocytic function as measured by ingestion

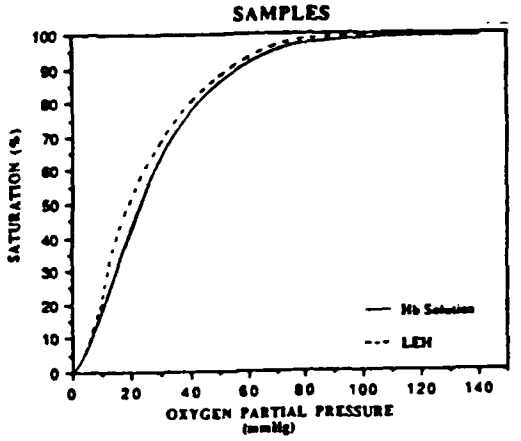
of latex beads. However, prediction of impact on host resistance should not be based solely on in vitro tests of the effect of test material on phagocytosis. Circulation half-life following 50% isovolemic exchange-transfusion in rats with LEH was about 20 hrs. Administration of LEH supported life in rats whose hematocrit had been reduced via isovolemic exchange transfusion to levels well below 5%, which was incompatible with survival when exchange transfusion was performed with the isotonic-isooncotic PBS solution. 97% exchange-transfusion demonstrated efficacy in life support.

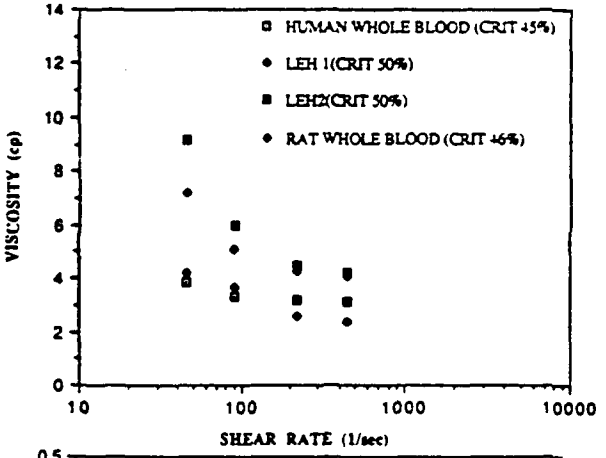
A novinvasive methodology for measuring the optical absorption spectra of LEH was developed as is presented in the attached final report "Optical Measurements on Intact LEH". Through this approach the stability of LEH through oxidative interactions between hemoglobin and membrane lipids can be assessed [60,62,67].

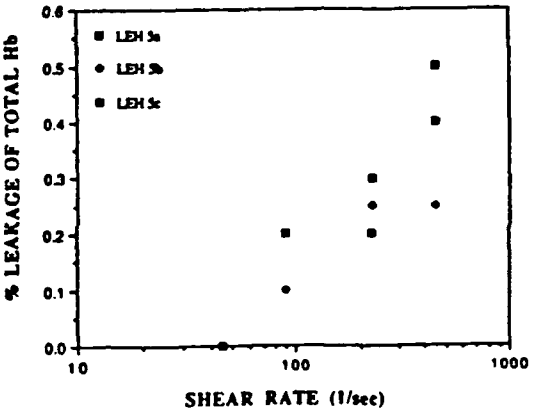

Hb Solution
LEH 5a
LEH 5b
OXYGEN CONTENT (ml O2/100ml soln)
PASS #

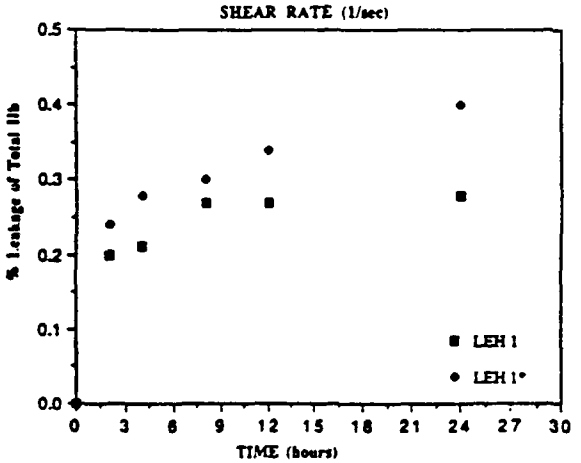

Starting Hb Conc.
Encapsulated Hb Conc.
Hemoglobin Concentration (wt%)
LEH1
LEH2
LEH3
LEH4
LEH5a
LEH5b
SAMPLES

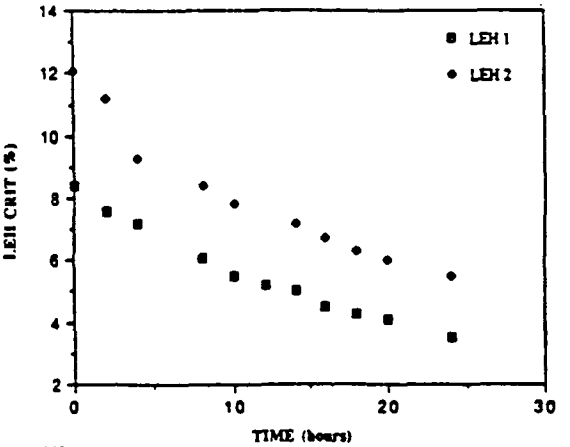


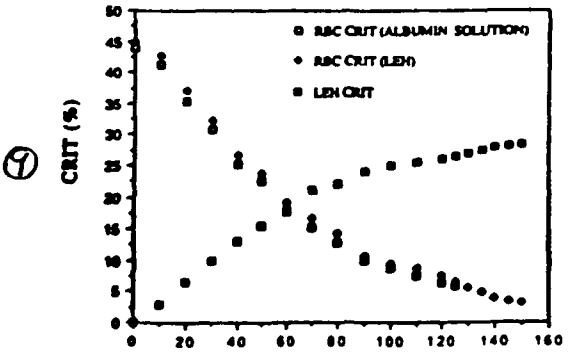

SATURATION (%)
Hb Solution
LEH
OXYGEN PARTIAL PRESSURE (mmHg)


HUMAN WHOLE BLOOD (CRIT 45%)
LEH 1(CRIT 50%)
LEH2(CRIT 50%)
RAT WHOLE BLOOD (CRIT 46%)
VISCOSITY (cp)
SHEAR RATE (1/sec)


LEH 5a
LEH 5b
LEH 5c
% LEAKAGE OF TOTAL Hb
SHEAR RATE (1/sec)


% Leakage of Total Hb
LEH 1
LEH 1*
TIME (hours)


LEH 1
LEH 2
LEH CRIT (%)
TIME (hours)


RBC CRIT (ALBUMIN SOLUTION)
RBC CRIT (LEH)
LEH CRIT
CRIT (%)

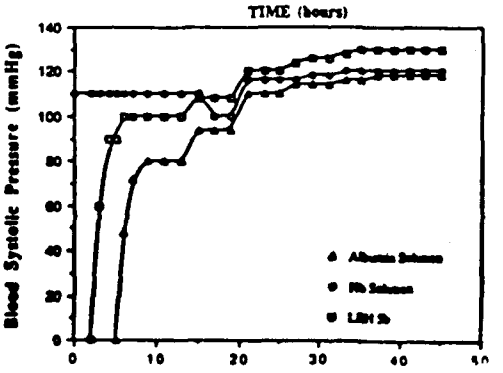

Albumin Solution
Hb Solution
LEH 5b
Blood Systolic Pressure (mmHg)

TABLE 1.

EFFECT OF ACUTE INTRAVENOUS CHALLENGE WITH LIPOSOME-ENCAPSULATED HEMOGLOBIN FORMULATIONS ON MORTALITY OF FEMALE CD-1® MICE FROM *Listeria monocytogenes* INFECTION

| Exposure Group | Lipocrit Conc. (%) | Dose Day[d] | N[e] | % Mortality | MST[f] |
|---|---|---|---|---|---|
| Vehicle[a] | -- | -3 | 20 | 20 | 8.85 |
| LEH1 | 25 | -3 | 19 | 21 | 9.05 |
| LEH1 | 50 | -3 | 19 | 11 | 9.42 |
| LEH2 | 25 | -3 | 18 | 39 | 7.78 |
| LEH2 | 50[b] | -3 | 20 | 60* | 7.10[g,h] |
| Vehicle | -- | 1 | 20 | 20 | 9.00 |
| LEH1 | 25 | 1 | 20 | 100*** | 2.60[i] |
| LEH1 | 50 | 1 | 19 | 100*** | 2.74[i] |
| LEH2 | 25 | 1 | 20 | 60* | 6.25[j,k] |
| LEH2 | 50[c] | 1 | 20 | 65** | 5.95[j,l] |

* $P < 0.01$; Chi square
** $P < 0.01$; Chi square
*** $P < 0.001$; Chi square
a Vehicle was albumin (0.5 ml/mouse)
b Dosed with 0.25 ml/mouse
c Dosed with 0.20 ml/mouse
d Mice dosed i.v. with 0.5 ml vehicle or LEH on Day -3 or Day 1 with i.v. *Listeria* challenge on Day 0
e Number of mice treated
f Mean survival time over a 10 day observation period
g $P < 0.05$ as compared to Vehicle; Dunnett's test
h $P < 0.01$ as compared to LEH1 50%; Dunnett's test
i $P < 0.001$ as compared to Vehicle: Dunnett's test
j $P < 0.01$ as compared to Vehicle: Dunnett's test
k $P < 0.001$ as compared to LEH1 25%: Dunnett's test
l $P < 0.001$ as compared to LEH1 50%; Dunnett's test

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## OPTICAL MEASUREMENTS ON INTACT LEH

### Introduction

The general objective of this project component was to develop a non-invasive methodology for measuring the optical absorption spectra of LEH. The concentrations of oxyhemoglobin ($HbO_2$) and methemoglobin (MetHb) are of most interest in connection with the oxygen-carrying capacity. Owing to high turbidity induced by light scattering, accurate absorption spectra of LEH cannot be measured in a conventional spectrophotometer. A similar difficulty obtains for optical measurements on intact red blood cell (RBC) suspensions. A procedure used in prior work with RBC involves the use of extremely thin samples in which light scattering is minimal. The major disadvantage of this approach is the measured absorbance is very low and subject to inaccuracies from spectroscopic scattered light and other instrumental artifacts. Furthermore, the calculation of Hb derivative concentrations requires measurements in the spectral regions of low extinction coefficient which are the most inaccurate. Diffuse reflection ($R_d$) measurements at several wavelengths is an alternative approach, as employed in devices for *in vivo* monitoring of blood oxygen. The $HbO_2$ concentration can be determined by means of an empirical calibration because RBC are highly uniform. However, the scattering centers in LEH have variable size, shape, and Hb content, requiring an absolute determination of the Hb absorbance ($A$). This can be accomplished by measuring the total reflection (R) and total transmission (T) at each wavelength of interest and calculating the dependence of the linear absorption coefficient k ($k = Log_e 10 \times A$) using a tissue optic model. The methodology for these measurements was developed in this project. Preliminary measurements were made on solution Hb and diluted RBC suspensions in order to develop the techniques, which was followed by measurements on LEH preparations.

### Materials and Methods

#### A. Diffuse Reflection and Transmission Spectra

The R and T spectra were measured with a computerized, two-channel, integrating sphere spectrophotometer. The present arrangement provides for measurements at 1 nm intervals compared to 5-10 nm in our prior work.[1] The light source was a 100 W tungsten-halogen lamp coupled to a 1/8 m monochromator with a stepper motor drive (Oriel Corp). The output beam was chopped, split into collimated 10 mm sample and reference beams, and transported to two 15 cm integrating spheres with a lens system. The samples were contained in a 5 mm path glass cuvette located in front of a 10 mm entrance port or behind a 10 mm exit port. The interior flux in each integrating sphere was sampled with a 3 mm glass optical cable coupled to a Hamamatasu R446 photomultiplier and measured with an Ithaco 3921 lock-in amplifier. The sample-to-reference signal ratio was stored in an HP Vectra computer which also controlled the stepper motor. Distilled water was used as the transmission reference and an aluminum plate painted with Kodak 6080 white coating was used as the reflection reference. The effects of beam size and collimation were evaluated by comparing the calculated values of k and s' for test systems with a 5 mW helium-neon laser. The measurements with the lamp-monochromator source for a 3 mm potato layer at 633 nm led to $k = 0.46\ cm^{-1}$

and s' = 5.5 $cm^{-1}$ compared to average values of k = 0.42±0.01 $cm^{-1}$ and s' = 4.5±0.7 $cm^{-1}$ for the laser measurements using 1 mm and 10 mm collimated beams. In a comparison with heterosized polystyrene spheres in aqueous methylene blue (MB) using 1 mm and 10 mm laser beams and 2 mm and 5 mm optical path cuvettes, the lamp-monochromator system led to k = 1.16±0.16 $cm^{-1}$ and s' = 24.1±1.9 $cm^{-1}$ for the two optical paths compared to k = 1.05±0.15 $cm^{-1}$ and s' = 21.1±1.9 $cm^{-1}$ for the laser with the two optical paths and two beam diameters.

**B. Flux Density Profiles**

The samples were located in a 4 cm path glass cuvette of 4 x 1 cm cross section. A 400 μm fused silica optical fiber with a 2 mm diameter spherical tip was inserted from the top and displaced in 1 mm increments. The front face was illuminated with chopped light from a 200 W high-pressure Hg-Xe arc through collimating lenses and a narrow-band interference filter. The incident beam diameter was approximately 3.5 cm with < 4% per cm radial variation in the incident irradiance. The probe signal was measured with a Hamamatasu R446 photomultiplier and an Ithaco model 3921 lock-in amplifier.

**C. Tissue Optic Calculations**

The Kubelka-Munk (KM) two-flux model and its variants were employed in earlier work owing to the computational convenience.[2] The KM model has inherent deficiencies, especially the inexact relationship of the KM absorption and scattering coefficients to the optical cross sections and the requirement for diffuse incident light. Semi-empirical methods have been proposed to improve KM results by comparisons to more exact calculations based on radiative transfer theory.[3] More recent tissue optic calculations utilize the photon diffusion approximation, a limiting form of radiative transfer that is applicable when scattering dominates over absorption.[4] The equation of transfer can be transformed to a diffusion equation for a two-term Legendre expansion of the scattering phase function (P1 approximation). The flux density distribution in the diffusion approximation is expressed in terms of the linear absorption (k) and scattering coefficients (s) and the mean cosine of the single particle scattering angle (g). The number of experimental measurements at each wavelength can be reduced from three to two by transforming the optical constants with "similarity" relations: $g \rightarrow 0$, $k \rightarrow k$, $s \rightarrow s(1 - g) \equiv s'$, where s' is the "reduced" scattering coefficient.[5] The accuracy of this transformation must be evaluated for each case. In this work, the 1DA calculation was carried out with P1 formulation of Jacques and Prahl[6] which uses an approximate correction for refractive index mismatch based on the boundary conditions of Groenhuis *et al.*[7] For the reduced 1DA calculation approximately 1000 (R,T) data sets were processed by a 33 Mhz "386" computer in a few minutes using an iterative program based on a two-dimensional Newtons's method.

**D. Sample Preparation**

LEH were prepared with Hb from fresh, defibrinated bovine blood. The RBC were separated by repeated centrifugation at 3000 rpm for 10 min at 4°C and resuspending in p*H* 7.4 phosphate buffered saline (PBS), followed by treatment with lysis buffer (p*H* 8.0 phosphate buffer plus 5 m*M* NaCl) and separation of the Hb by centrifugation at 15,000 rpm for 35 min. The Hb solution was

concentrated to 14 g/100 ml in dialysis tubing surrounded by Aquacide II (Calbiochem). The lipid mixture (1 g hydrogenated soy phosphatidylcholine, 125 mg cholesterol, 93 mg dicetylphosphate, and 4 mg $\alpha$-tocopherol) was dissolved in 1:1 chloroform-diethyl ether and evaporated to dryness under vacuum. The concentrated Hb solution (20 ml) was added to the lipid film and held at 4°C for 15 h with occasional swirling, followed by bath sonication for 4 h under nitrogen, centrifugation at 1500 rpm for 6 min at 4°C to remove large fragments, and repeated centrifugation at 15,000 rpm for 45 min and resuspending in PBS to remove excess Hb. $HbO_2$ in LEH was converted to deoxyHb by adding 2 mg of sodium dithionite to 5 ml of LEH. HbCO was obtained by blowing CO over the surface of a cuvette containing $HbO_2$ for 5 min. Hemiglobin nitrite ($HbNO_2$) was obtained by adding 2 mg sodium nitrite crystals to 5 ml of $HbO_2$. The LEH were lysed by mixing 0.5 ml of LEH with 9.5 ml of 1% D-glucopyranoside, stirring for 30 min, and centrifuging at 15,000 rpm for 35 min at 4°C. The human RBC suspensions were prepared from freshly drawn blood from a female volunteer. The RBC were hemolyzed by dilution in the lysing buffer. Absorption spectra were measured with a Beckman DU-7 spectrophotometer (referred to as "DU-7").

## Results

The detailed results of this project component are provided in Quarterly Reports. A brief summary of the results is presented here with representative data.

### A. Optical Measurements on Mixtures of Hb Derivatives in Solution

The optical spectra of bovine Hb derivatives measured with the "DU-7" are shown in **Figure 1**. (All Figures are located at end of this report.) Mixture of $HbO_2$ and MetHb were prepared in different ratios and the absorbance was measured in the "DU-7" at 21 wavelengths. The concentrations were then calculated from the corresponding extinctions coefficients of each derivative using the method of single value decomposition (SVD) as described in Quarterly Report No. 6 (23 May 1990). The results are summarized in **Table 1**.

**Table 1. Analysis of Oxyhemoglobin and Methemoglobin Mixtures**

| | [$HbO_2$]:[MetHb] | | | | |
|---|---|---|---|---|---|
| *As Mixed* | 1:1.39 | 1:2.78 | 1:4.17 | 1.44:1 | 2.16:1 |
| *Calculated* | 1:1.39 | 1:2.93 | 1:4.19 | 1.33:1 | 1.87:1 |

The good agreement indicates that the SVD method should be applicable for similar calculations with LEH.

### B. Optical Measurements on Intact Red Blood Cells

The reduced 1DA methodology was tested by measuring R and T for a bovine RBC suspension in PBS from 380 to 1050 nm and calculating the optical coefficients. The results are shown in **Figure 2**. The k spectrum accurately reproduces the visible absorption band of $HbO_2$. The band near 980 nm is a water absorption. The s' spectrum is very weak and approximately constant as expected.

## C. Optical Measurements on LEH

**Figure 3** shows the 1DA spectra of LEH incorporating 0.17 g/100 ml Hb. The points are the "DU-7" spectrum of the lysed LEH on the same scale. The agreement is good except for the higher amount of MetHb in the lysed LEH as indicated by the 630 nm absorption. The MetHb fraction estimated from k at 577 and 630 nm is 13% in the intact LEH and 26% in the lysed LEH. The spectral parameters of bovine $HbO_2$ in PBS are very close to human $HbO_2$ from lysed RBC (**Table 2**) and the literature values.[8] The lower peak-to-valley ratios for $HbO_2$ in intact and lysed LEH compared to solution $HbO_2$ are attributed to the overlapping absorption of MetHb. The s' spectrum shows deviations from smooth curves that "mirror" the k spectrum. This effect has been observed with other tissue phantoms in which colorless scattering particles are suspended in a homogeneous absorber. It is attributed to removal of photons by "inner filtering" prior to reaching a scattering center. This effect occurs also for LEH in which a significant fraction of the liposomes do not incorporate Hb. The LEH chromophore spectra were modified *in situ*. **Figure 4** shows the reduced 1DA spectra of Hb derivatives in LEH. The general spectral features are similar to measurements on solution Hb.[8,9] The band maxima are compared with literature values for human Hb in **Table 3**.

**TABLE 2. Absorbance Parameters of Oxygenated Hemoglobin**

| *Sample* | $\lambda_1$ [a] | $\lambda_2$ [a] | $\lambda_0$ [b] | $R_{12}$ [c] | $R_{10}$ [d] | $R_{20}$ [d] |
|---|---|---|---|---|---|---|
| Lysed human RBC[e] | 576 | 541 | 560 | 1.07 | 1.82 | 1.71 |
| Intact human RBC[f] | 576 | 540 | 560 | 1.05 | 1.65 | 1.57 |
| Intact human RBC + ADS[f] | 576 | 541 | 560 | 1.07 | 1.80 | 1.69 |
| Bovine $HbO_2$ in PBS[e] | 577 | 541 | 560 | 1.06 | 1.82 | 1.72 |
| Intact bovine LEH[f] | 576 | 541 | 559 | 1.06 | 1.50 | 1.43 |
| Lysed bovine LEH[f,g] | 577 | 541 | 560 | 0.98 | 1.46 | 1.49 |

[a] absorption peak
[b] absorption valley
[c] peak-to-peak absorbance ratio
[d] peak-to-valley absorbance ration
[e] measured in Beckman DU-7 spectrophotometer
[f] calculated from reduced 1DA analysis
[g] in 1% D-glucopyranoside

**TABLE 3. Absorption Spectra of Hemoglobin Derivatives Calculated with the Reduced 1DA Methodology**

| *Derivative* | *Wavelength Maxima (nm)* | |
|---|---|---|
| | *Figure 3* | *Solution Hb*[a] |
| $HbO_2$ | 576, 542 | 577, 542 |
| HbCO | 570, 540 | 569, 539 |
| $HiNO_2$ | 625, 540 | 625, 538 |
| deoxyHb | 555 | 555 |

[a] human Hb in solution; from van Assendelft [8].

## C. Flux Density Profiles

The object of these measurements was to compare the flux density profiles calculated with the reduced 1DA for 5 mm layers with experimental measurements on 4 cm depth layers for equivalent media. The theoretical asymptotic diffusion length ($\delta$) is given by:

$$\delta = 1/\sqrt{[3k(k + s')]}$$

**TABLE 4. Attenuation constants of tissue phantoms**

| *System* | *Wavelength* (nm) | $k$ ($cm^{-1}$) | $s'$ ($cm^{-1}$) | $\delta$[a] (cm) | $\delta_{exp}$[b] (cm) |
|---|---|---|---|---|---|
| LEH[c] | 505 | 1.48 | 6.56 | 0.17 | 0.17 |
| RBC[d] | 505 | 0.47 | 0.37 | 1.17 | 1.13 |
| RBC + ADS[e] | 505 | 0.66 | 6.83 | 0.26 | 0.25 |

[a] calculated diffusion length
[b] extrapolated attenuation depth measured in 4 cm layer
[c] 0.17 g/100 ml Hb
[d] $2.4 \times 10^7$/ml RBC
[e] $2.4 \times 10^7$/ml RBC + 2.4% ADS

The values of $\delta$ calculated with the reduced 1DA values of k and s' are in good agreement with the experimental results (**Table 4**). In one case artificial dairy substitute was added to the RBC suspension in order to increase the turbidity for the same Hb concentration. The good agreement for all systems

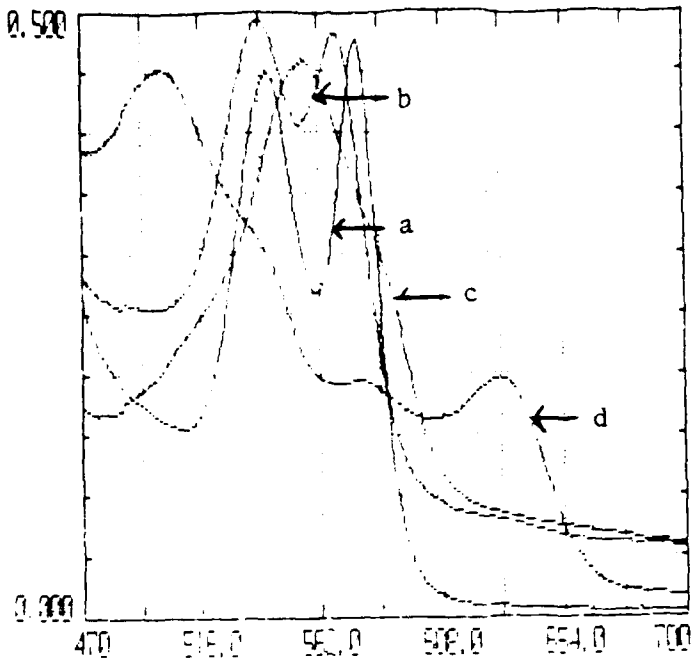


Figure 1. Optical spectra of bovine hemoglobin in PBS measured in "DU-7": (a) $HbO_2$; (b) HbCO; (c) deoxyHb; (d) $HiNO_2$.

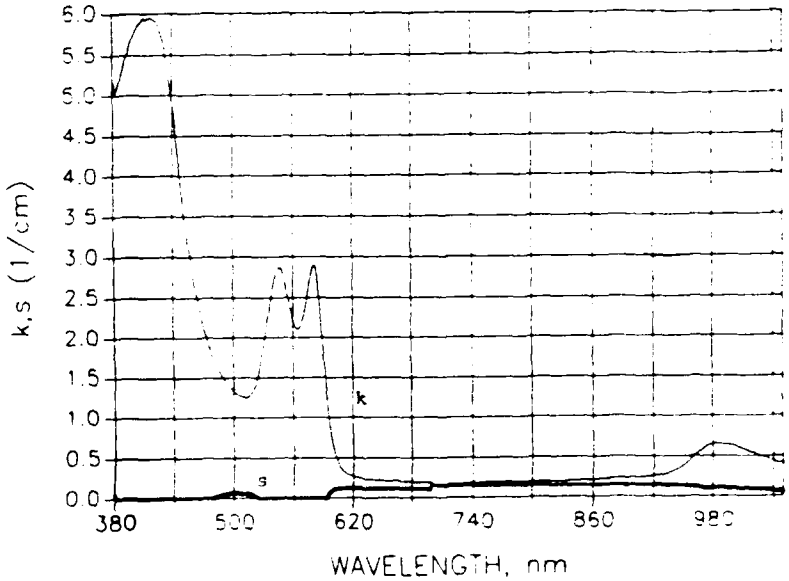


Figure 2. Calculated absorption (k) and scattering (s) spectra of a dilute RBC suspension in PBS.

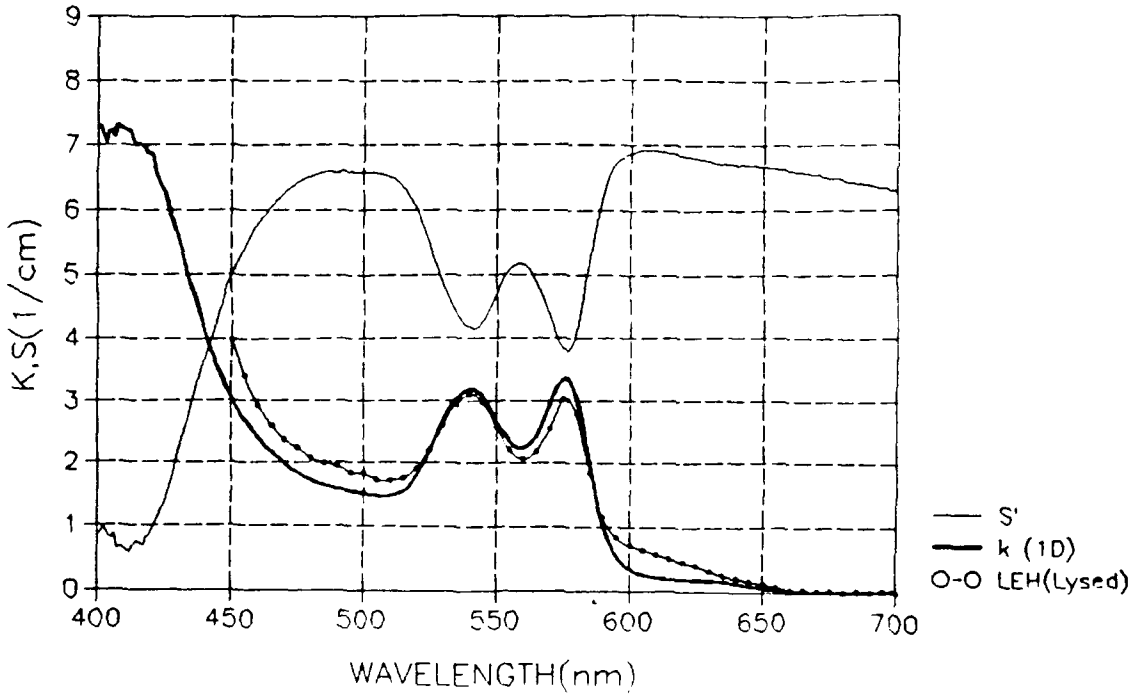


Figure 3. Calculated absorption (k) and scattering (s) spectra of LEH. The points were measured in the "DU-7" after lysis.
the LEH spectrum

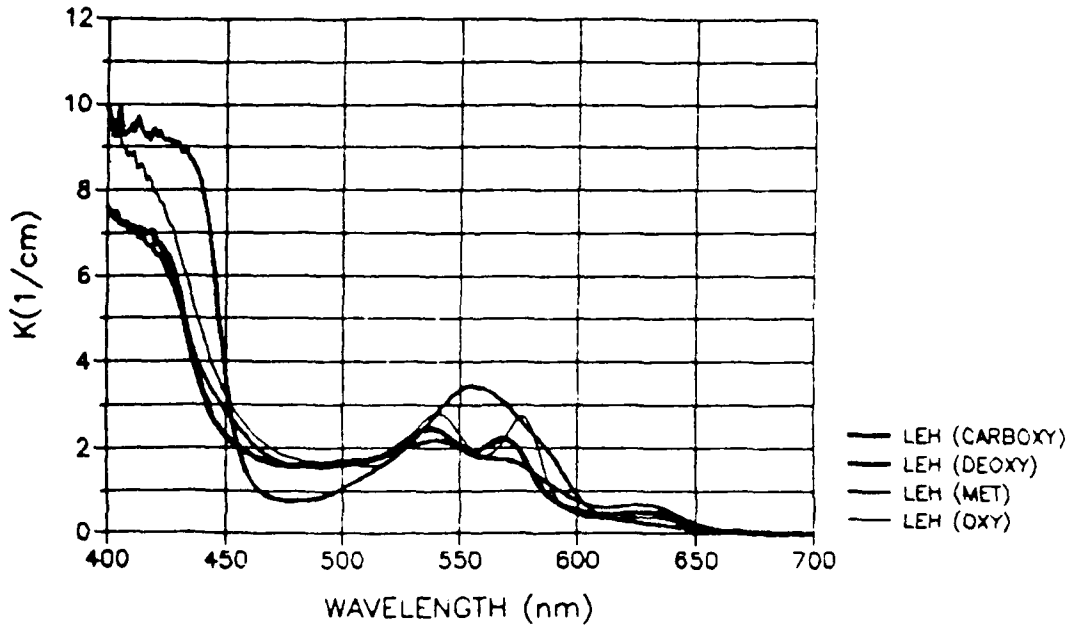


Figure 4. Calculated absorption (k) spectra of Hb derivatives.

indicates that the optical constants calculated with the reduced 1DA methodology leads to approximately correction light intensity profiles in an optically dense, highly attenuating layer.

## Conclusions

The results of this investigation demonstrate that accurate absorption spectra of $HbO_2$ and other Hb derivatives can be measured in RBC and LEH with a non-invasive diffuse optics methodology based on the reduced 1-D diffusion approximation. The accuracy of the results was shown by comparisons of the calculated extinction coefficients to literature results for solution Hb. An independent evaluation was carried out by calculating the attenuation depth of optically thick samples at selected monochromatic wavelengths and comparing to measurements made with an inserted optical fiber probe. Single value decomposition analysis was used to calculate the fraction of active $HbO_2$ in known mixtures of $HbO_2$ and metHb. This method based on measurements at 10 and 25 wavelengths is far superior to usual spectral analysis procedures utilizing 3 wavelengths.

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# PREPRINTS

**DIFFERENTIAL EFFECTS ON HOST RESISTANCE OF LIPOSOME-ENCAPSULATED HEMOGLOBIN BLOOD SUBSTITUTES MADE WITH CONVENTIONAL OR STEALTH LIPIDS.** R.L. Sherwood*[1], D.L. McCormick[1], S. Zheng[2], and R.L. Beissinger[2]. [1]IIT Research Institute, Chicago, IL and [2]Ill. Inst. Tech., Chicago, IL.

Transfusion with liposome-encapsulated hemoglobin (LEH) can maintain survival in experimental animals whose hematocrits have been reduced to levels which cannot otherwise support life. However, the quantity of LEH required for life support may overload the reticuloendothelial system, thereby compromising host immunity. To determine if changes in LEH lipid composition can modify immunotoxicity, blood substitutes based on conventional (LEH1) and "stealth" (LEH2; gift of Liposome Technology Inc.) lipids were tested for effects on host resistance. On Day 0, groups of 18 to 20 female CD-1 mice were given an i.v. infectious challenge with a 20% lethal dose of *Listeria monocytogenes*; all mice received an i.v. dose of LEH1, LEH2, or albumin vehicle on Day -3 or Day +1. Mice dosed on Day +1 with LEH1 died rapidly from *Listeria* infection; mice dosed with LEH2 on Day +1 also died more rapidly than controls, but lived significantly longer than did mice dosed with LEH1. By contrast, when administered on Day -3, LEH1 had no effect on host immunity, while LEH2 increased susceptibility to *Listeria* infection. LEH1 and LEH2 both caused significant reduction of phagocytic activity as measured by rat alveolar macrophage (AM) ingestion of latex microspheres. Following a 5 hr incubation with LEH1 or LEH2, fewer AM ingested beads, and the number of beads per cell was also decreased in a dose dependent manner. These results suggest that LEH1 challenge on Day +1 caused immunosuppression resulting in fulminant *Listeria* infection and death, probably as a result of overload of host phagocytes responsible for *Listeria* clearance from the blood. LEH2 had significantly less adverse impact on host immunity. These results demonstrate that changes in lipid composition can modulate the immunotoxicity of LEH; the differential effects of LEH1 and LEH2 appear to result from differences in both half-life and intrinsic immunotoxic activity. Replacement of conventional lipids in LEH with stealth lipids may provide a mechanism for *in vivo* oxygen delivery with less adverse impact on host resistance and immunity.

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# INTRODUCTION

Several reports have suggested that acute (Merion, 1985; Nugent, 1984) or chronic (Allen et al., 1987) administration of liposomes or lipid-containing materials causes overloading of the reticuloendothelial system (RES). It is well known that liposomes tend to accumulate in mononuclear cells of the RES (Allen et al., 1987). Empty and formulated liposomes and lipid emulsions have been shown to decrease phagocytic activity (Nugent, 1984; Wassef et al., 1984; Allen et al., 1987; Merion, 1985). Another class of chemicals used as blood substitutes (perfluorochemicals) have also been implicated in depression of RES function (Bucala et al., 1983; Virmani et al, 1983, 1984; Lane and Lamkin, 1984). Normal functioning of RES clearance pathways is crucial to the maintenance of proper immune responsiveness; thus, RES overload and resulting alterations in phagocytic cell number and/or activity may increase host susceptibility to infectious agents (Nugent, 1984). Host phagocytes (monocytes, macrophages) are crucial in inactivating and removing bacteria, fungi, viruses and other foreign material from the body (Eisen, 1980). They also interact extensively with lymphocytes in modulating normal immune responses. Thus, impairment of host phagocyte functions could result in increased susceptibility to pathogenic or opportunistic infections or enhance improper immunomodulation resulting in immunosuppression or allergy. These studies were designed to determine if LEH formulations would adversely affect host immunity and to determine whether depression of phagocytosis was responsible for the immunotoxic effect.

## MATERIALS AND METHODS

**Experimental Design:**

In vivo: Mice received an i.v. infectious challenge with a 10-30% lethal dose of *L. monocytogenes* on Day 0. On Day -3 or Day +1, groups of 18-20 mice received 7.5 g% albumin control solution or 0.2 to 0.5 ml of LEH1 or LEH2 suspensions prepared to 25% or 50% lipocrit concentrations. Mice were observed for survival for 10 days after infectious challenge. Percent mortality and mean survival time for each dose group were calculated.

In vitro: LEH1 and LEH2 formulated to 30% lipocrits were tested for effect on phagocytosis of rat alveolar macrophages (AM). AM were obtained by tracheobronchial lavage with warm, sterile saline (Sherwood et al., 1989). LEH formulations were incubated with $1x10^5$ viable AM/ml for 4 hrs prior to addition of latex beads (3.06 $\mu$m diameter; Polysciences, Inc., Warrington, PA) at a 50:1 bead to cell ratio. After 1 hr, trypan blue dye was added and cells were cytocentrifuged onto glass slides. Cells were fixed in formalin vapor and unengulfed beads dissolved in methylene chloride. One hundred viable cells per slide were microscopically counted and the number of beads ingested per cell recorded. Numbers of cells ingesting beads and numbers of beads per cell were determined.

**Animals:** Female, outbred CD-1® mice (Charles River Laboratories, Portage, MI) weighing 16-18 g upon arrival were used for host defense studies. Male CD® rats (CRL) were used for phagocytosis assays. Animals were housed in polypropylene cages with hardwood chip bedding. Cages were changed twice each week. Purina rodent chow 5001 and city of Chicago water in drinking bottles was provided *ad libitum*. Following a one week quarantine period to verify the health of the animals, mice were randomized and placed into exposure groups based on body weights. The mean body weight of each exposure group was 22.2 g and the body weight of individual mice did not vary by more than 20% of the group mean.

**Bacteria:** *L. monocytogenes* (American Type Tissue Collection strain 13932 serotype 4b) was used. Stock cultures were obtained from 24 hr petri plate cultures on Brain Heart Infusion Agar incubated at 35°C. Bacteria were resuspended in sterile nutrient broth containing 15% v/v of glycerol as a cryoprotective agent. Frozen stock cultures were thawed and resuspended in sterile 0.85% saline. A volume of 0.2 ml (approximately $2.66 \times 10^5$ colony forming units per mouse) was injected i.v. into the tail vein using a 1 ml syringe with a 25g 5/8" needle. Following infection, mice were observed at least once daily for a 10-day period.

**LEH Formulations:** Human stroma-free hemoglobin (Hb) was used in LEH formulations. LEH formulations contained hydrogenated soy or egg phosphatidylcholine, cholesterol, phosphatidylinositol or the "stealth" phospholipid polyethylene glycol distearoyl phosphatidyl ethanoloamine (gift of Liposome Technology Inc.), and $\alpha$-tocopherol. Double emulsion methods were used for preparation of LEH (Zheng et al., in press). LEH was made through formation of multilammellar vescicles which were reduced in size by a microfluidizer, washed in isotonic PBS and centrifuged to remove unencapsulated Hb. LEH emulsions were then prefiltered through 5, 3, and 1 $\mu$m filters before final processing through 0.8, 0.6 and 0.45 $\mu$m filters.

**Statistics:** Percent mortality and mean survival time were determined in the *Listeria* challenge study. Significance between exposure groups was assessed using life table analysis (BMDP Statistical Software, Inc., Los Angeles, CA). Differences in phagocytic index (% of cells ingesting at least one bead) and number of beads per cell were tested by ANOVA and Dunnett's test. Probabilities of $P \leq 0.05$ were accepted as significant.

## CONCLUSIONS

1. The results suggest that liposomes formulated with conventional lipids were rapidly cleared from the bloodstream by the RES system as has been demonstrated previously. The fact that no difference was observed in mortality due to *Listeria* infection between controls and LEH1 mice dosed on Day -3 suggests that LEH1 was cleared and adequately processed prior to infectious challenge on Day 0. However, liposomes formulated with stealth lipids adversely affected host defenses when LEH2 was administered on Day -3; these results suggest a delayed clearance pattern of LEH2 as compared to LEH1.
2. Similar arguments for LEH clearance may be made from data obtained after infectious challenge on Day 0 with LEH treatment on Day +1. However, in this instance, rapid RES clearance of LEH1 appeared to result in massive immunosuppression while the delayed clearance of LEH2 allowed some immune response against the microbial infection.
3. Both LEH formulations depressed phagocytic function as measured by ingestion of latex beads. A 5 hr incubation period failed to differentiate between LEH formulations in this *in vitro* test.
4. The results demonstrate that changes in lipid composition can modulate the immunotoxicity of LEH. The differential effects of LEH1 and LEH2 appeared to result from differences in both half-life and intrinsic immunotoxic activity.

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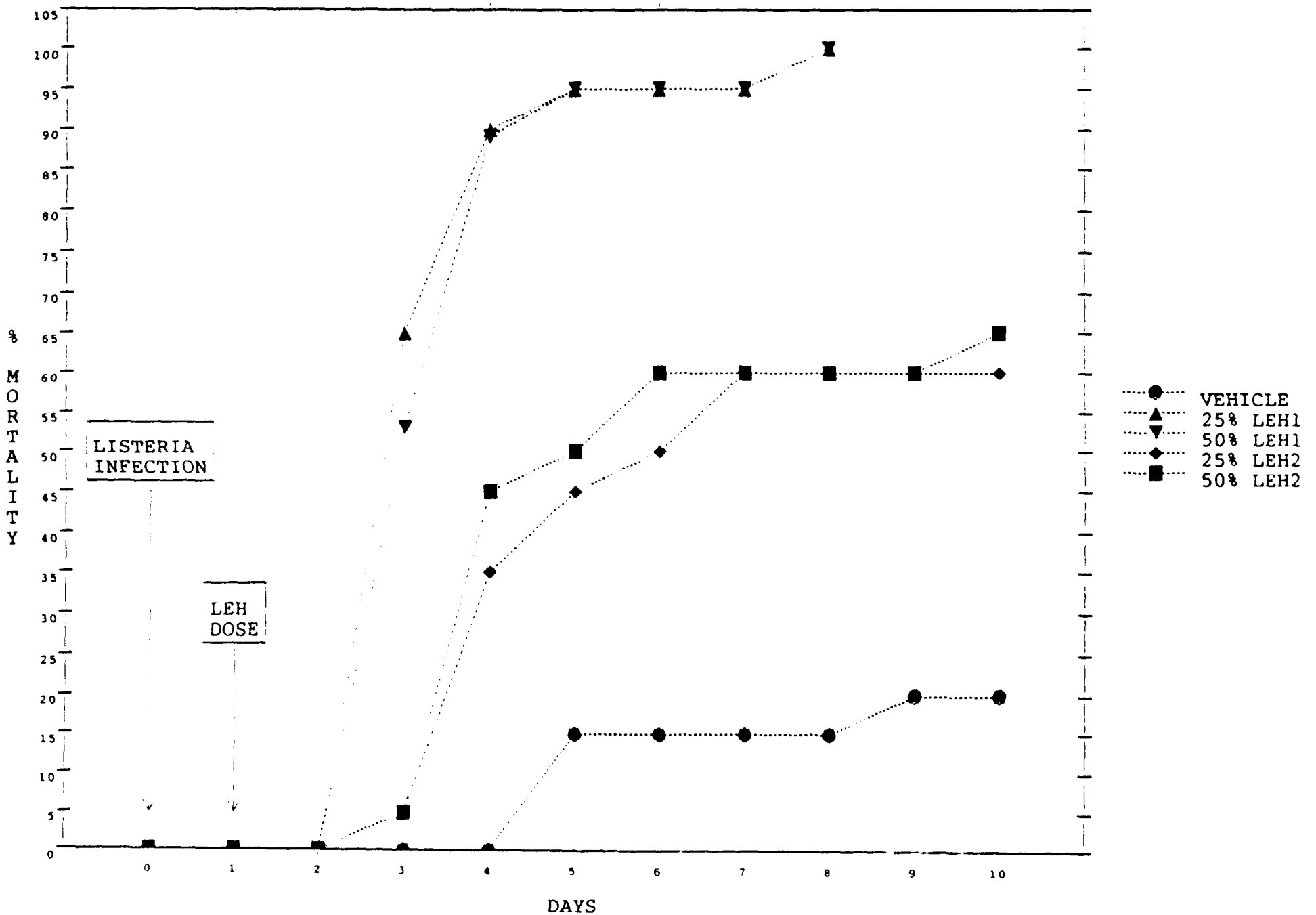
TABLE 1.

EFFECT OF ACUTE INTRAVENOUS CHALLENGE
WITH LIPOSOME-ENCAPSULATED HEMOGLOBIN FORMULATIONS
ON MORTALITY OF FEMALE CD-1® MICE
FROM *Listeria monocytogenes* INFECTION

| Exposure Group | Lipocrit Conc. (%) | Dose Day[d] | N[e] | % Mortality | MST[f] |
|---|---|---|---|---|---|
| Vehicle[a] | -- | -3 | 20 | 20 | 8.85 |
| LEH1 | 25 | -3 | 19 | 21 | 9.05 |
| LEH1 | 50 | -3 | 19 | 11 | 9.42 |
| LEH2 | 25 | -3 | 18 | 39 | 7.78 |
| LEH2 | 50[b] | -3 | 20 | 60 *,### | 7.10 $,&& |
| Vehicle | -- | 1 | 20 | 20 | 9.00 |
| LEH1 | 25 | 1 | 20 | 100 *** | 2.60 $$$ |
| LEH1 | 50 | 1 | 19 | 100 *** | 2.74 $$$ |
| LEH2 | 25 | 1 | 20 | 60 *,## | 6.25 $$,&&& |
| LEH2 | 50[c] | 1 | 20 | 65 **,## | 5.95 $$,&&& |

[a] Vehicle was albumin (0.5 ml/mouse)
[b] Dosed with 0.25 ml/mouse
[c] Dosed with 0.20 ml/mouse
[d] Mice dosed i.v. with 0.5 ml vehicle or LEH on Day -3 or Day 1 with i.v. *Listeria* challenge on Day 0
[e] Number of mice treated
[f] Mean survival time over a 10 day observation period
*, **, *** $P < 0.05$, $P < 0.01$, $P < 0.001$ vs. Vehicle; Chi square
## $P < 0.01$ vs. similar LEH1 formulation, Chi square
### $P < 0.001$ vs. LEH1 50%, Chi square
$, $$, $$$ $P < 0.05$, $P < 0.01$, $P < 0.001$ vs. Vehicle; Dunnett's test
&& $P < 0.01$ vs. LEH1 50%; Dunnett's test
&&& $P < 0.001$ vs. similar LEH1 formulation; Dunnett's test

EFFECT OF IV LEH CHALLENGE
ON MURINE LISTERIA INFECTION



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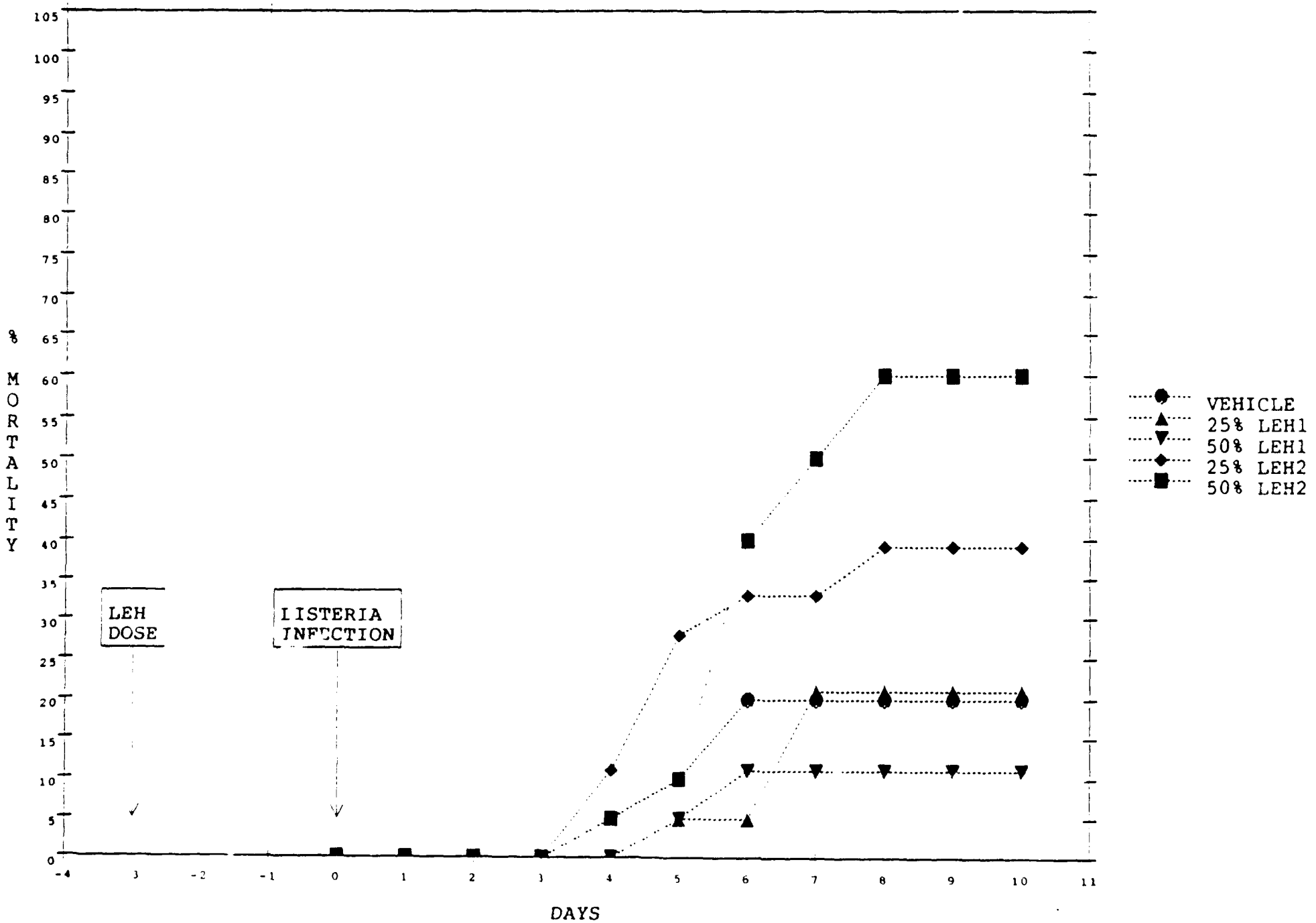
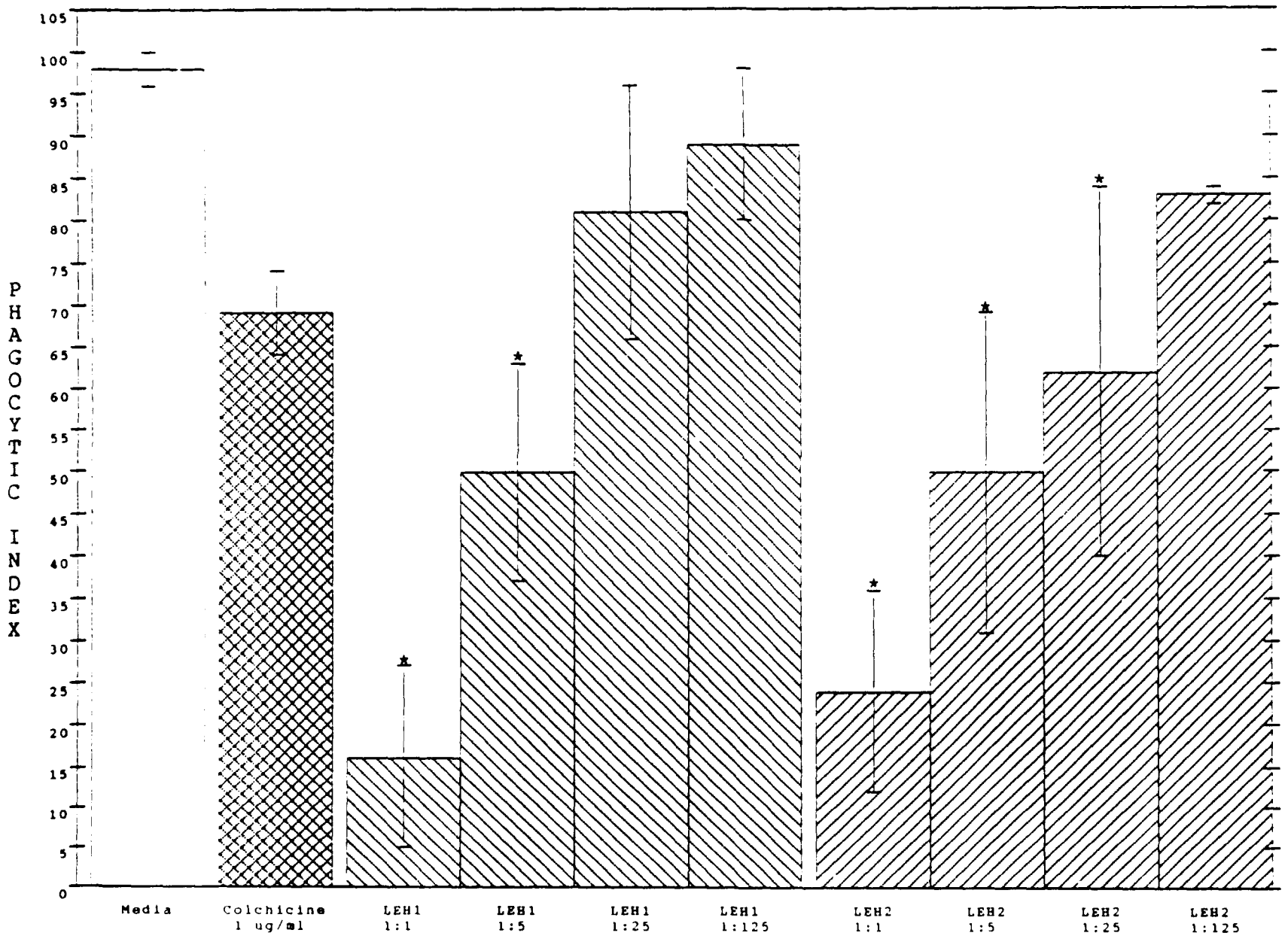


EFFECT OF 5 HR IN VITRO INCUBATION
OF 30% LIPOCRIT LEH FORMULATIONS ON
RAT ALVEOLAR MACROPHAGE PHAGOCYTIC ACTIVITY



EFFECT OF 5 HR IN VITRO INCUBATION
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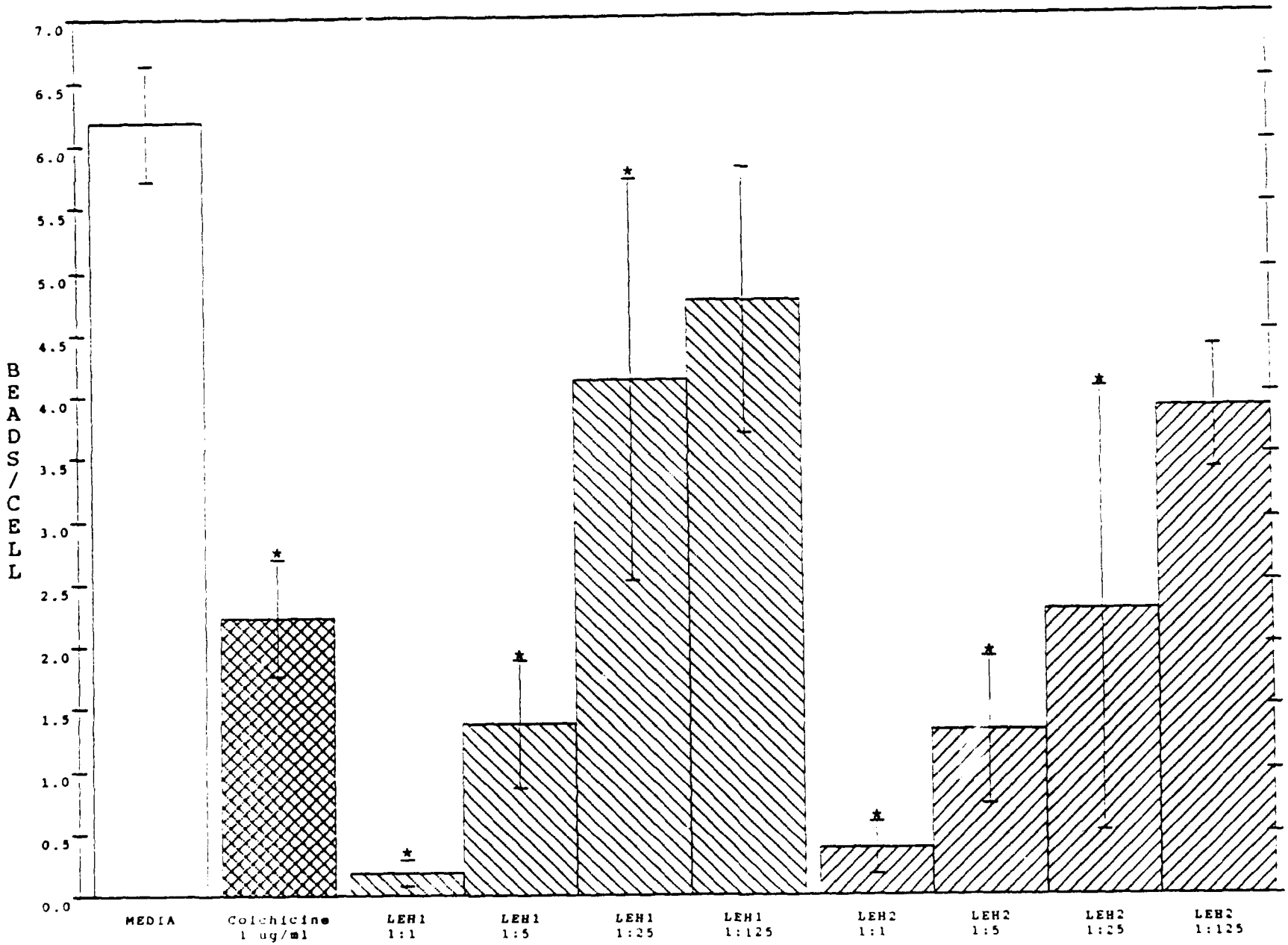
RAT ALVEOLAR MACROPHAGE PHAGOCYTOSIS OF LATEX BEADS



# "STEALTH" LIPOSOME-ENCAPSULATED HEMOGLOBIN

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## ABSTRACT

This study encompasses the further development, characterization and efficacy in life support obtained for hemoglobin encapsulated within lipid vesicles (LEH). "Stealth" phospholipid (polyethyleneglycol distearoylphosphatidylethanolamine), egg PC, cholesterol, and alpha-tocopherol are used to form LEH; results are compared to conventional liposomes, which are prepared with phosphatidyl inositol in place of the "Stealth" phospholipid. Met-Hb generation accompanying LEH processing (below 10°C) and 1 month storage (-20°C) appeared to be small with only a 3% and less than a 10% increase for encapsulated over precursor, respectively. Oxygen affinity and cooperativity and steady shear viscosity values for LEH suspensions (30% by volume) appeared to be near the normal values expected for whole blood. Incubation in plasma at 37°C resulted in only a small amount of Hb release from LEH, i.e. 0.5% or less; also shear had very little effect on causing Hb leakage from LEH. Circulation half-life following 50% isovolemic exchange-transfusion in rats with LEH was about 20 hrs; 97% exchange-transfusion demonstrated efficacy in life support.

KEY WORDS: polyethyleneglycol distearoylphosphatidylethanolamine, liposome-encapsulated hemoglobin, blood, mononuclear phagocyte system, viscosity, shear

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## 1. INTRODUCTION

There is an urgent need for a universally transfusable (nonallergenic), oxygen-carrying blood replacement fluid that can be used in emergency situations to provide temporary life support until an adequate supply of whole blood becomes available. Numerous approaches have been taken in the attempt to develop materials which can deliver oxygen effectively and are safe for use as a red blood cell substitute; liposome-encapsulated Hb (LEH) has been developed more recently [1-9]. Liposome technology provides a mechanism for encapsulation and in vivo delivery of drugs, proteins, etc. which probably would otherwise be degraded, cleared rapidly, or toxic to the host.

The overall goal of our work is the development of a safe, efficacious and economically viable oxygen-carrying red blood cell substitute composed of hemoglobin solution encapsulated in a liposome. In these studies "Stealth" liposomes (liposomes containing polyethyleneglycol distearoylphosphatidylethanolamine, PEG-DSPE) that ARE designed to evade recognition and rapid uptake by the mononuclear phagocyte system (i.e. the reticuloendothelial system (RES) [10-14] are used. Although hemoglobin encapsulated in more conventional liposomes, such as those containing phosphatidylinositol [15], have been shown to provide an effective means of oxygen delivery in vitro and in experimental animals [7,16-18], our recent experiments [19] suggest that such LEH systems, by overloading the RES [20,21], cause alterations in phagocytic activity and increase host susceptibility to infectious challenge [22]. Our recent results with "Stealth" LEH suggest that they are less immunotoxic as they cause less adverse effects when treated animals were tested by infectious challenge [19]. This study encompasses the further development, characterization and efficacy in life support of LEH using "stealth" lipids.

## 2. Materials and Methods

Human stroma-free hemoglobin solutions were prepared at 4 ℃ following aseptic techniques as described elsewhere [3,5]. All equipment used in processing Hb and LEH is depyrogenated and all water used (for example in the washing and lysing steps and in the preparation of phosphate-buffered saline) was bacteria and pyrogen-free. To maximize oxygen-carrying capacity high concentrations of Hb solution up to 35 g% (i.e. 35 grams Hb per 100 ml solution) were prepared.

Pyridoxal-5-phosphate (P-5-P) (Sigma Chemical Co.) was added to the hemoglobin solutions to control oxygen affinity of the LEH to a value similar to that of fresh red blood cells. The antioxidant catalase, which acts as a scavenger of free radicals, was added to the Hb solution [23]. Also it is known that other constituents of the red blood cell hemolysate from which the hemoglobin solution is prepared protect against oxidation of hemoglobin and phospholipid [23].

The membrane lipids used to encapsulate Hb solution included hydrogenated soy or egg phosphatidylcholine (Soy-PC or Egg-PC), cholesterol (CHOL), phosphatidylinositol (PI) or the "stealth" phospholipid polyethylene glycol distearoyl phosphatidyl ethanolamine (PEG-PE), and α-tocopherol (α-Tc) to make conventional (C-LEH) and "Stealth" (S-LEH) liposomes, respectively. Soy-PC, Egg-PC (IV-40) and PEG-PE were a gift from Liposome Technology Inc. (Menlo Park, CA). The rest of the lipids were obtained from Sigma Chemical Co. (St. Louis, MO). All these materials were used as obtained without further purification. The liposome membrane was formulated to contain the lipid molar ratios for Soy-PC or Egg-PC:CHOL:PI or PEG-PE:α-T of 1.0:1.0:0.1:0.02. The LEH were prepared at a lipid to Hb loading of about 150 μmols per ml of precursor Hb solution.

Aseptic techniques were followed in the preparation of LEH. Double emulsion methods were used for preparation of LEH [18]. These include formation of a water-in-oil-in-water type multiple emulsion [24] using egg PC (IV-40) and cholesterol as primary and secondary emulsion surfactants, respectively, followed by organic solvent removal in a rotary evaporator operating under partial vacuum. As the organic solvents are removed, LEH spontaneously form in the excess lipid system. The evaporation procedure is continued until dryness using a single stage vacuum pump to maximize removal of all organic solvent and water so that the Hb concentration within the LEH is as high as possible. This results in the deposition of anapparently dry Hb/LEH film on the walls of the round bottom flask. Concentrated Hb solution is then added under agitation to rehydrate and resuspend the LEH. Homogenization and size reduction of the LEH in a Microfluidizer follows. The LEH so formed were washed at least three times in isotonic PBS and centrifuged at 30,000 x g for 30 mins to remove all unencapsulated Hb solution and any remaining organic solvent (if any). Final processing of the LEH samples was performed so that ultimately the LEH could be made to pass (using a 400cc, 76mm diameter pressure filtration cell, Nucleopore, Plesantville, CA) through a 0.45μ sterilizing filter. Prior to addition of the LEH sample to the filtration cell, it was first prefiltered and then filtered under vacuum through 5, 3, and 1μ CF filters (Nucleopore). The resulting LEH sample was then reloaded into the pressure cell and using nitrogen as the pressure source (at pressures well below 100 psi) it was filtered through 0.8, 0.6 and 0.45μ CF filters. If desired all filtration steps can be done in the filtration cell

Encapsulated Hb concentration was determined by dissolving the liposome membrane with n-Octyl β-D-glucopyranoside detergent solution as described elsewhere [3,18]. Then the resulting Hb solution concentration was measured for oxy, reduced and met-Hb components by the method of Benesch et al. [25], modified using the extinction coefficient values provided by Van Assendelft and Zijlstra [26]. Oxygen content in terms of ml $O_2$ per ml of the LEH suspension sample was determined using a Lex-$O_2$-Con (Hospex Fiberoptics, Chestnut Hill, MA) [27].

Steady shear viscosity of the suspension samples was measured in a uniform shear field with a Wells-Brookfield Syncro-Lectric Microviscometer (Model LVT) equipped with a 0.80° cone (Model CP-40, Soughton, MA). Shear rates from 45 to 450 $s^{-1}$ at 37°C were evaluated. The cone-and-plate geometry is very useful as it gives a good approximation of viscometric flow with constant shear rate throughout the flow field [28]. The fragility of the LEH to shear, i.e. leakage of Hb, was evaluated by shearing the LEH samples for 30 mins in the viscometer as a function of shear rate. The effect of shear rate on leakage of encapsulated Hb was obtained for freshly prepared LEH samples in either 7.5 g% egg albumin/PBS or human plasma, both at 30% by volume. Following centrifugation (13,600 x g, Microcentrifuge Model 235C, Fisher Scientific) the concentration of total Hb in the supernatant of the sheared sample was compared with that of the unsheared sample. The benzidine method [29] was used for determining plasma Hb concentration at low concentration levels (i.e., in the mg/dl range) as a result of leakage of Hb from liposomes. It is known to be accurate down to concentrations of 1 mg/dl. The effect of 1 day incubation at 37° on leakage of encapsulated Hb was obtained for freshly prepared LEH samples in either 7.5 g% egg albumin/PBS or human plasma (both at 30% by volume LEH). Also the effect of cholesterol content in the liposome membrane on leakage of encapsulated Hb was evaluated. The benzidine method was also used for determination of plasma Hb concentration.

The Oxy-hemoglobin equilibrium dissociation and association curves for the various LEH samples, measured as a function of oxygen partial pressure, were obtained using a Hemox-Analyzer (TCS Medical Products Co., Huntingdon Valley, PA). Oxygen affinity ($P_{50}$) and cooperativity (Hill exponent n) were determined from the generated curves.

Circulation half-life of LEH and efficacy in life support was evaluated on unconscious rats in Illinois Institute of Technology's Small Animal Lab. Female rats (Harlan Sprague, Indianapolis, IN) weighing 225 to 275g (8 to 12 weeks of age) were used. Cannulation of the femoral artery and vein was carried out based on the model developed by Keipert and Chang [30]. Using a doubly cannulated rat and a peristaltic pump (Manostat, New York, NY), exchange transfusions were performed by removing blood at a constant rate of about 0.2 ml/min, coupled with its simultaneous isovolemic replacement with either LEH suspension or control. The decrease in hematocrit levels was recorded during the exchange-transfusion.

## 3. RESULTS AND DISCUSSION

Resulting S-LEH encapsulated Hb concentrations of about 25 g% were greater than 80% of precursor Hb solution concentration. LEH processing resulted in encapsulation efficiencies of about 50%. For example, Hb and

lipid (i.e. total lipid including cholesterol) in the "Stealth" LEH formulations were loaded at about 3.1 μmoles per 150 μmoles, respectively and resulted in LEH containing 0.9 μmoles encapsulated Hb per 82 μmoles of total lipid. These results were determined from a total phosphorous analysis in conjuction with Bligh-Dyer extraction on 1 ml samples of "Stealth" LEH at a lipocrit of 30% containing an encapsulated Hb solution concentration of 20 g%. The phosporous analysis was performed at LTI's quality control laboratory. Met-Hb generation accompanying LEH processing appeared to be small with only a 3% increase for encapsulated over precursor. These results correspond to an oxygen content for an LEH suspension sample (50% by volume LEH) of 15 volume% oxygen. Storage of the LEH for up to one month at -20°C resulted in percent met-Hb concentrations to levels of about 9%. Additions of various components to the lipid phase of LEH systems appeared to reduce the oxidative interactions between hemoglobin and membrane lipid. Some of these included the addition of cholesterol to the membrane phase to protect Hb from oxidation [31,32]. Also oxidation of Hb to met-Hb may have been inhibited by using saturated PC instead of unsaturated PC [3].

The size of S-LEH are expected to be similar to that reported in a previous study [18] in which thin-section electron micrographs of C-LEH made using double emulsion processing and passed 10 times through a Microfluidizer™ resulted in a particle size range of a 3μ filtered sample from 50 nm to a little greater than 1μ with a median particle size of about 300 to 400 nm. The oxygen saturation curve obtained for C-LEH or S-LEH, the precursor Hb solution, and a whole blood sample using a Hemox Analyzer is shown in Figure 1. Oxygen affinity (based on $P_{50}$) and cooperativity (as characterized by the Hill coefficient) for S-LEH suspensions appeared to be near the normal values seen for whole blood.

Steady shear viscosity results were obtained for S-LEH suspension samples (in PBS containing 7.5 g% albumin at isooncotic levels) for shear rates to about 500 $s^{-1}$. The viscosity results for both C-LEH and S-LEH suspension samples (both prepared at 50% lipocrit) were higher than those obtained for human and rat whole blood (both at 45-46% hematocrit), however viscosity results obtained for LEH suspension samples of 30% lipocrit were slightly lower than that measured for the whole blood samples. All measurements were made at 37°C. The effect of shear rate on leakage of encapsulated Hb was obtained for freshly prepared LEH samples in either 7.5 g% egg albumin/PBS or human plasma, both at 30% by volume. Although leakage was highest for the C-LEH and S-LEH samples suspended in plasma, only 0.5% leakage of the encapsulated Hb was measured at the highest shear rate value tested, i.e. about 500 $s^{-1}$.

The effect of storage of either C-LEH or S-LEH at 37°C on leakage of encapsulated Hb was obtained for freshly prepared LEH samples in either 7.5 g% egg albumin/PBS or human plasma (both at 30% by volume LEH). Again, less than 0.5% leakage of the encapsulated Hb was observed for either LEH sample in plasma even after 24 hours of incubation (see Figure 2a). As shown in Figure 2b, cholesterol content lower than that used in the standard formulation, which is a 1:1 molar ratio of cholesterol to egg-PC, resulted in substantial leakage of Hb from LEH into 7.5% egg albumin/PBS. For example, a molar ratio of 0.4:1.0 is accompanied by 35% leakage of hemoglobin. It is well known that high cholesterol content in the membrane reduces membrane permeability in serum and plasma [33].

Circulation half-life following 50% isovolemic exchange-transfusion typically was about 15 to 20 hrs for both of the C-LEH and S-LEH samples tested (see Figure 3). These times are desirably long and compare very favorably to results reported in another study for a similar phospholipid-lipid formulation [5]. Also, high cholesterol content in the membrane, as is the case for both C-LEH and S-LEH formulations, prolongs liposome stability in circulation [33]. Other recent studies have also found that the "stealth" lipid, PEG-PE, significantly increased the blood circulation time of liposomes formulated with it [10-14,34]. Also those studies have shown "stealth" liposomes to have greatly decreased uptake by the reticuloendothelial system and thus enhanced circulation time compared to many other conventional phospholipids formulations. A possible explanation for this behavior is that the PEG polymer sterically stabilizes the LEH [34,35] like that previously reported for ionic surfactant coating of colloidal particles [36]. This results in "limited accessibility" [13] of the liposome surface to adsorption by plasma proteins [37] such as immunoglobulins and high density lipoproteins, which could cause vesicular breakdown [38] and opsonization followed by RES uptake [12].

Recently it was found that replacement of conventional lipids in LEH with "Stealth" lipids may provide a mechanism for in vivo oxygen delivery with less adverse impact on host resistance and immunity [19]. It was also found that both conventional and "Stealth" LEH caused significant reduction of phagocytic activity [19]. Yet, as shown in this study both types of LEH have approximately the same clearance rates. Therefore, it appears that prediction of impact on host resistance should not be based solely on in vitro tests of the effect of test material on phagocytosis.

As shown in Figure 4, nearly total (97%) isovolemic exchange transfusion demonstrates efficacy of S-LEH suspension samples, since administration of LEH supported life in rats whose hematocrit had been reduced to levels below 5% which are incompatible with survival when performing exchange transfusion with isotonic/isooncotic PBS containing 7.5 g% albumin. Note that exchange transfusion using the hypotonic Hb control solution (7.5 g% stroma-

free Hb) was not lethal even at 0% hematocrit. These results concerning efficacy of LEH are consistent with those found in other recent studies for terminal hematocrit obtained for control and LEH-exchanged animals [7,16,17].

**ACKNOWLEDGEMENTS**

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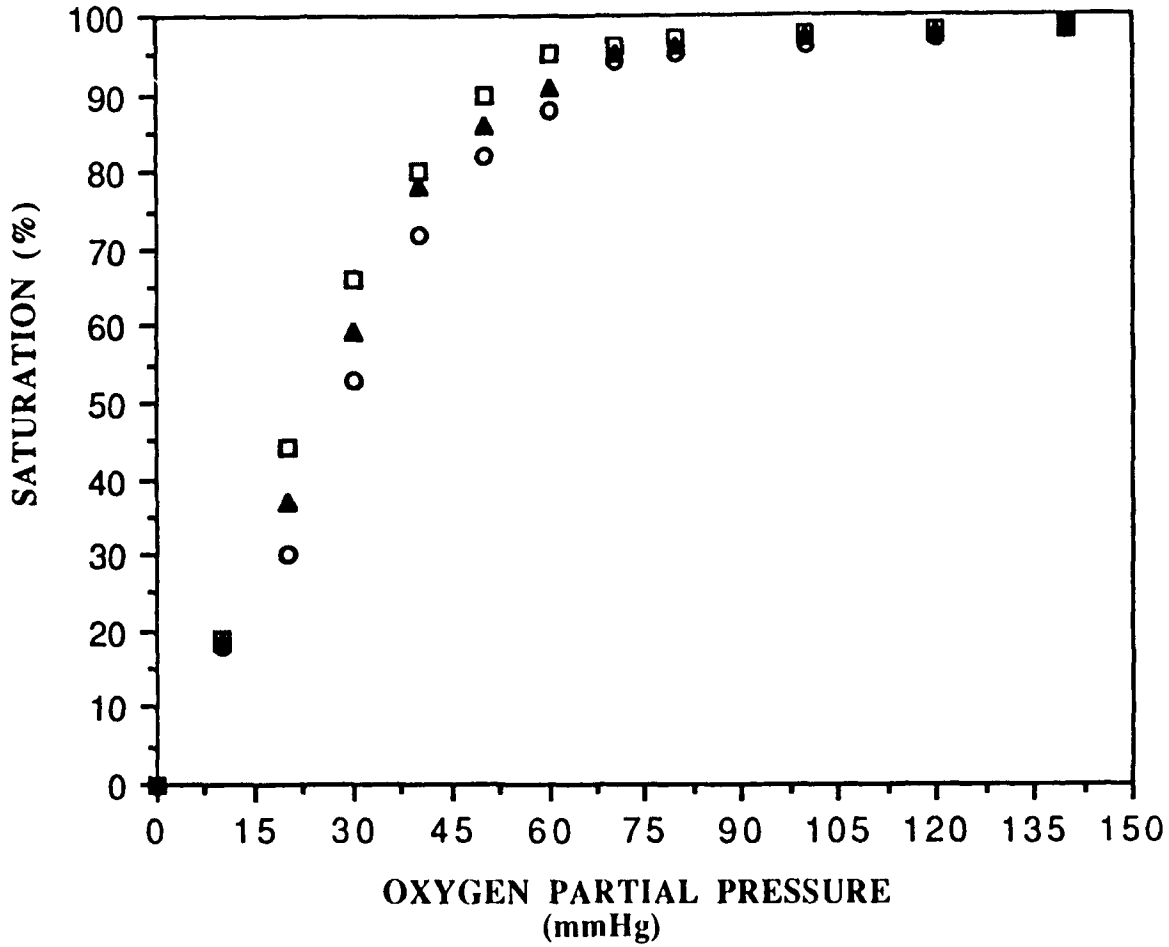
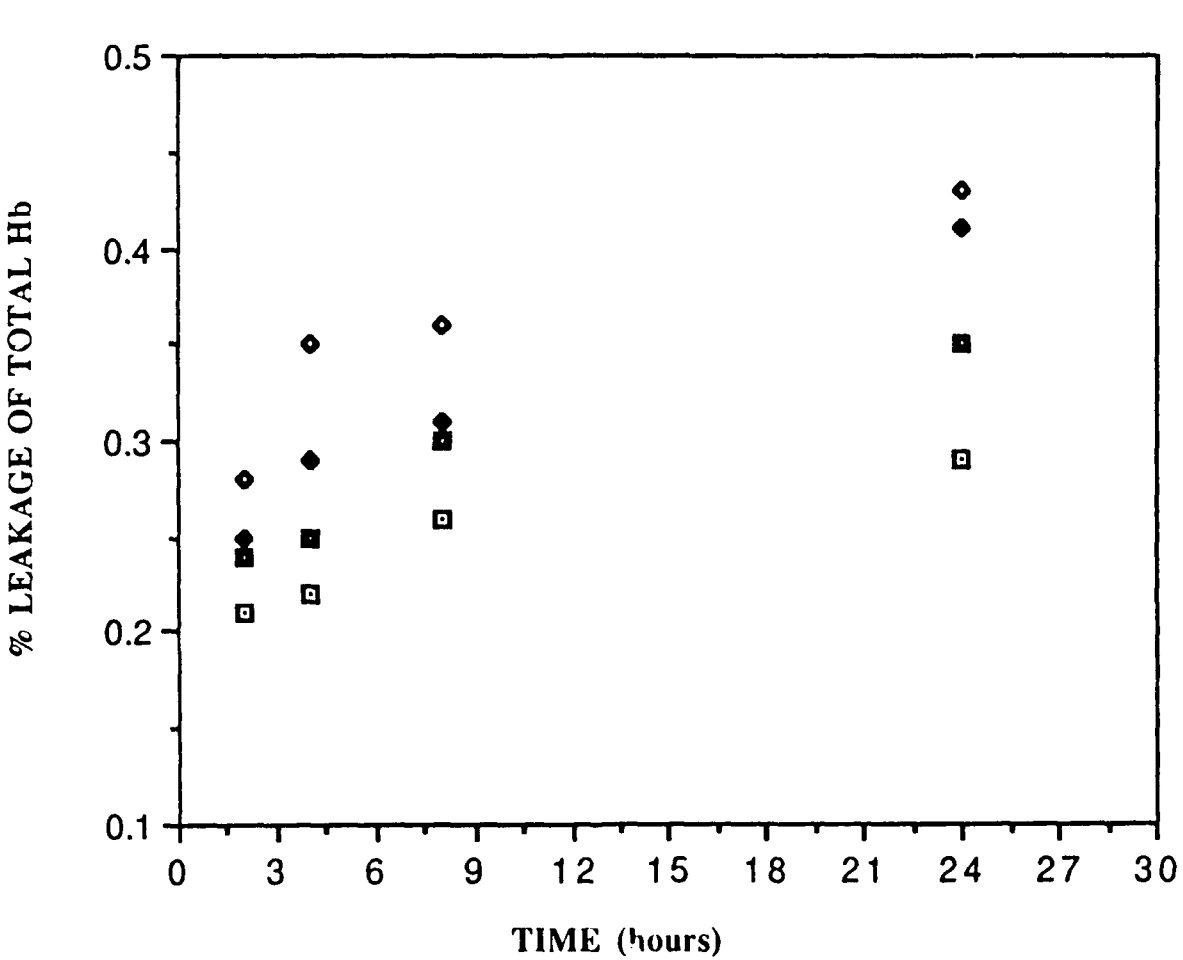
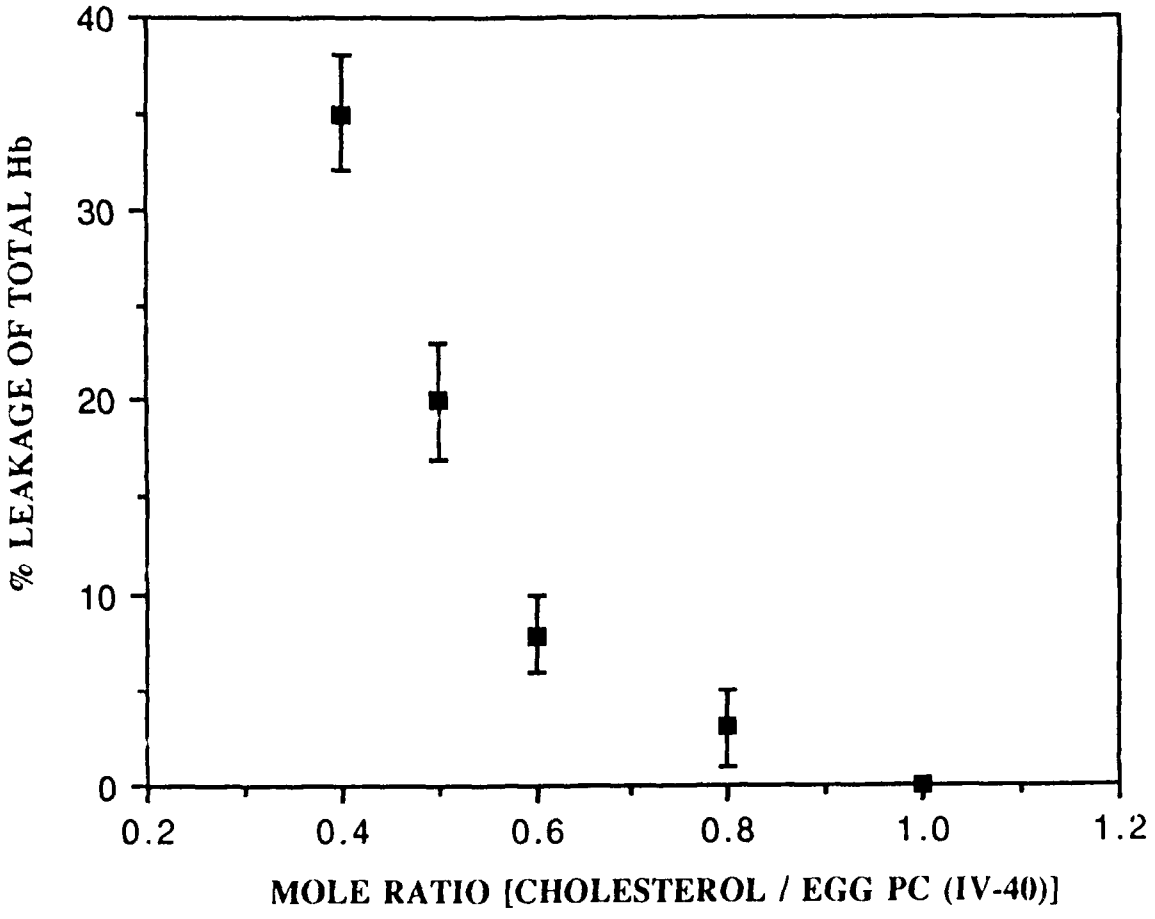
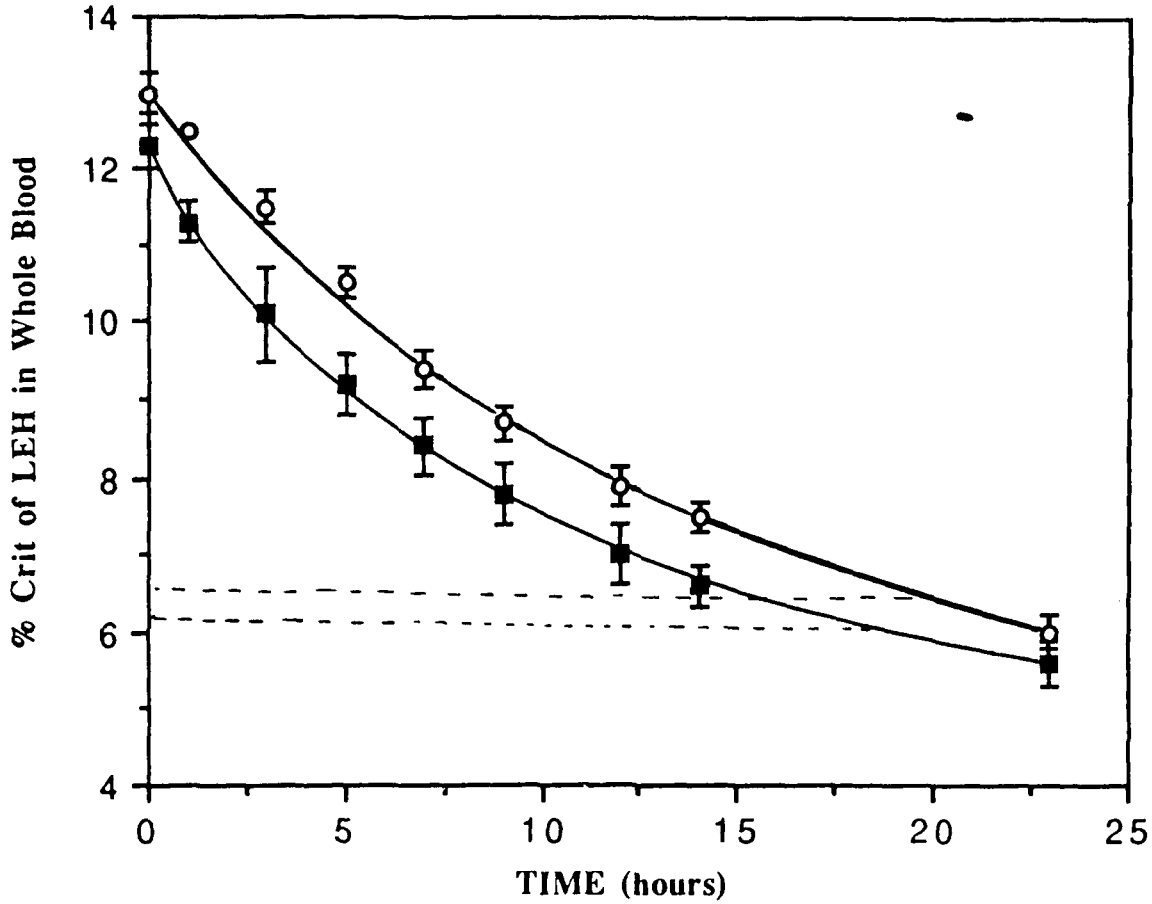
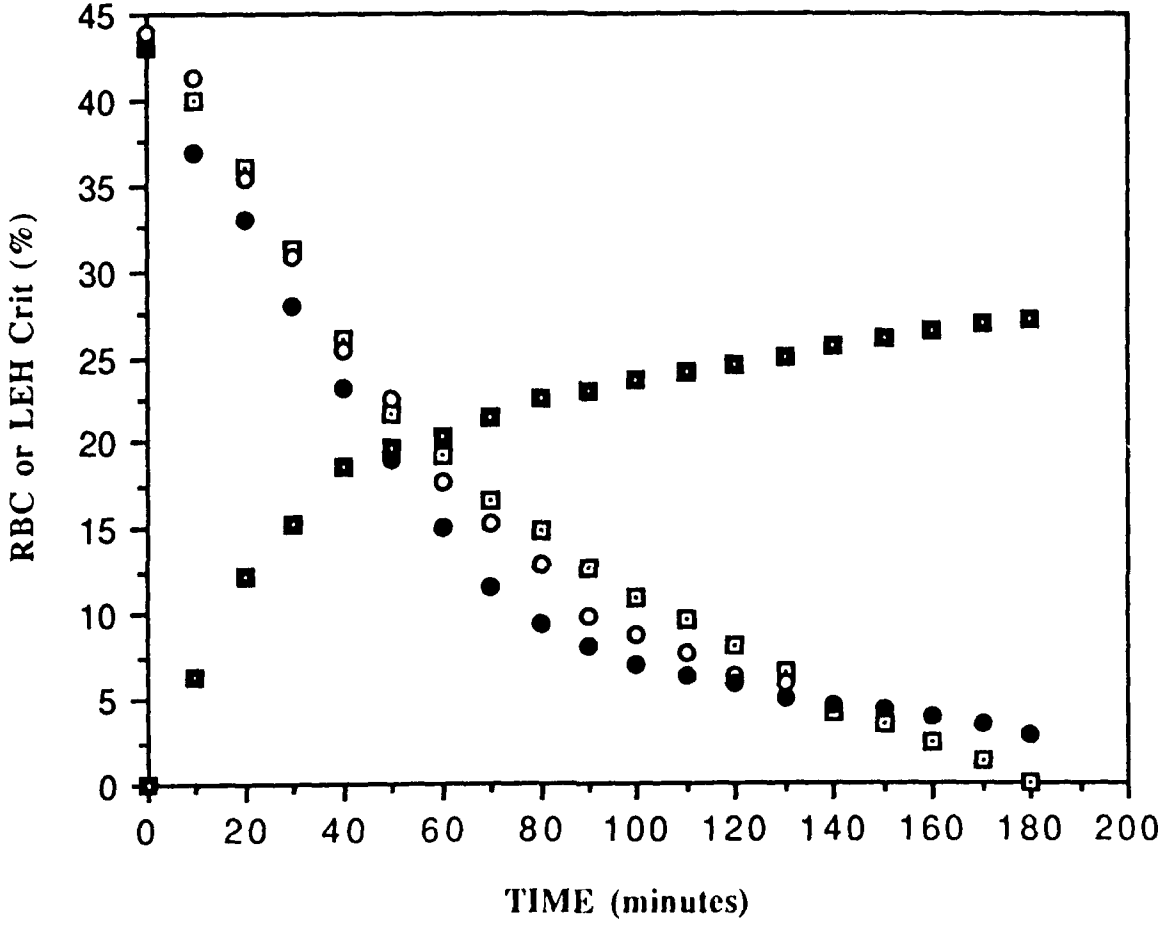
## LIST OF FIGURES


SATURATION (%)
100
90
80
70
60
50
40
30
20
10
0
0
15
30
45
60
75
90
105
120
135
150
OXYGEN PARTIAL PRESSURE
(mmHg)


0.5
0.4
0.3
0.2
0.1
% LEAKAGE OF TOTAL Hb
0
3
6
9
12
15
18
21
24
27
30
TIME (hours)


40
30
20
10
0
0.2
0.4
0.6
0.8
1.0
1.2
% LEAKAGE OF TOTAL Hb
MOLE RATIO [CHOLESTEROL / EGG PC (IV-40)]


% Crit of LEH in Whole Blood
14
12
10
8
6
4
0
5
10
15
20
25
TIME (hours)


RBC or LEH Crit (%)
45
40
35
30
25
20
15
10
5
0
0
20
40
60
80
100
120
140
160
180
200
TIME (minutes)

# LIPOSOME-ENCAPSULATED HEMOGLOBIN PROCESSING METHODS

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## ABSTRACT

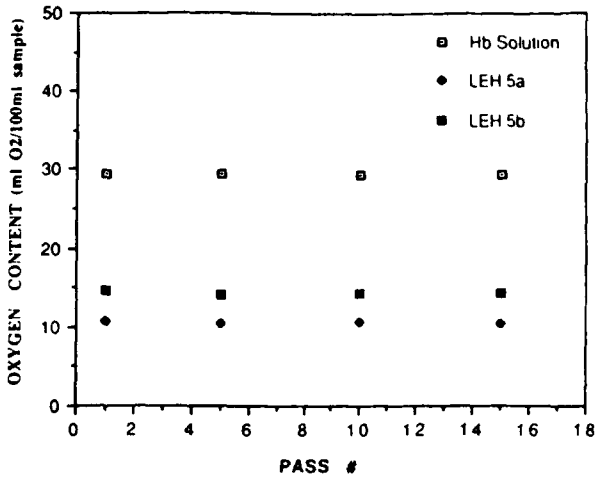
An effective and safe red blood cell substitute is being developed based on double emulsion/evaporation techniques followed by high pressure homogenization to form liposome-encapsulated hemoglobin (LEH). Formulations are made up of hydrogenated phosphatidylcholine (PC, soy or egg), cholesterol, phosphatidylinositol (PI), and α-tocopherol in a molar ratio of 1:1:0.2:0.02, respectively. Resulting LEH-encapsulated hemoglobin (Hb) concentrations are greater than 80% of precursor Hb solutions. Met-Hb generation accompanying LEH processing appears to be small with only a 3% increase for encapsulated over precursor. These results correspond to an oxygen content for an LEH suspension sample (50% by volume LEH) of 15 volume% oxygen. Oxygen affinity and cooperativity values for LEH suspensions appear to be near the normal values expected for whole blood. The viscosity of LEH suspension samples (50% by volume LEH in phosphate-buffered saline containing 7.5 wt% albumin) were slightly higher than that of whole blood. The effect of shear rate on leakage of encapsulated Hb from LEH was small, i.e. 0.5% or less. Nearly total isovolemic exchange transfusion using a cannulated rat model demonstrates efficacy of LEH suspension samples. There appears to be no difference in rat internal organ weights between rats exchanged with control compared to rats exchanged with LEH. Circulation half-life following 50% isovolemic exchange-transfusion is about 15 to 18 hours.

## INTRODUCTION

Numerous approaches have been taken toward the development of a blood replacement fluid resulting in three classes of synthetic oxygen-carrying blood substitutes: 1) perfluorochemical emulsions [1-9]; 2) hemoglobin (Hb) free in solution [10-19]; and more recently 3) encapsulated Hb that involves various techniques for liposome and even emulsion-based encapsulation of Hb ([20-34]. By encapsulation of Hb in phospholipid vesicles, i.e. liposome-encapsulated Hb (LEH), the toxic effects which accompany the use of some of these blood substitutes may be eliminated [35]. LEH formulations can provide reasonable stability of encapsulated Hb at room temperature by inclusion of antioxidants; longer retention times in circulation by adjustment of the phospholipid formulation; normal COP with large oxygen carrying capacity, low oxygen affinity, and cooperativity; and adequate oxygen/carbon dioxide mass transport. Still, clinical concerns with respect to cardio-vascular infusion of LEH exist. These include tissue/organ toxicity and immunotoxicity [6,7], effect on coagulation system and platelets [36-41], and effect on reticuloendothelial (i.e. the mononuclear phagocyte)/host defense system [42-44]. This study is concerned with the development of an effective and safe red blood cell (RBC) substitute using double emulsion/evaporation techniques to form multilamellar vesicles followed by reduction in size by Microfluidization to form LEH. Various in-vitro and in-vivo (rats) properties of the LEH are evaluated.

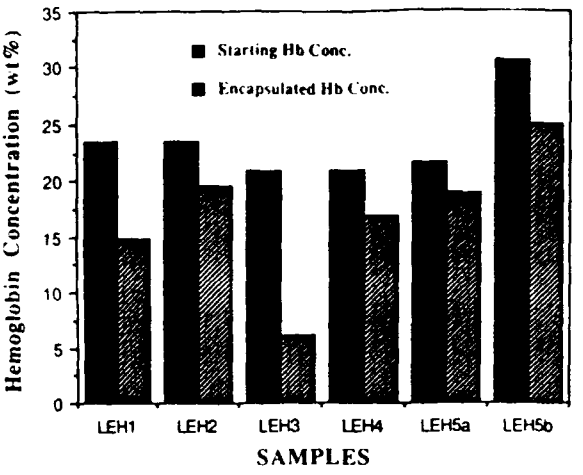
## MATERIALS

Human stroma-free hemoglobin solutions were prepared at about 4 °C following aseptic techniques. All equipment used in the processing was depyrogenated and all water used (for example in the washing and lysing steps and in the preparation of phosphate-buffered saline) was bacteria and pyrogen-free. To maximize oxygen-carrying




**Effect of Number of Microfluidizer (MFZ) Passes on Oxygen Content at 4 °C for Hb Solution and LEH samples**

**FIGURE 1.**




**Starting and Encapsulated Hb Concentration of LEH Samples**

**FIGURE 2.**

capacity high concentrations of Hb solution up to 35 g% (i.e. 35 grams Hb per 100 ml solution) were prepared via a multistep procedure similar to that as has been described previously [22,24,26]. The Hb solution was stored either frozen (-20°C) or at 4 °C until use.

In these studies pyridoxal-5-phosphate (P-5-P) (Sigma Chemical Co.) was added to the hemoglobin solutions to control oxygen affinity of the LEH to a value similar to that of fresh red blood cells. The antioxidant catalase was added to the Hb solution. Also, other constituents of the red blood cell hemolysate from which the hemoglobin solution was prepared appear to protect against oxidation of hemoglobin and phospholipid [45].

The membrane lipids used to encapsulate Hb solution included hydrogenated soy or egg phosphatidylcholine (Soy-PC or Egg-PC), cholesterol (CHOL), phosphatidylinositol (PI) and α-tocopherol (α-T). Hydrogenated Soy-PC was obtained from the American Lecithin Co. (Atlanta, GA) and hydrogenated Egg-PC (IV-40) was supplied by Liposome Technology Inc. (Menlo Park, CA). The rest of the lipids were obtained from Sigma Chemical Co. (St. Louis, MO). All these materials were used as obtained without further purification. The liposomal membrane was formulated to contain lipid molar ratios for Soy-PC or Egg-PC:CHOL:PI:α-T of 1.0:1.0:0.2:0.02, respectively. Phosphatidylinositol was chosen as the negatively charged phospholipid because it has been found that its inclusion in certain formulations results in a liposome membrane with significantly enhanced circulation time [46]. The LEH were prepared at a lipid to Hb loading of about 150 μmols per ml of precursor Hb solution (from 20 to 30 g%).

## METHODS

Aseptic techniques were followed in the preparation of LEH; all equipment used in the processing was depyrogenated and all water used was bacteria and pyrogen-free. Three methods were used for producing liposome-encapsulated hemoglobin (LEH): film hydration, reverse evaporation and double emulsion.

**Film hydration** following procedures as fully described elsewhere [24,26] was used to form multilamellar vesicles, which were reduced in size by a Microfluidizer™ M110 (Microfluidics, Newton, Ma). Most recently we have modified this process (prior to Microfluidization) by rotary evaporation (operating under partial vacuum at room temperature) of as much water as possible from the formed LEH in Hb solution. This resulted in a somewhat dry Hb/LEH film that deposited on the walls of a round bottom flask. Hb solution was added to rehydrate and resuspend the LEH. The Microfluidization processing and washing procedures at 5 °C to obtain the LEH were as described recently [24,26]. The washed liposomes were then resuspended in isotonic PBS containing 7.5 g% egg albumin.

**Reverse-phase evaporation** methods [30,47] were modified as described below to form LEH. Sonication was not used as it damages the hemoglobin. The approach used in this study involved formation of an emulsion of 30 ml aqueous concentrated Hb solution in an organic solvent mixture of 20 ml diethyl ether and 12 ml trichlorotrifluoroethane, which contained in dissolved form the formulated phospholipids/lipids. Organic solvents were

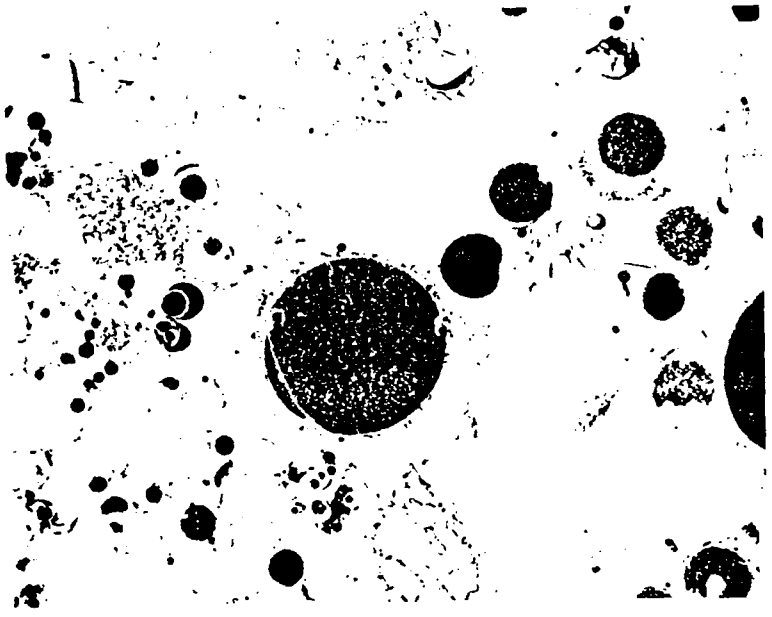


**FIGURE 3. Thin-section electron micrograph for LEH passed 10 times through the Microfluidizer™.**

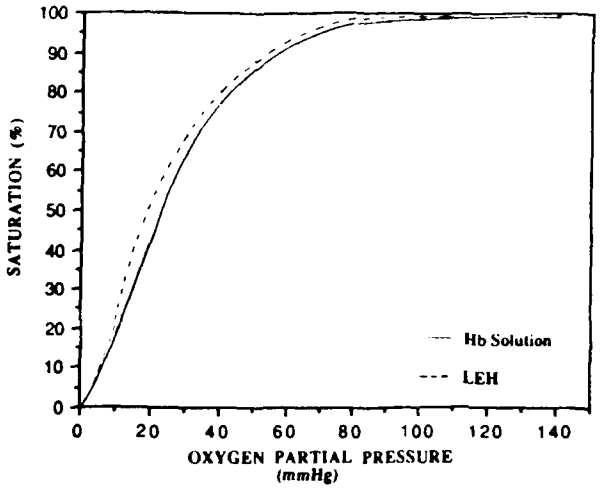
slowly evaporated at room temperature in a rotary evaporator operating under partial vacuum. As the organic solvents were removed, LEH spontaneously formed in the excess lipid system. The evaporation procedure was continued until dryness to maximize removal of all organic solvent and water so that the Hb concentration within the LEH was as high as possible. This resulted in the deposition of an apparently dry Hb/LEH film on the walls of the round bottom flask. Concentrated Hb solution was then added under agitation to rehydrate and resuspend the LEH. Microfluidization, washing and resuspension procedures used for LEH were the same as described above for film hydration.

**Double emulsion** methods included formation of a water-in-oil-in-water type multiple emulsion [48] followed by organic solvent removal and then size reduction to form LEH. A primary emulsion was formed of Hb solution droplets dispersed in an organic solvent blend containing in dissolved form the formulated lipids/phospholipids. Components and compositions are exactly the same as used above for reverse evaporation. The primary emulsion was then well-dispersed into precursor Hb solution to form the double emulsion. The remaining steps in the process to form LEH that involve organic solvent removal, microfluidization, and washing are similar with that reported above for the reverse evaporation method. Thin-section electron micrographs of the LEH were obtained with a Philips model 201 electron microscope.

The encapsulated Hb concentration was determined by dissolving the liposomal membrane with n-Octyl β-D-glucopyranoside detergent solution [22] and then measuring the resulting Hb solution concentration for oxy, reduced and met-Hb components. A 0.1 ml volume of LEH sample (at a lipocrit of about 30%) was dissolved in 5 ml of concentrated detergent solution (30 mg/ml). The resultant material was stirred for 40-45 mins at room temperature, until the liposome membrane dissolved (or formed micelles) and the solution lost all its turbidity. The solution was then filtered through a 0.1 micron filter. The quality of the encapsulated Hb as a function of storage (4°C) time was determined by measuring the met-Hb concentration in the LEH stored sample.

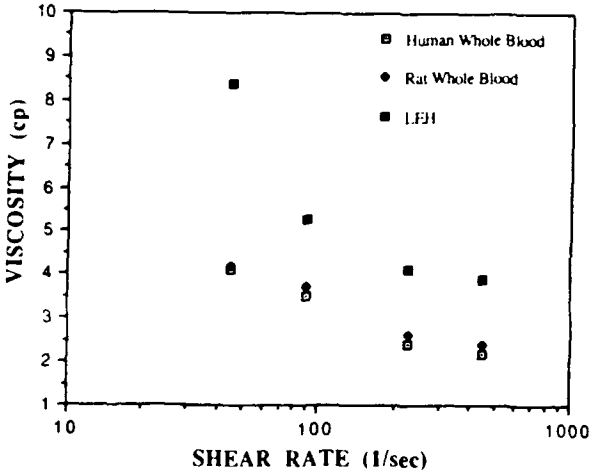
Oxy, reduced and met-Hb concentrations in a given Hb solution were determined by the method of Benesch et al. [49], modified by using the extinction coefficient values provided by Van Assendelft and Zijlstra [50]. A sensitive method that was used for determining Hb solution concentration at low concentration levels (i.e., in the mg/dl range) is the benzidine method [51] and was used to determine plasma Hb concentration in the studies on leakage of Hb from liposomes. It is known to be accurate up to ! mg/dl.

Steady shear viscosity of the suspension samples was measured in a uniform shear field with a Wells-Brookfield Syncro-Lectric Microviscometer (Model LVT) equipped with a 0.80° cone (Model CP-40, Soughton, MA). Shear rates from 45 to 450 $s^{-1}$ at 37°C were evaluated. The cone-and-plate geometry is very useful as it gives a good approximation of viscometric flow with constant shear rate throughout the flow field [52]. The fragility of the LEH, i.e. leakage of Hb, was evaluated by shearing the LEH samples for 10 mins in the viscometer as a function of shear rate. Following mild centrifugation (150 x g) the concentration of total Hb in the supernatant of the sheared sample




Oxygen Dissociation Curve of Starting Hb Solution and LEH

FIGURE 4.




Viscosity of LEH (50% Crit), Rat Whole Blood (46% Crit) and Human Whole Blood (45% Crit).

FIGURE 5.

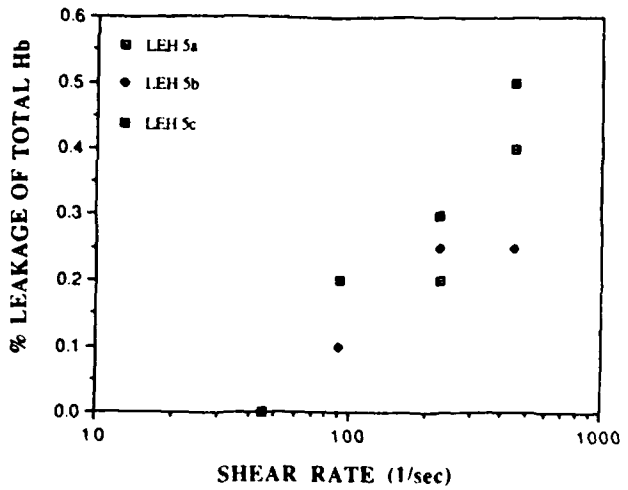
was compared with that of the unsheared sample.

Efficacy and circulation half-life of LEH was evaluated on unconscious rats in Illinois Institute of Technology's Small Animal Lab. Female rats (Harlan Sprague, Indianapolis, IN) weighing 225 to 275g (8 to 12 weeks of age) were used. Cannulation of the femoral artery and vein was carried out based on the model developed by Keipert and Chang [53]. Using a doubly cannulated rat and a peristaltic pump (Manostat, New York, NY), exchange transfusions were performed by removing blood at a constant rate of about 0.2 ml/min, coupled with its simultaneous isovolemic replacement with either LEH suspension or control. The decrease in hematocrit levels was recorded during the exchange-transfusion.

## RESULTS AND DISCUSSION

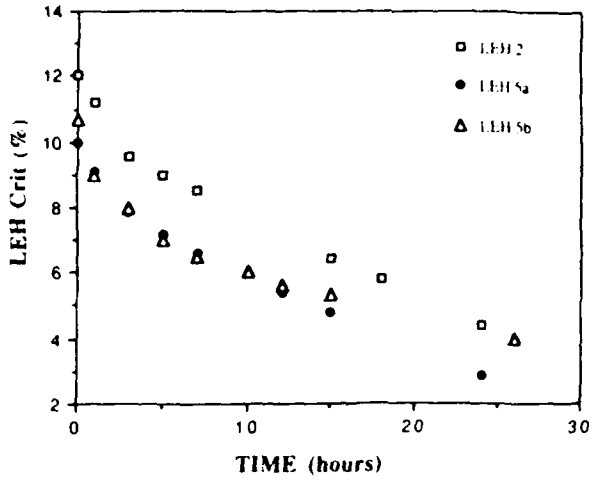
Results obtained in this study suggest that Microfluidizer™ processing at temperatures below 9°C (preferably at 4°C), have little or no effect on met-Hb generation. Oxygen content, which is primarily a measure of oxy-Hb concentration, was determined using a Lex-$O_2$-Con. Neither an oxygen content reduction in precursor Hb solution or in LEH encapsulated Hb solution was found as either material was passed up to 15 times through the Microfluidizer™ (see Figure 1). However, oxygen content was found to be reduced when processing temperatures were above 10°C. This most likely represents generation of met-Hb. Encapsulated met-Hb concentrations of from about 2 to 5% of the total Hb concentration were obtained when all processing steps in the formation of the LEH product were taken in account. Storage of the LEH for up to one month at -20°C resulted in percent met-Hb concentrations to levels of about 9%. Although catalase had been added to the Hb solution encapsulated in the LEH, results have been reported recently that suggest that the addition of glutathione and NADH results in reduction of met-Hb LEH concentration during storage at 4°C [54]. Our future studies will look into this Hb stabilizing feature.

We have evaluated three general methods for producing LEH. These include film hydration followed by size reduction using a Microfluidizer™ M110 (LEH 3 is unmodified, LEH 4 is modified), reverse evaporation (LEH 1 is unmodified, LEH 2 is modified) and double emulsion (LEH 5a uses Soy-PC, LEH 5b uses Egg-PC, and LEH 5c is Soy-PC based and suspended in human plasma instead of 7.5% albumin/PBS). Note that LEH 1, LEH 2, LEH 3 and LEH 4 are made with Soy-PC. Figure 2 shows the resulting encapsulated Hb concentrations for typical batches produced following the various methodologies. LEH made by double emulsion processing resulted in the highest percentage of precursor Hb solution concentration encapsulated, i.e. about 80% for LEH 5b. These results correspond to an oxygen content for an LEH suspension sample (50% by volume LEH) of 15 volume% oxygen. For double emulsion processing Soy-PC or Egg PC appeared to give similar results on the percentage of precursor Hb solution concentration encapsulated, i.e. either were equally good. The remainder of this paper refers to results obtained for double emulsion-produced LEH.




Effect of Shear Rate on Hb Leakage of Freshly Prepared LEH

FIGURE 6.




Half-life of LEH Samples in Rats after 50% Blood Exchange

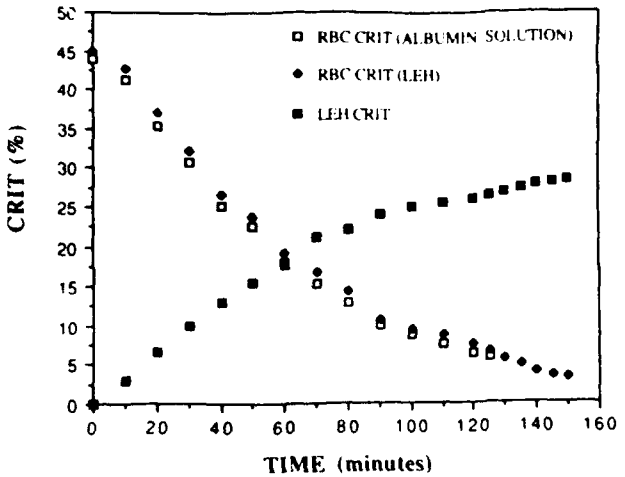
FIGURE 7.

A thin-section electron micrograph for LEH passed 10 times through the Microfluidizer™ is shown in Figure 3 at a magnification of 25,000 times. The particle size range of the 3-micron-filtered sample appears to be from as small as 50 nm to a little greater than 1 micron. However, the average particle size is about 300 to 400 nm. The oxygen saturation curve obtained for LEH and the precursor Hb solution using a Hemox Analyzer is shown in Figure 4. The $P_{50}$ for the LEH and Hb solution samples (both containing P-5-P) are 22 and 19 mm Hg, respectively. Oxygen cooperativity values for LEH appear to be near the normal values expected for whole blood.

Steady shear viscosity results were obtained (see Figure 5) for LEH suspension samples (in PBS containing 7.5 g% albumin at isooncotic levels). The viscosity results of the LEH suspension sample were slightly higher than that of human and rat whole blood (both at comparable hematocrit). Measurements were made at 37°C. The effect of shear rate on leakage of encapsulated Hb was obtained for freshly prepared LEH samples in either 7.5 g% egg albumin/PBS or human plasma, both at 30% by volume LEH (see Figure 6). Although leakage was highest for the LEH sample suspended in plasma, only 0.5% leakage of the encapsulated Hb was measured at the highest shear rate value tested, i.e. about 500 $s^{-1}$. Egg-PC based-LEH suspended in 7.5% albumin/PBS (LEH 5b) showed only 0.25% leakage at the highest shear rate compared to 0.4% for the Soy-PC based-LEH suspension sample.

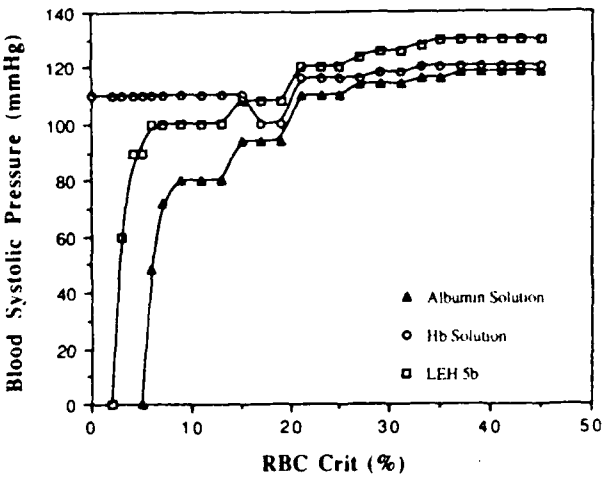
Circulation half-life following 50% isovolemic exchange-transtusion was about 15 to 18 hours for the various LEH samples shown in Figure 7. These times are desirably long and compare very favorably to results reported in another study for a similar phospholipid/lipid formulation [24]. As shown in Figure 8a, nearly total (97%) isovolemic exchange transfusion demonstrates efficacy of the LEH suspension samples, since administration of LEH supported life in rats whose hematocrit had been reduced to levels below 5% which are incompatible with survival when performing exchange transfusion with isotonic/isooncotic PBS containing 7.5 g% albumin. These results concerning efficacy of LEH are consistent with those found in other recent studies for terminal hematocrit obtained for the control-exchanged animal and the LEH-exchanged animal [25,28,30]. The effect of nearly total (97%) isovolemic exchange transfusion of blood with LEH on rat arterial blood pressure is reported in Figure 8b as a function hematocrit dilution. Note that exchange transfusion using the Hb control solution was not lethal even at 0% hematocrit. The corresponding arterial blood pressure was not much reduced from the value at the start of exchange, i.e. 45% hematocrit. Rat internal organs were harvested at terminal crit following exchange with the albumin/PBS control and the LEH suspension sample. There appeared to be no apparent difference in organ weights between rats exchanged with control compared to rats exchanged with LEH.

We are interested in developing LEH formulations with prolonged circulation time, corresponding to slow removal by the reticuloendothelial system along with avoidance of impairment of the host/defense system [55,56]. We are currently investigating the negatively charged phospholipid polyethyleneglycol distearoyl phosphatidylethanolamine. Use of the Microfluidizer™ is expected to permit preparation of adequate quantities of LEH for total and partial blood replacement in larger animals, i.e., not just limited to mice and rats [25,28,30].




Terminal Crit in Rats Exchange with either 7.5 wt% Albumin in PBS or LEH with 7.5 wt% Albumin in Outer Phase

FIGURE 8a.




Effect of Blood Exchange with 7.5 wt% albumin Solution, 7.5 wt% Hb Solution and LEH 5b on Blood Systolic Pressure

FIGURE 8b.

## ACKNOWLEDGMENTS

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Optical measurements on tissue phantoms

**Leonard I. Grossweiner, Linda Ramball Jones, James L. Karagiannes, Joshua Phiri, and Velu Senthil**

High-resolution reflection and transmission spectra were measured for tissue phantoms using an integrating sphere spectrophotometer. The systems studied consisted of light scattering particles in aqueous dyes and gelatin, liposome-encapsulated hemoglobin, and red blood cells. The absorption spectra calculated with the "reduced" 1-F diffusion approximation (1DA) are in approximate agreement with measurements on the separated chromophores. The reduced 1DA scattering spectra show deviations from smooth curves in regions of high absorption, which are attributed to departures of the physical systems from a random particle model. The diffusion lengths calculated with the reduced 1DA model agree with experimental measurements on optically thick layers.

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(s) and the mean cosine of the single particle scattering angle (g). Implementation of the P1 and DE models requires three experimental measurements, typically the total transmission (T) and reflection (R) coefficients and the on-axis transmission. The number of measurements can be reduced to two by transforming the optical constants with "similarity" relations: $g \rightarrow 0$, $k \rightarrow k$, $s \rightarrow s(1 - g) \equiv s'$, where s' is the "reduced" scattering coefficient.[8] The accuracy of this transformation must be evaluated for each case. Monte Carlo (MC) simulation is an alternative approach that has been applied to tissues in recent work.[7,9,10] The lengthy computational times required for useful accuracy limits MC to the "direct" problem of calculating the flux density distribution and macroscopic parameters from input values of k, s, and g.

The 1-D diffusion approximation with the similarity transformation (referred to as "reduced 1DA") was used by Karagiannes *et al.*[11] to calculate k and s' spectra of tissues and tissue phantoms from measurements of R and T. Widely divergent optical constants are reported in the literature for various tissues, which make it difficult to evaluate the accuracy of the results.[6] Tissue phantoms consisting of known absorbers and scatterers provide a better opportunity for evaluation of theoretical models. The present paper reports results obtained with different tissue phantoms, including suspensions of light scattering particles in aqueous dyes and gelatin, liposome-encapsulated hemoglobin derivatives, and red blood cells. The diffusion lengths calculated with the reduced 1DA methodology are compared with experimental measurements on optically thick samples. An unexplained anomaly in our earlier work is the occurrence of deviations in the s' spectra from smooth curves, which are more pronounced for tissue phantoms than tissues.[11] Additional results reported in this paper suggest that the effect originates from departures of the physical

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for the laser with the two optical paths and two beam diameters.

## B. Flux Density Profiles

The samples were located in a 4 cm path glass cuvette of 4 x 1 cm cross section. A 400 $\mu$m fused silica optical fiber with a 2 mm diameter spherical tip was inserted from the top and displaced in 1 mm increments. The front face was illuminated with chopped light from a 200 W high-pressure Hg-Xe arc through collimating lenses and a narrow-band interference filter. The incident beam diameter was approximately 3.5 cm with < 4% per cm radial variation in the incident irradiance. The probe signal was measured with a Hamamatasu R446 photomultiplier and an Ithaco model 3921 lock-in amplifier.

## C. Tissue Optic Calculations

The 1DA calculation was carried out with P1 formulation of Jacques and Prahl[12] which uses an approximate correction for refractive index mismatch based on the boundary conditions of Groenhuis *et al.*[13] For the reduced 1DA calculation 500-1000 (R,T) data sets were processed by a 33 Mhz "386" computer in a few minutes using an iterative program based on a two-dimensional Newtons's method. The DE model was implemented with the transformation: $k \rightarrow k$; $s \rightarrow s(1 - g^2)$; $g \rightarrow g/(1 + g)$.[14] The internal reflection coefficient ($r_i$) for an air-glass-water interface was taken as 0.493[15] and specular reflection coefficient ($R_{sp}$) was taken as 0.04. The reduced 1DA results were augmented by MC calculations based on the model of Prahl, which utilizes the Henyey-Greenstein phase function, variable photon stepsize, and an implicit capture technique.[7,10] Equivalent results were obtained by inserting the photon bundle at a single point and by assuming a large diameter circular beam. A typical thick-layer calculation for 5,000 passes of 5,000 photons with 40 intermediate flux density steps required 30 min to 6 h on a 33 Mhz "386" computer depending on the optical constants.

**D. Tissue Phantoms**

Liposome encapsulated hemoglobin (LEH) was prepared with hemoglobin (Hb) from fresh, defibrinated bovine red blood cells (RBC). The RBC were separated by repeated centrifugation at 3000 rpm for 10 min at 4°C and resuspending in pH 7.4 phosphate buffered saline (PBS), followed by treatment with lysis buffer (pH 8.0 phosphate buffer plus 5 mM NaCl) and separation of the Hb by centrifugation at 15,000 rpm for 35 min. The Hb solution was concentrated to 14 g/100 ml in dialysis tubing surrounded by Aquacide II (Calbiochem). The lipid mixture (1 g hydrogenated soy phosphatidylcholine, 125 mg cholesterol, 93 mg dicetylphosphate, and 4 mg α-tocopherol) was dissolved in 1:1 chloroform-diethyl ether and evaporated to dryness under vacuum. The concentrated Hb solution (20 ml) was added to the lipid film and held at 4°C for 15 h with occasional swirling, followed by bath sonication for 4 h under nitrogen, centrifugation at 1500 rpm for 6 min at 4°C to remove large fragments, and repeated centrifugation at 15,000 rpm for 45 min and resuspending in PBS to remove excess Hb. Oxyhemoglobin ($HbO_2$) in LEH was converted to deoxyhemoglobin (deoxyHb) by adding 2 mg of sodium dithionite to 5 ml of LEH. Carbonyl hemoglobin (HbCO) was obtained by blowing CO over the surface of a cuvette containing $HbO_2$ for 5 min. Hemiglobin nitrite ($HbNO_2$) was obtained by adding 2 mg sodium nitrite crystals to 5 ml of $HbO_2$. The LEH were lysed by mixing 0.5 ml of LEH with 9.5 ml of 1% D-glucopyranoside, stirring for 30 min, and centrifuging at 15,000 rpm for 35 min at 4°C. The human RBC suspensions were prepared from freshly drawn blood from a female volunteer. The RBC were hemolyzed by dilution in the lysing buffer. Methylene blue (C.I. 52015) and eosin Y (C.I. 45380) were obtained from Sigma Chemical and used without purification. Polystyrene microspheres (PSM) were obtained from Duke Scientific. The artificial dairy substitute (ADS) was

Coffeemate (Carnation Co.). The colored gelatin was strawberry Jell-O (General Foods). Absorption spectra were measured with a Beckman DU-7 spectrophotometer (referred to as "DU-7").

## III. Results

### A. Calculated k and s' Spectra of Tissue Phantoms

Dye-Microsphere Systems: **Figure 1** shows the k and s' spectra obtained for 2.0 $\mu$m PSM ($1.5 \times 10^9$ $cm^{-3}$) in 8 $\mu M$ eosin Y (EY). The calculated k spectrum is in good agreement with the EY absorption measured in the "DU-7" converted from optical density (OD) to k. A similar measurement at a lower PSM concentration ($3 \times 10^8$ $cm^{-3}$) led to an almost identical k spectrum and a weaker, featureless s' spectrum (**Table I**). The s' spectrum shows a "negative" deviation near the peak of the k spectrum. A tentative explanation for this effect involves the loss of potentially scattered photons by absorption in the external medium. The Mie oscillations in the blue-green region are present in the s' spectrum of PSM without the dye. The results for 27 $\mu M$ methylene blue (MB) with 2.0 $\mu$m PSM ($1.2 \times 10^9$ $cm^{-3}$) are shown in **Fig. 2**. The k spectrum is red-shifted by 11 nm from the "DU-7" spectrum which is attributed to adsorption of the cationic dye by the PSM. The s' spectrum has a strong "positive" deviation in the region of the 673 nm absorption band and a weaker "negative" deviation at the 619 nm band. The comparison with **Fig. 1** indicates that the composition of the phantom system has a significant effect on the magnitude and direction of the s' deviations.

Gelatin-ADS Phantoms: Measurements were made on tissue phantoms consisting of pink gelatin incorporating 1.6%, 3.2%, and 7.7% (w/w) ADS. **Figure 3** shows the results for 3.2% ADS. The magnitude of k at 496 nm is constant for the three ADS concentrations at $11.1 \pm 1.1$ $cm^{-1}$. The corresponding values of s' at 675 nm are 0.7, 2.9, and 6.0 $cm^{-1}$ which are approximately proportional to the

ADS concentration. The s' spectra have increasingly large "negative" deviations with higher ADS (**Table 1**). The microstructure of this system is similar to EY-plus-PSM in which colorless scattering particles are suspended in a homogeneous absorbing medium.

Red Blood Cells: Most prior measurements on intact RBC were made with optically thin layers in the far-red spectral region, e.g., Reynolds.[16] **Figure 4** shows the k and s' spectra of an RBC suspension ($2.4 \times 10^7$ $cm^{-3}$) in PBS. The spectral results are summarized in **Table 2**. The wavelength peaks and valley agree with literature values for solution human $HbO_2$[17] and lysed RBC (points). The peak-to-valley ratios are lower in the intact RBC and the overall absorption is 20-30% lower than measured for the lysed RBC in the "DU-7". The difference can be explained by a smaller effective absorption cross section when all chromophores are located within the scattering centers. The s' spectrum is almost flat for intact RBC from 425-700 nm. The addition of a high ADS concentration to the RBC suspension led to "negative" s' deviations in regions of high k (**Figure 5**). The modified RBC system resembles EY-plus-PSM in which colorless scattering particles are distributed in a dispersed chromophore. The presence of ADS also led to a small increase of the k spectrum. This may be an example of the "detour" effect in which a longer photon path increases the probability of absorption.

Liposome Encapsulated Hemoglobin: **Figure 6** shows the IDA spectra of LEH incorporating 0.17 g/100 ml Hb and the "DU-7" spectrum of the lysed LEH on the same scale. The agreement is good except for the higher amount of MetHb in the lysed LEH as indicated by the 630 nm absorption. The MetHb fraction estimated from k at 577 and 630 nm is 13% in the intact LEH and 26% in the lysed LEH. The spectral parameters of bovine $HbO_2$ in PBS are very close to human $HbO_2$ from lysed RBC (**Table II**) and the literature values for human

solution $HbO_2$.[17] The peak-to-valley ratios for $HbO_2$ in intact and lysed LEH are lower than solution $HbO_2$ owing to the overalapping absorption of MetHb. The "negative" deviations in the s' spectrum are very pronounced for LEH. This system resembles RBC-plus-ADS in which most of the liposomes are not in proximity with Hb. **Figure 7** shows the k spectra of Hb derivatives in LEH. The general features are similar to measurements on solution Hb.[17,18] The band maxima are compared with literature values for human Hb in **Table III**.

### B. Flux Density Profiles

The object of these measurements was to compare the flux density profiles calculated with the reduced 1DA for 5 mm layers with experimental measurements on 4 cm depth layers for equivalent media. Typical flux density profiles are shown in **Figure 8**. The asymptotic diffusion length ($\delta$) is given by:

$$\delta = 1/\sqrt{[3k(k + s')]} \qquad (1)$$

The values of $\delta$ calculated with the reduced 1DA values of k and s' are in good agreement with the experimental results (**Table IV**). A more detailed analysis was made for the RBC-plus-ADS tissue phantom at 505 nm. **Figure 9** compares the experimental flux density profile with calculations based on the reduced 1DA model and the DE and MC models for $g = 0.9$, $k = 0.66\ cm^{-1}$ and $s = s'/(1 - g) = 68.3\ cm^{-1}$. The three results are in approximate agreement with the data except near the front face where the calculated flux density is low. MC calculations at other values of $0 < g < 0.99$ led to very similar profiles, which supports the accuracy of the similarity transformation for this data set.

## IV. Discussion

The reduced 1DA methodology led to high-resolution k spectra which are in good agreement with the separated chromophores allowing for perturbing factors, e.g., binding of MB to PSM and presence of MetHb in LEH. Accurate

results were obtained for the EY-plus-PSM system at 515 nm with $s'/k \approx 0.2$ and $g \approx 0.93$[19] suggesting that the limitation of 1DA accuracy to $s'/k >> 1$ and "low g" may be too conservative for calculations of k spectra. The integrated absorption (A) in a thick layer is a useful quantity for many biological applications. Wilson and Jacques[20] have shown that the diffuse reflection ($R_d$) of a semi-infinite layer is fully determined by $s'/k = N'$ for a broad range of optical constants. A convenient expression for the reduced 1DA value of $R_d$ is:

$$R_d = \frac{N'}{(1 + N' + \sqrt{3(1 + N')}\left\{1 + \frac{(2b)}{\sqrt{3(1 + N')}}\right\}} \qquad (2)$$

where $b = (1 + r_i)/(1 - r_i)$.[21] For given values of $N'$ and $r_i$, the thick layer absorption $A = 1 - R$ may be calculated from Eq. 2 and $R = R_{sp} + (1 - R_{sp})R_d$. Equation (2) gives $A = 0.679$ for the RBC-plus-ADS system of **Fig. 4** at 505 nm compared to 0.675±0.001 for two MC runs at $g = 0$ with 5000 passes of 5000 photons. The accuracy of the reduced 1DA calculation of A was tested further by comparison with DE and MC calculations for different values of g and $s = s'/(1 - g)$. Some results are: $g = 0.5$ (0.692, 0.690±0.001); $g = 0.9$ (0.699, 0.706±0.002); $g = 0.95$ (0.699, 0.701±0.003; $g = 0.99$ (0.700, 0.706±0.0.006), where the first values of A in parentheses were obtained with the DE model and the second value is the average of two MC runs. The agreement supports the accuracy of the DE model for $0 < g < 0.99$ and indicates that A can be calculated to practical accuracy with the reduced 1DA optical constants.

The direction and extent of the s' deviations show a rough correlation with the departure of the microstructure of the system. **Table I** compares the values of $N'$ at the absorption maxima to the direction and extent of s'

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TABLE I. Magnitude of s' deviations for tissue phantoms and tissues

| *Sample* | λ (nm) | $s'/k^a = N'$ | *Deviations*[b] | *Ref* |
|---|---|---|---|---|
| LEH | 576 | 2.1 | - - - | this work |
| RBC | 576 | 0.3 | o | this work |
| RBC + ADS | 576 | 2.4 | - - | this work |
| EY + PSM ($3 \times 10^8$) | 516 | 0.2 | o | this work |
| EY + PSM ($1.5 \times 10^9$) | 515 | 2.6 | - - | this work |
| MB + PSM ($1.2 \times 10^9$) | 673 | 1.5 | + + | this work |
| | 619 | 2.6 | - - | |
| gelatin + 1.6% ADS | 496 | 0.07 | - | this work |
| gelatin + 3.2% ADS | 496 | 0.23 | - - | this work |
| gelatin + 7.7% ADS | 495 | 0.47 | - - - | this work |
| white potato | 410 | 2.2 | - | c |
| sweet potato | 490 | 1.2 | - | [11] |
| bovine muscle | 450 | 0.3 | - | [11] |
| bovine liver | 460 | 0.7 | o | [11] |
| calf brain | 430 | 0.2 | - | [11] |
| rat liver | 1950 | 0.3 | + + | [22] |

[a] s' measured adjacent to absorption band.

[b] ---, strong negative deviations; --, negative deviations; -, weak negative deviations; o imperceptible deviations; +, weak positive deviations; ++, positive deviations.

[c] unreported result.

**TABLE II. Absorbance parameters of oxygenated hemoglobin**

| Sample | $\lambda_1$[a] | $\lambda_2$[a] | $\lambda_o$[b] | $R_{12}$[c] | $R_{10}$[d] | $R_{20}$[d] |
|---|---|---|---|---|---|---|
| | (nm) | | | | | |
| Lysed human RBC[e] | 576 | 541 | 560 | 1.07 | 1.82 | 1.71 |
| Intact human RBC[f] | 576 | 540 | 560 | 1.05 | 1.65 | 1.57 |
| Intact human RBC + ADS[f] | 576 | 541 | 560 | 1.07 | 1.80 | 1.69 |
| Bovine $HbO_2$ in PBS[e] | 577 | 541 | 560 | 1.06 | 1.82 | 1.72 |
| Intact bovine LEH[f] | 576 | 541 | 559 | 1.06 | 1.50 | 1.43 |
| Lysed bovine LEH[f,g] | 577 | 541 | 560 | 0.98 | 1.46 | 1.49 |

[a] absorption peak

[b] absorption valley

[c] peak-to-peak absorbance ratio

[d] peak-to-valley absorbance ration

[e] measured in Beckman DU-7 spectrophotometer

[f] calculated from reduced 1DA analysis

[g] in 1% D-glucopyranoside

**TABLE III. Absorption spectra of hemoglobin derivatives in LEH calculated with reduced IDA methodology**

| *Derivative* | *Wavelength Maxima (nm)* | |
|---|---|---|
| | *Figure 6* | *Solution Hb*[a] |
| $HbO_2$ | 576, 542 | 577, 542 |
| Hb | 570, 540 | 569, 539 |
| $HbNO_2$ | 625, 540 | 625, 538 |
| deoxyHb | 555 | 555 |

[a] human Hb in solution; from van Assendelft [17].

**TABLE IV. Diffusion length in tissue phantoms**

| *System* | *Wavelength* (nm) | $k$ ($cm^{-1}$) | $s'$ ($cm^{-1}$) | $\delta$[a] (cm) | $\delta_{exp}$[b] (cm) |
|---|---|---|---|---|---|
| LEH[c] | 505 | 1.48 | 6.56 | 0.17 | 0.17 |
| EY + PSM[d] | 543 | 0.17 | 5.39 | 0.59 | 0.57 |
| RBC[e] | 505 | 0.47 | 0.37 | 1.17 | 1.13 |
| RBC + ADS[f] | 505 | 0.66 | 6.83 | 0.26 | 0.25 |

a diffusion length calculated with Equation (1)

b extrapolated attenuation depth measured in 4 cm layer

c 0.17 g/100 ml Hb

d 8.3 $\mu$M eosin Y + 1.5 x $10^9$/ml PSM

e 2.4 x $10^7$/ml RBC

f 2.4 x $10^7$/ml RBC + 2.4% ADS

## FIGURE CAPTIONS

**Figure 1**. *Curves (a)* and *(c)*, respectively, are the calculated k and s' (+ 2) spectra of eosin Y (EY) plus polystyrene microspheres (PSM). *Curve (b)* is the absorption spectrum of the same dye concentration measured in the Beckman DU-7 spectrophotometer and converted from OD to k. *Curve (d)* is the calculated s' spectrum (+ 1.5 ) of the PSM without dye.

**Figure 2**. *Curves (a)* and *(c)*, respectively, are the calculated k and s' spectra of methylene blue (MB) plus PSM. *Curve (b)* is the dye absorption measured in the spectrophotometer. The red shift in *Curve (a)* is attributed to dye binding.

**Figure 3**. *Curves (a)* and *(b)*, respectively, are the calculated k and s' spectra of colored gelatin plus artificial dairy substitute (ADS).

**Figure 4**. *Curves (a)* and *(c)*, respectively, are the calculated k and s' spectra of a dilute red blood cell (RBC) suspension. *Curve (b)* is the absorption spectrum after lysis measured in the spectrophotometer.

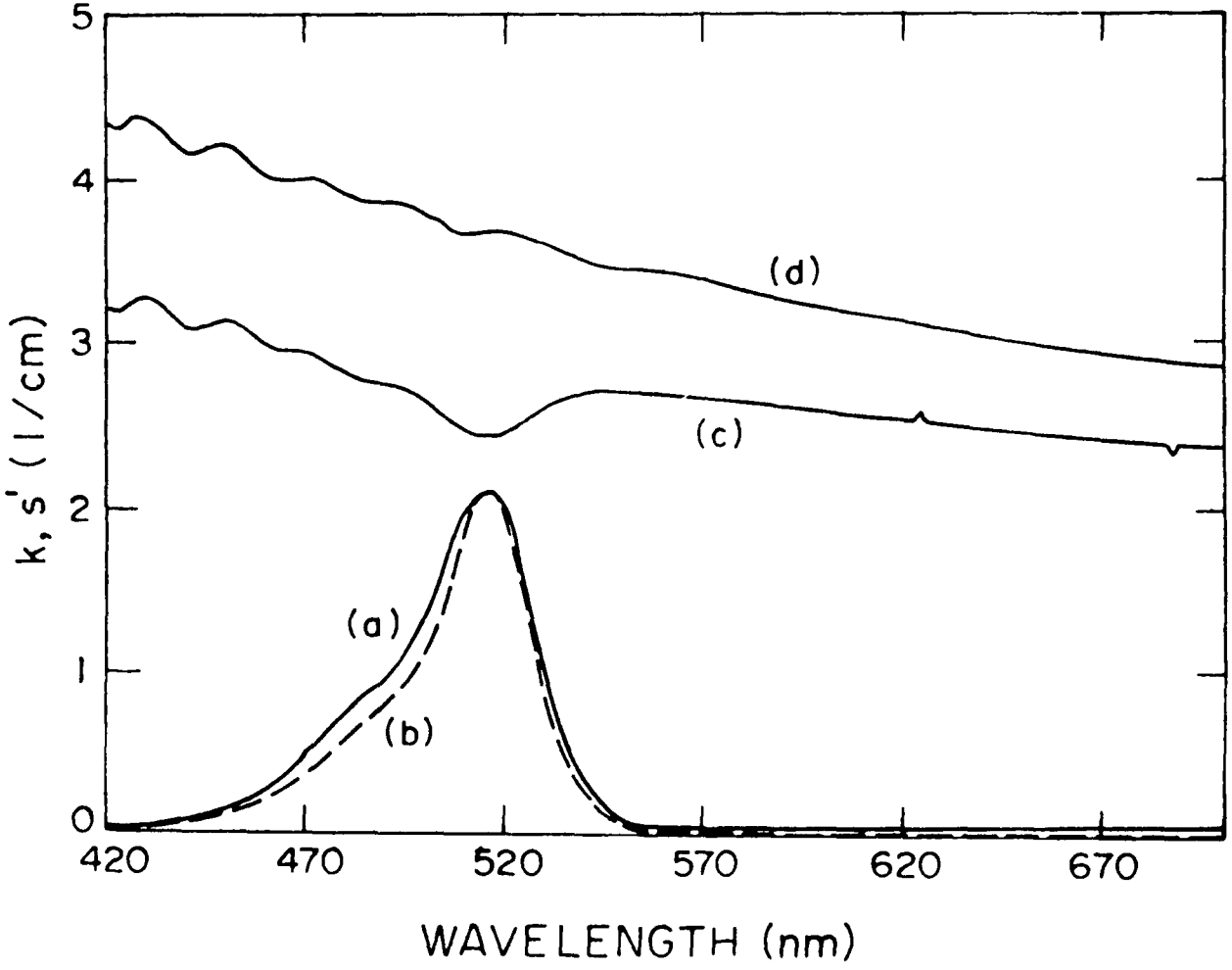
**Figure 5**. *Curves (a)* and *(b)*, respectively, are the calculated k and s' spectra of the RBC suspension plus ADS. The presence of ADS leads to s' deviations.

**Figure 6**. *Curves (a)* and *(b)*, respectively, are the calculated k and s' spectra of liposome encapsulated hemoglobin (LEH). *Curve (c)* is the absorption spectrum of the lysed preparation measured in the spectrophotometer. The absorption band near 630 nm is attributed to methemoglobin.

**Figure 7**. Calculated k spectra of LEH containing hemoglobin derivatives: *Curve (a)* oxyhemoglobin; *Curve (b)* deoxyhemoglobin; *Curve (c)* carbonylhemoglobin; *Curve (d)* hemiglobin nitrite.

**Figure 8.** Experimental flux density distributions measured in 4 cm optical path cuvette: *Curve (a)* RBC at 505 nm; *Curve (b)* EY plus PSM at 543 nm; *Curve (c)* RBC plus ADS at 505 nm; *Curve (d)* LEH at 505 nm. The experimental diffusion length is compared to 1DA calculations in Table IV.

**Figure 9.** Flux density distribution for LEH at 505 nm based on the similarity relations: ● experimental data; ○ reduced 1DA calculation (g = 0); □ delta-Eddington calculation for g = 0.9; △ Monte Carlo calculation for g = 0.9.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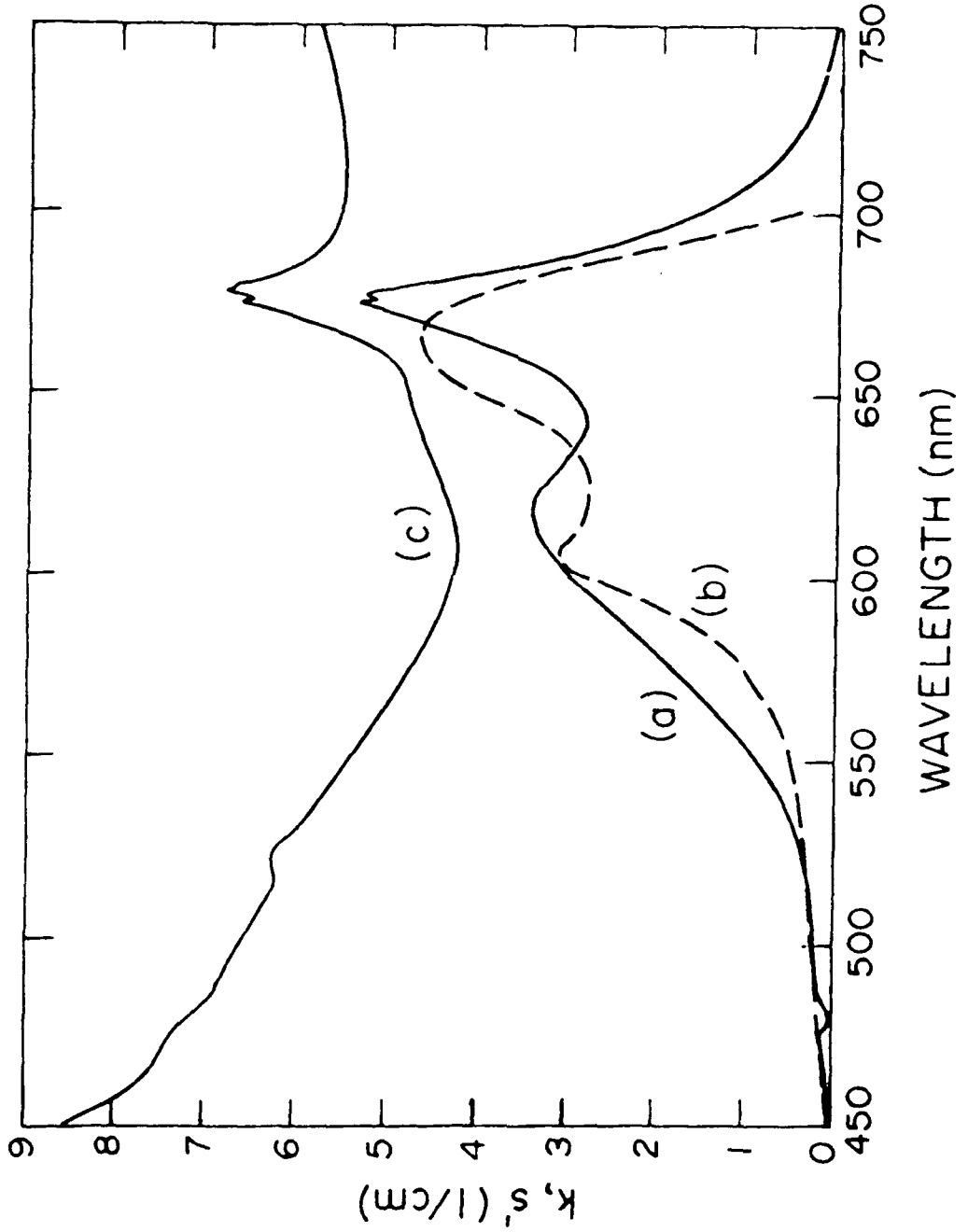
WAVELENGTH (nm)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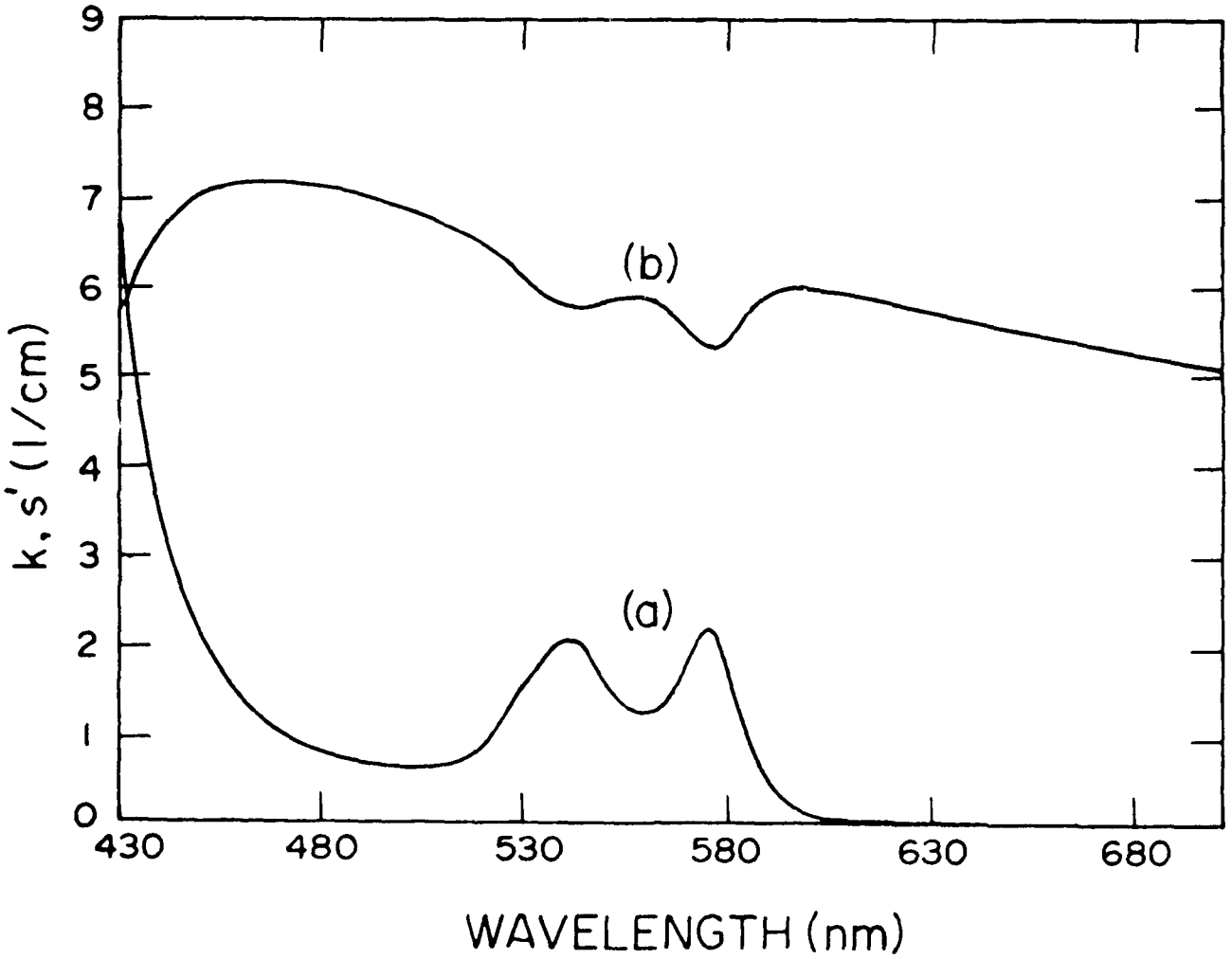
(c)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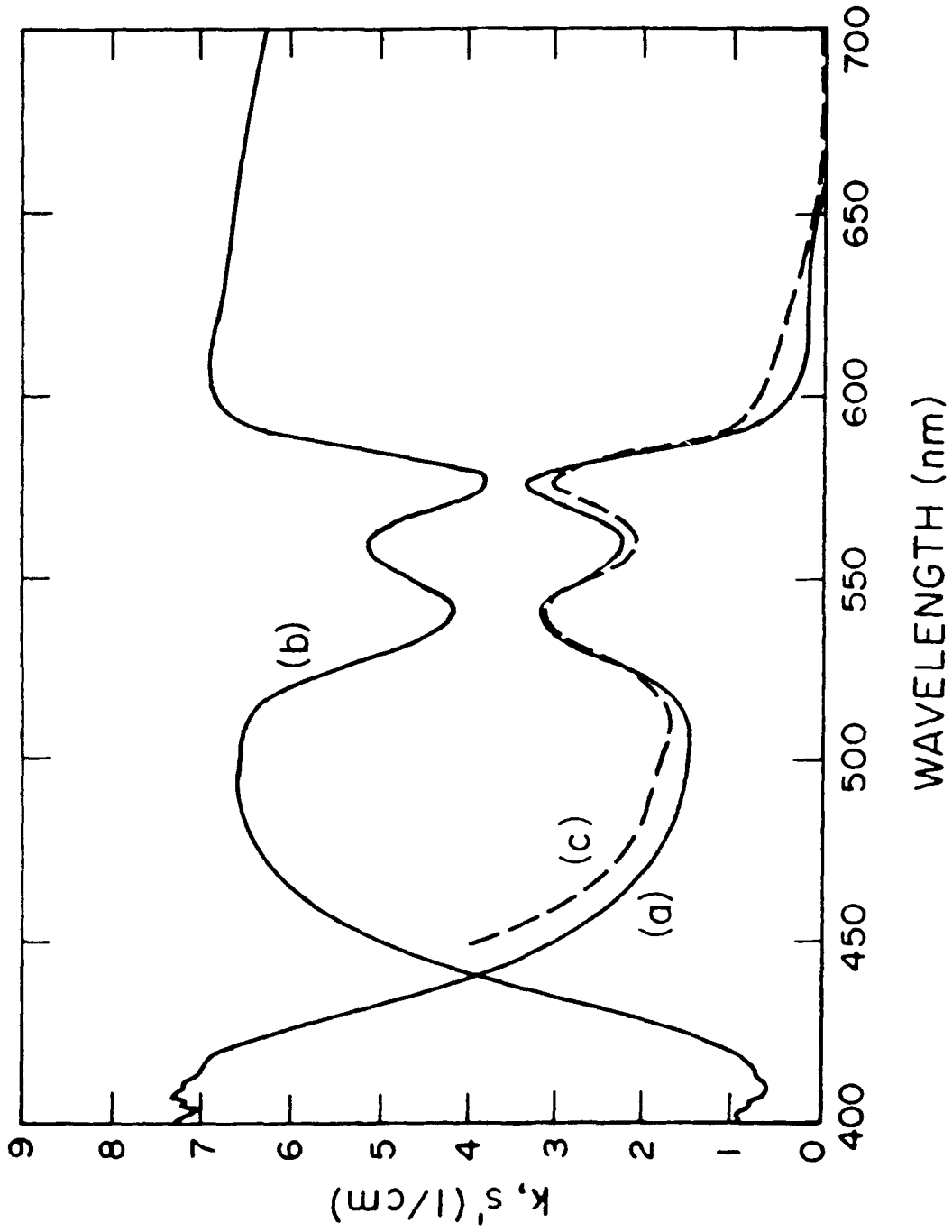
K, s' (1/cm)
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650
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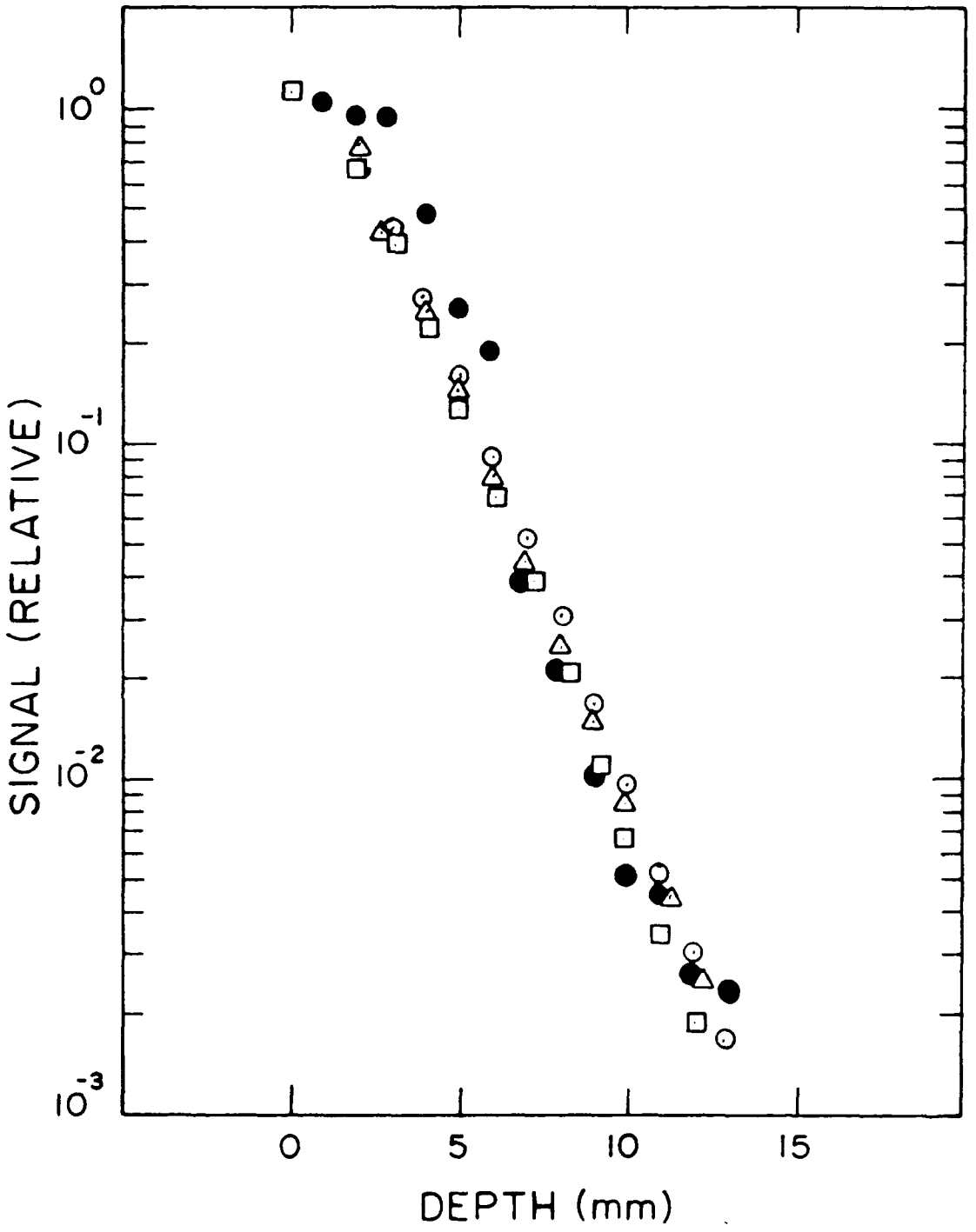
SIGNAL (RELATIVE)
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10
15
DEPTH (mm)

# LIPOSOME-ENCAPSULATED HEMOGLOBIN PROCESSING TO FORM ARTIFICIAL RED BLOOD CELLS

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## ABSTRACT

Process parameters using a Microfluidizer™ M110 to produce liposome-encapsulated hemoglobin (LEH) were further studied to examine their effect on hemoglobin (Hb) encapsulation efficiency (yield), steady shear viscosity, mechanical stability, and oxygen delivery. Liposome formulation loading ratios of up to 300 μmol of lipid per ml of Hb solution were evaluated; a maximum yield was obtained at 300 μmol/ml. Liposomes containing encapsulated Hb concentrations as high as 15.5 g/100 ml were prepared. LEH particle size distribution, determined from negatively stained whole mount preparations using transmission electron microscopy, resulted in average vesicle sizes for optimal batches of about 155 nm. Steady shear viscosity of LEH (up to 40% by volume) in an isotonic-isooncotic solution of PBS containing either albumin or dextran were evaluated for shear rates to 2000 $s^{-1}$. Values obtained were generally higher than those of whole blood at all shear rates tested. Little leakage of Hb from liposomes stored in isotonic PBS was observed as a function of storage time and shear rate. Administration of LEH supported life in rats whose hematocrit had been reduced via isovolemic exchange transfusion to levels well below 5%, which was incompatible with survival when exchange transfusion was performed with the isotonic-isooncotic PBS solution.

## INTRODUCTION

One of the greatest threats to human life is severe blood loss due to traumatic injury and/or surgical procedure as well as the pathological changes following them, unless the intravascular volume and oxygen delivery to tissues can be adequately restored. The most widely used blood replacement therapy is the transfusion of whole blood or some of its components. Several problems associated with this approach include hemolytic transfusion

reactions, allergic reactions, circulatory overload, embolism, citrate toxicity, coagulation disturbances and the transmission of blood borne diseases like AIDS and hepatitis [1,2]. Also, whole blood has a short shelf life of 21 days outside the body, which limits its use. The limitations and logistics of distribution of blood of desired type causes additional problems. According to Miller [3], millions of units of blood collected every year are either unused or unaccounted for.

All the above limitations have necessitated the search for an acceptable red cell substitute. The fact that no product has yet been marketed in the U.S. shows that the problem of finding a suitable red cell substitute is not a simple one. Numerous approaches have been taken toward development of blood substitutes, which can deliver oxygen effectively and are safe for use, resulting in three still-experimental classes of synthetic oxygen-carrying blood substitutes: 1) perfluorochemical emulsions [3-6]; 2) hemoglobin (Hb) solution: either normal, polymerized intermolecularly or crosslinked intramolecularly but acellular [7-12]; and more recently 3) microencapsulated Hb [13-23]. However, toxic effects including nephrotoxicity have been found in the in-vivo usage of some of these oxygen delivery vehicles [7,24-26].

Liposome technology provides a mechanism for encapsulation of Hb with reasonable stability; thus permitting in vivo delivery of Hb-bound oxygen, which probably would otherwise be degraded, cleared rapidly, or toxic to the host. However, potential problems still exist in the use of LEH. One of these involves the solubilization of phospholipid membrane by human plasma high density lipoproteins [27]. Other aspects of clinical concern with respect to cardio-vascular infusion of LEH include tissue/organ toxicity [28,29], effect on coagulation system and platelets [30-34], and effect on reticuloendothelial (i.e. the mononuclear phagocyte)/host defense system [35-38].

This study further characterizes film hydration processing using a high pressure extrusion device, i.e. the Microfluidizer™ M110, to form a red blood cell substitute. Previous work has been reported elsewhere [16,39]. This study determines the effect of process parameters on various biochemical and biophysical properties of the prepared LEH and evaluates efficacy in life support. Use of the Microfluidizer™ in processing LEH is expected to permit preparation of adequate quantities of LEH for total and partial blood replacement in larger animals, i.e., not just limited to mice and rats [14,40,41].

## MATERIALS AND METHODS

### Hemoglobin Solutions

Human stroma-free hemoglobin solutions were prepared at 4 °C following techniques as described elsewhere [16,39,42]. All equipment used in the processing was depyrogenated and all water used (for example in the washing and lysing steps and in the preparation of phosphate-buffered saline) was bacteria and pyrogen-free. To maximize oxygen-carrying capacity high concentrations of Hb solution up to 35 g% (i.e. 35 grams Hb per 100 ml solution) were prepared.

Pyridoxal-5-phosphate (P-5-P) (Sigma Chemical Co.) was added to Hb solution at a molar ratio of 2:1 (P-5-P:Hb) to control oxygen affinity of the LEH to a value similar to that of fresh red blood cells. The antioxidant catalase, which acts as a scavenger of free radicals, was added to the Hb solution [43]. Also it is known that other constituents of the red blood cell hemolysate from which the hemoglobin solution is prepared protect against oxidation of hemoglobin and phospholipid [43]. To inhibit bacterial growth, 50,000 units of penicillin, 25,000 units of polymixin, 50 mg of streptomycin, and 40 mg of gentamycin were added per liter of Hb or PBS solution.

### Lipids

The membrane lipids used to encapsulate Hb solution included hydrogenated soy-phosphatidylcholine (HSPC), cholesterol (CHOL), dicetyl phosphate (DCP) and, α-tocopherol (α-T) [16,39]. HSPC was obtained from American Lecithin Co., Atlanta, GA (as Phospholipon 100-H), and the rest of the lipids were obtained from Sigma Chemical Co., St. Louis, MO. HSPC, a neutral phospholipid, contains 80-85% distearoyl-PC (DSPC), 10-15% dipalmitoyl-PC and traces of lysolecithin [42]. It is felt that this is an adequate and less

expensive alternative to other expensive synthetic saturated phosphatidylcholines (PC). The rationale for use of various components in the lipid mix to form the LEH membrane have been fully described elsewhere [42]. These especially include cholesterol at 50% of the total molar ratio, DCP and a small amount of α-tocopherol. All these materials were used as obtained without further purification. The liposomal membrane was formulated to contain lipid molar ratios for HSPC:CHOL:DCP:α-T of 10:9:0.9:0.1. Liposomes were prepared with varying lipid to Hb loading ratios. In this study, lipid loading, defined as the total number of micromoles (μmol) of lipid used to make an LEH batch per ml of precursor Hb solution added, was varied from 50 to 300μmol/ml to evaluate its effect on yield and average encapsulated hemoglobin concentration.

Buffer Solutions

The lysing buffer used is a low-salt buffer which contains 0.7 mM $Na_2HPO_4$, 0.13 mM $NaH_2PO_4$, 12.1 mM NaCl and 0.012 mM KCl. These concentrations were calculated on the basis of the actual $Na^+$ and $K^+$ concentrations in the red blood cell as reported by Miale [44]. 0.01 M phosphate buffered saline (PBS) was prepared by adding to one litre of distilled DI water 8.5 gm NaCl (0.145 mole), 1.2 gm of $Na_2HPO_4$ (0.0085 mole) and 0.22 gm of KCl (0.003 mole). The pH was adjusted to 7.4 by adding a small amount of $NaH_2PO_4$ (about 0.22 gm). PBS was used for washing of the liposomes and also as a suspending medium for liposomes. Two different suspending media were used for preparing LEH suspensions. The first, 7.5 wt% albumin/PBS was prepared from bovine albumin (Sigma Chemical Company). At this concentration level of albumin the colloid osmotic pressure of this "artificial plasma" approximately balances with that of the blood plasma and is isooncotic with respect to whole blood. The other isotonic-isooncotic suspending medium used was 5.0 wt% (Dex-40, 38,000 MW) in PBS.

LEH Production

The film-hydration method used in this study for LEH production was similar to that fully described in previous reports [16,39]. The major steps of the process were: Hb solution preparation, preparation of a lipid film, hydration of film with Hb solution, microfluidization, washing, filtration and resuspension of liposomes.

Encapsulation efficiency was determined for two different pressure drops across the interaction chamber of the Microfluidizer 110™ system (kindly provided by Microfluidics Corp. Newton, MA). The air-pump used to pressurize the microfluidizer had a multiplier of about 140. Therefore, the inlet air pressures of 40 and 70 psig used in the study, corresponded to liquid pressure drops across the interaction chamber of about 5600 and 9800 psi, respectively. All batches were prepared using four true passes as four passes had previously been found to be adequate [16].

After microfluidization and washing, the liposome suspension was filtered using vacuum created by a water aspirator, through 5 and 3 μm Nuclepore polycarbonate membrane filters with hydrophilic surfaces (Nuclepore Corp., Pleasanton, CA). The volume fraction of liposomes during filtration was maintained at about 5-10%, to provide for easy and rapid filtration. Filtering, first through 5 μm and then through 3 μm filters, was required to remove large LEH particles which probably would not pass through the microcirculation of rats. In order to increase the efficiency of filtration and extend the life of membrane filters, thick prefilters or depth filters (Nuclepore Corp.) were used to retain particles larger than 5 μm; and thus preventing these from clogging the membranes. The depth filters are fibrous materials which provide series of channels with no clearly defined or regular pore structure. Their thicker construction and high porosity resulted in high flow rates; the larger particles are randomly trapped within the body of the filter media. The depth filters used for this purpose were glass fiber prefilters for 1.2-5.0 μm membranes.

After filtration, the liposomes were pelleted by centrifuging at 16,000g (10,500 RPM) for 20 mins, followed by resuspension in suspending buffer to a desired lipocrit. "Lipocrit" was defined in our previous studies [16,39] as the volume fraction of LEH in a prepared sample.

**Measurement of LEH Encapsulated Hb Concentration**

The encapsulated Hb concentration was determined by dissolving the liposomal membrane with a detergent solution and then measuring the resulting Hb solution concentration for oxy, reduced and met-Hb concentration using the method of Benesch et. al [45], which was modified using the extinction coefficient values provided by Van Assendelft and Zijlstra [46], which was $e_{HiCN}(540) = 11.5$. These values gave a better estimate of met-Hb in this assay. As this method is very sensitive to pH changes, the Hb solution was carefully

diluted with a 0.01 M PBS buffer (pH 7.4).The detergent used was n-Octyl β-D-glucopyranoside [42]. A 0.1 ml volume of LEH sample (at a lipocrit of about 30%) was dissolved in 5 ml of concentrated detergent solution (30 mg/ml). The resultant material was stirred for 40-45 mins at room temperature, until the liposomal membrane dissolved (or formed micelles) and the solution lost all its turbidity. The solution was then filtered through 0.22 μm syringe filters and absorbance was recorded to determine oxy, reduced and met-Hb concentrations. From these total encapsulated Hb concentration in 0.1 ml LEH sample was calculated. It was assumed for this calculation that the volume of the lipid membrane was small. If lipid volume is not a small fraction of LEH volume, then this approach overestimates the encapsulated Hb concentration.

To determine if the detergent had an effect on the quality of the encapsulated Hb, the effect of the detergent on the quality of Hb was measured by adding an appropriate amount of detergent solution to a small sample of Hb solution and recording the resulting level of met-Hb in solution. Since no detectable change in met-Hb was observed in the first hour of the addition of the detergent, this method was considered to be reliable for met-Hb determination in LEH. The quality of the encapsulated Hb was determined by measuring the concentration of met-Hb in a sample stored at 4°C as a function of storage time. This was done as described above and by recording the resulting met-Hb level in the LEH sample.

Viscosity Measurement

The viscosity of various LEH samples was evaluated in the different suspending media (e.g., 5% Dex-40, 7.5% albumin buffers). After the final LEH wash the liposome pellet was resuspended in the desired buffer. Liposome suspensions were then prepared at a desired lipocrit. Viscosity of these suspensions was measured with a Weissenberg Rheogoniometer (Model 16/18, Carri-Med Ltd., Dorking, England) as a function of shear rate and lipocrit using a 10 cm diameter cone and plate arrangement with a cone angle of 0.5°. The cone-and-plate geometry is very useful as it gives a good approximation of viscometric flow with constant shear rate throughout the flow field [47].

Leakage of Hb from Liposomes

The fragility of the LEH as a function of shear rate and storage time was evaluated by shearing the LEH samples for 10 mins in the viscometer at three different shear rates.

The concentration of total Hb in the supernatant of the sheared samples was measured following mild centrifugation (150 x g) [45,46] and compared with both the unsheared samples, i.e., the load/unload control (the sample was loaded, held between the viscometer platen without shearing for 10 min, and then removed) and the control (the sample had not been subjected to any handling).

Particle Size

The procedure used for preparing negatively stained whole mount preparations of LEH samples for transmission election microscopy (EM) was based on standard techniques used for biological specimens [48,49]. A primary fixation with glutaraldehyde followed by secondary fixation with osmium tetroxide ($OsO_4$) was used to preserve the shape and size of the lipid vesicles. Grids were observed using a Zeiss EM9S transmission electron microscope with an accelerating voltage of 60 kV. In order to obtain a good estimate of average particle size, photomicrographs of several fields of the grids were taken. The images from these negatives were projected onto a digitizing pad (Scriptel Corporation) and the diameters of the particles were input into a computer to obtain a histogram for the size distribution. A grating replica with 54,800 lines per inch (Ernest F. Fullam, Inc., Schenectady, NY) was used to obtain a calibration for particle size determination.

Efficacy Testing

Preliminary efficacy testing was performed on unconscious rats (Harlan Sprague, Indianapolis, IN) Rats used in all exchange studies were 8 to 12 weeks old and 275 to 300 grams. One day prior to exchange of blood with LEH, rats were anesthetized with ketamine (80 mg/kg body weight) and acepromazine (1 mg/kg). Following the introduction of anesthesia, the jugular vein and femoral artery were exposed and isolated from the surrounding tissue. The rats were then surgically implanted with a silastic cannulae of 0.025 in I.D. x 0.047 in O.D. for the jugular vein and 0.010 in I.D. x 0.023 in O.D. for the femoral artery. The cannulae were filled with sterile hep rin solution throughout the procedure, and were sealed with a smooth wire plug. The animals were allowed to recover overnight after the surgical implantation of the cannualae, and the next day the LEH exchange-transfusion experiments were performed.

A peristaltic pump (Manostat, New York, NY) was used to maintain an isovolemic blood exchange flow rate of 0.2 ml/min. Blood was removed from the femoral artery of the animal and simultaneously replaced with the same amount of LEH sample or a control solution (5% Dex-40 in PBS). The hematocrit of each blood sample was determined in duplicate by collecting the blood from the artery and centrifuging it in microhematocrit tubes. The decrease in hematocrit levels was recorded until the animal ceased to breathe.

Efficacy of LEH was demonstrated by assessing its ability to deliver oxygen in an anesthesized animal whose hematocrit had been reduced via isovolemic exchange transfusion to levels well below 5%, which was found to be incompatible with survival when performing exchange transfusion with isotonic/isooncotic PBS containing 5 g% Dex-40. Further, because of the nature of efficacy testing, an LEH sample that is shown to be efficacious is also considered to be nontoxic, in an acute sense.

## RESULTS AND DISCUSSION

### Encapsulation Efficiency

In an attempt to increase the amount of functional encapsulated hemoglobin in the liposomes and thus, increase the oxygen-carrying capacity of the LEH suspension, a systematic study was performed to evaluate the effect of lipid loading on encapsulation efficiency. The encapsulation efficiency (% ENC) is defined herein as the percentage of added Hb that is encapsulated in the liposomes. The pressure drop across the interaction chamber of the Microfluidizer was also varied to study its effect on encapsulation. Three different types/sizes of interaction chambers were used to see their effect on yield. The results of these experiments are presented in Figure 1. It is noted that yield increases with increasing lipid loading for both pressure drops used: 40 and 70 psig, which corresponds to pressure drop across the interaction chamber of about 6,000 and 10,000 psi, respectively. This may be because there is more lipid surface area available for formation of vesicles as the amount of lipid is increased for the same volume of Hb solution (40 ml).

In a previous study percent capture for film hydration processed liposome-encapsulated cytosine arabinoside at a lipid loading of 300 μmol/ml was found to be as high as 78% [50]. Figure 1 also shows that the increase in pressure drop has a negative effect on

encapsulation. This result may be due to the reduced residence time of the fluid in the interaction chamber and/or the expected reduced particle size for the larger pressure drop.

Yield was also found to depend on the size of the interaction chamber. The largest chamber (H230Z) used in this study, gave a higher yield than the other two smaller chambers. For the larger interaction chamber the yield increases, as the magnitude of shear forces is lowered, consequently, larger particles may be produced and therefore result in an increased encapsulated volume of Hb solution (see Appendix). Since the difference between encapsulation values for the largest and the intermediate (B-50) size interaction chambers were not substantial, the B-50 interaction chamber was used for all the remaining LEH studies reported below.

**Effect of Precursor Hb Solution**

The amount of the precursor solution (Hb solution) encapsulated by the liposomes appears to depend on the pH and the ionic strength of the precursor solution (see Table 1). It is also observed that the method of Hb solution preparation had an effect on the encapsulated Hb concentration in the LEH preparations. Hb solution was prepared by three different methods: 1) RBCs were lysed with deionized water and the final pH was adjusted to about 7.2-7.4 via an overnight dialysis against a pH 7.8 PBS buffer; 2) RBCs were lysed with DI water, but final dialysis was not performed (Hb solution had pH 6.5) and 3) RBCs were lysed with low salt buffer with the final Hb solution having a pH 7.2. As shown in Table 1, encapsulated Hb concentration depends both on the ionic strength and precursor Hb solution concentration. The higher the precursor Hb concentration, the higher was the resulting encapsulated concentration. The highest encapsulated Hb concentration occurred for batches with precursor Hb solution concentrations of 34 g%, prepared using the low salt buffer. The vesicular Hb concentration in this case was approximately 15.5 g%. Low salt buffer lysis produces a Hb solution that is lower in salt concentration than the suspending isotonic medium. The gradient in salt concentration leads to a difference in osmotic pressure between the interior of the liposomes and the outer buffer phase. Since the osmotic pressure is higher inside the liposomes than outside, water is forced out of the liposomes, thereby leaving a higher concentration of Hb in the liposomes that is maintained temporarily as equilibrium is readjusted. Results such as this have been reported previously [16].

**Viscosity of LEH Suspensions**

Steady shear viscosities of LEH in different suspending media at various lipocrits were determined at physiologically relevant shear rates. Figures 2a and 2b show viscosity versus shear rate for LEH preparations suspended in 0.01M PBS containing either 5% Dex-40 or 7.5% bovine albumin. These results are comparable although higher than those obtained for the viscosity of human whole blood (45% hematocrit). It is desirable that RBC substitutes should have rheological properties not too dissimilar to those of whole blood. Pure albumin PBS buffer has a lower viscosity than does the Dex-40/PBS buffer; and likewise the LEH suspended in albumin/PBS had a lower viscosity than the LEH suspended in Dex-40/PBS. Although bovine albumin buffer gave a lower viscosity, it may be species-specific and therefore immunogenic on subsequent exchange transfusions in the same rat. Dextran may therefore be a better substitute as it is not expected to be species-specific.

**LEH Particle Size**

An LEH batch with a lipid formulation loading of 300 μmol/ml Hb solution prepared using an inlet air pressure of 70 psig was analyzed for particle size. The particle size distribution obtained for this batch using transmission electron microscopy is presented in Figure 3. The sample showed a large variation in particle size. Particles ranging from 70 nm up to 320 μm were observed. The average particle size for this preparation was about 155 nm. These results are similar to those reported previously using laser light scattering [16].

The above results may be compared with the results obtained by Mayhew et al. [50,51] in which the Microfluidizer was used to prepare using film hydration techniques liposomes encapsulating small molecules such as cytosine arabinoside and adriamycin. The particle sizes obtained from these preparations were about 200 nm and were found to depend on lipid composition, nature of the substance being encapsulated, pressure drop and the number of passes the material was run through the interaction chamber. Their lipid loading range (2-40 μmol/ml) did not have a significant effect on particle size [50]. Also for pressure drops higher than 4000 psig across the interaction chamber, particle size did not decrease substantially. However, liposome diameters decreased with increasing phosphatidylcholine (PC) content in the liposomal membrane. The liposomes containing adriamycin showed a larger particle size (211 nm) for the ten pass material than the preparations containing

cytosine arabinoside (100 nm). The lipids used for preparing these liposomes were added in a molar ratio of CHOL:PC:PG::5:4:1. Microfluidization of dehydration-rehydration liposomes at a formulated lipid loading of 66 μmol/ml resulted in liposomes with sizes of less than 200nm for the ten pass material at a pressure drop across the interaction chamber of about 6000 psig [52].

**LEH Storage**

The met-Hb concentration levels in LEH samples were recorded as a function of storage time as shown in Figure 4. The samples were stored at 4°C and catalase was coencapsulated with the Hb to inhibit Hb oxidation. A freshly prepared LEH batch had about 6-8% of the total encapsulated Hb in the form of met-Hb. These levels increased to 18% following 15 days of storage at 4°C.

There can be several reasons for the oxidation of functional Hb to nonfunctional met-Hb. These include oxidation due to peroxides produced from membrane lipids, autoxidation of Hb and complex lipid-heme interaction [53-55]. For LEH to have a long shelf life, it is important to maintain low levels of met-Hb in the preparation. Therefore, there is a need to coencapsulate antioxidants/Hb preservatives, i.e. more effective than catalase, in the liposomes in order to reduce met-Hb generation. Antioxidants, such as glutathione and NADH, were tested in a recent study and were shown to stabilize the rate of met-Hb generation during storage at 37°C and even to reduce the amount of met-Hb concentration at 4°C [56]. Also, lyophilization procedures using protective carbohydrates (such as sucrose and trehalose) to replace water are being developed for the preservation of LEH in the dry state at room temperature [56,57].

**Leakage under Shear**

The stability (or fragility) of LEH under shear was investigated as a function of sample age by imposing shear on an LEH sample and recording the leakage of Hb into the external buffer phase. Total leakage of Hb for two batches of LEH with lipid loadings of 100 and 200 μmol/ml, respectively showed that over a three week period was small and remained constant at levels between 0.3 to 0.6%, independent of shear rates to about $2000^{-1}$. The low percentage of leakage of the encapsulated Hb from the liposomes demonstrates the integrity of the LEH membrane. Overall the percent leakage of Hb appeared to be a little lower for

the 200 μmol/ml LEH batch. There appeared to be about a 10% increase in steady shear viscosity during the 22-day storage period. These results are similar to those found for whole blood as reported previously [58].

**Efficacy Studies**

Preliminary efficacy testing using isovolemic exchange transfusions of LEH (about a 30% lipocrit) with rat whole blood in unconscious rats showed that the animals could survive down to a hematocrit as low as 2.5%, thus indicating the acute nontoxic nature of the product (see Figure 5). The key factor in these studies appears to be the filtration of the product using Nuclepore prefilters. It may be very important to remove the large particles (>3μm) present in these LEH preparations. For these studies the control solution used was 5% Dex-40 in PBS, filter sterilized using 0.22 μm syringe filters. The LEH sample was prepared by suspension of the liposomes in the 5% Dex-40 PBS buffer. Viscosity as a function of shear rate for this sample is shown in Figure 2b (30% lipocrit). Figure 5 shows a typical hematocrit dilution curve for an exchange transfusion. The solid circles show the hematocrit levels in the control animal; the squares show the results for the experimental animal. The total crit (solid squares) refers to the volume fraction of cells (RBC + LEH) in the animal's blood. The lipocrit refers to the cellular volume of only LEH per total sample volume, i.e., either LEH exchange solution or transfused blood. The control animal in this experiment was a 277 gm male rat, which survived the exchange transfusion to a terminal hematocrit of 4%. The animal exchanged with the 30% lipocrit LEH sample was a 245 g male rat, which survived the exchange transfusion to a terminal hematocrit of 2.5%.

These preliminary results demonstrate efficacy since administration of LEH supported life in rats whose hematocrit had been reduced via isovolemic exchange transfusion to levels well below 5% which are incompatible with survival when performing exchange transfusion with isotonic/isooncotic PBS containing 5 g% dextran 40. From this data the oxygen-carrying capacity can be estimated to be about 3.5 vol% oxygen at the terminal hematocrit of 2.5%. This batch was prepared with a 300 μmol/ml lipid loading and had a vesicular Hb concentration of 8.5 g%, which corresponded to an oxygen content of 6.4 volume % $O_2$ for the 30% lipocrit feed sample. These results concerning the efficacy of LEH are consistent

with those found in other recently reported studies for terminal hematocrit obtained comparing the control animal and the LEH exchanged animal [14,40,41]

As shown in Figure 5, the lipocrit in the animal begins to level off at 20%, although a sample of 30% crit is being injected into the blood stream. This may indicate that the liposomes are being taken up by the reticuloendothilial system [38] and/or being solubilized by plasma high density lipoprotiens [27], thus, reducing the amount of liposomes available for circulation. Results, using an LEH formulation much the same as that used herein, on LEH circulation half-life and oxyhemoglobin equilibrium saturation as a function of oxygen partial pressure have been previously reported [16,42,59]. Circulation half-life for the hydrogenated soy PC-based LEH was high and in the range of 15 to 20 hrs. Oxygen affinity, as represented by the oxygen partial pressure for which oxy-Hb was 50% saturated, is greater than 20 psi. Cooperatively for the LEH product resulted in a value of $n = 2.8$.

Issues with regard to safety of the LEH for use as a red blood cell substitute will be addressed in future reports. These will include: evaluation of effect on coagulation and thrombosis, impairment/blockade of the reticuloendothelial (i.e. the mononuclear phagocytic) system associated with effect on the host defense system, and general toxic effect on various body tissues and organs (e.g. kidney).

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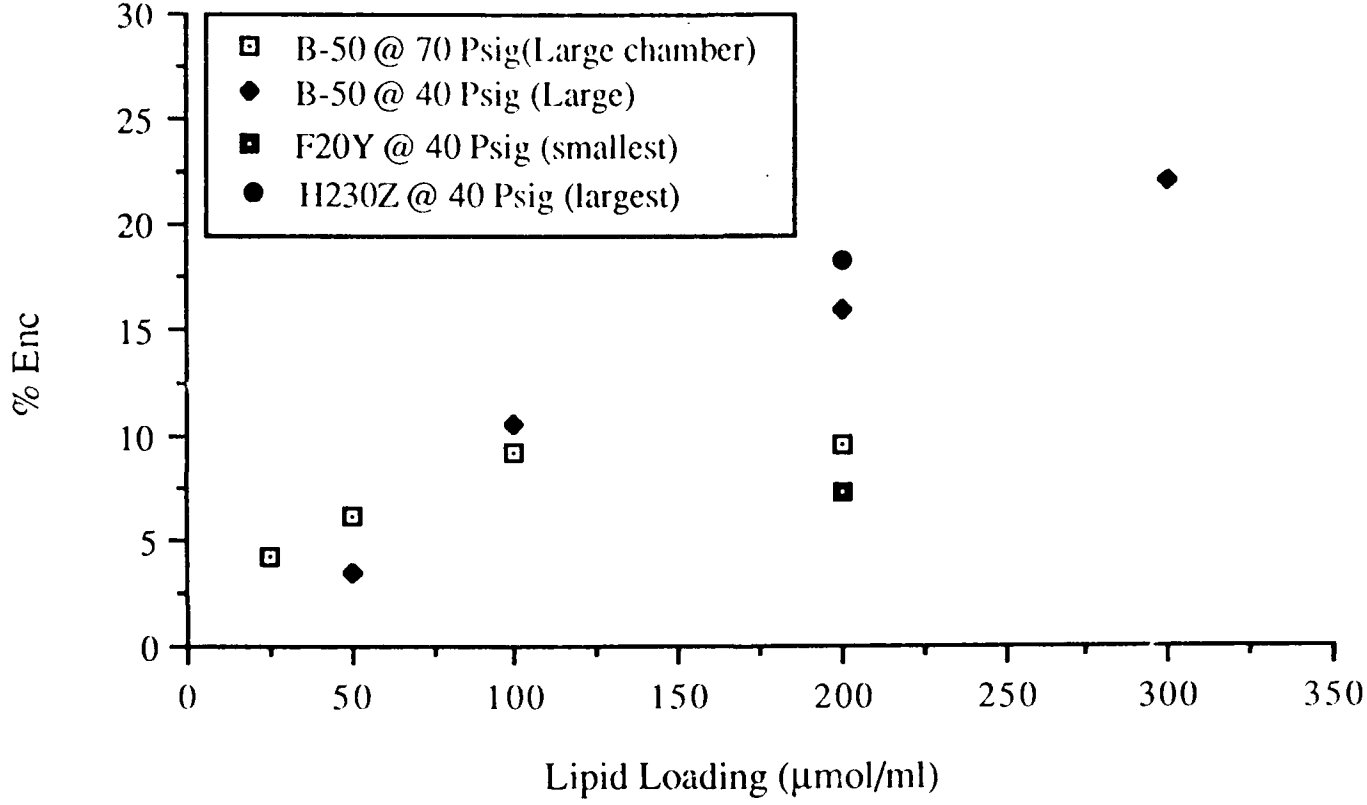
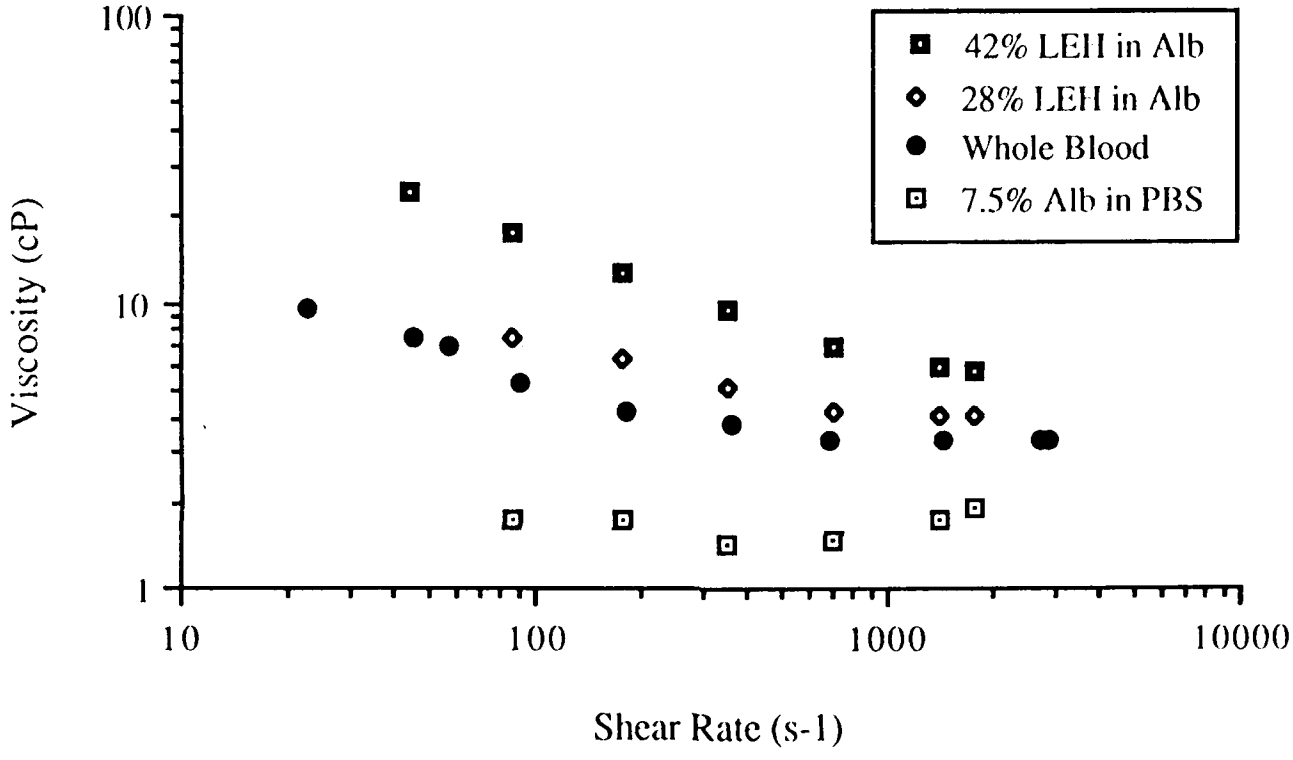
Table 1. Effect of Method of Preparing Precursor Hb Solution on Encapsulated Hb Concentration

| Method of preparation | Solution pH | Precursor Hb Conc. (g/dl) | Encap. Hb Conc. (g/dl) |
|---|---|---|---|
| Low salt buffer lysis | 7.2<br>10.9 | 1) 19.0 | |
| | | 2) 34.0 | 15.5 |
| DI water lysis<br>(with dialysis) | 7.2-7.4 | 21.8 | 8.5 |
| DI water lysis<br>(withough dialysis) | 6.5 | 22.2 | 6.3 |

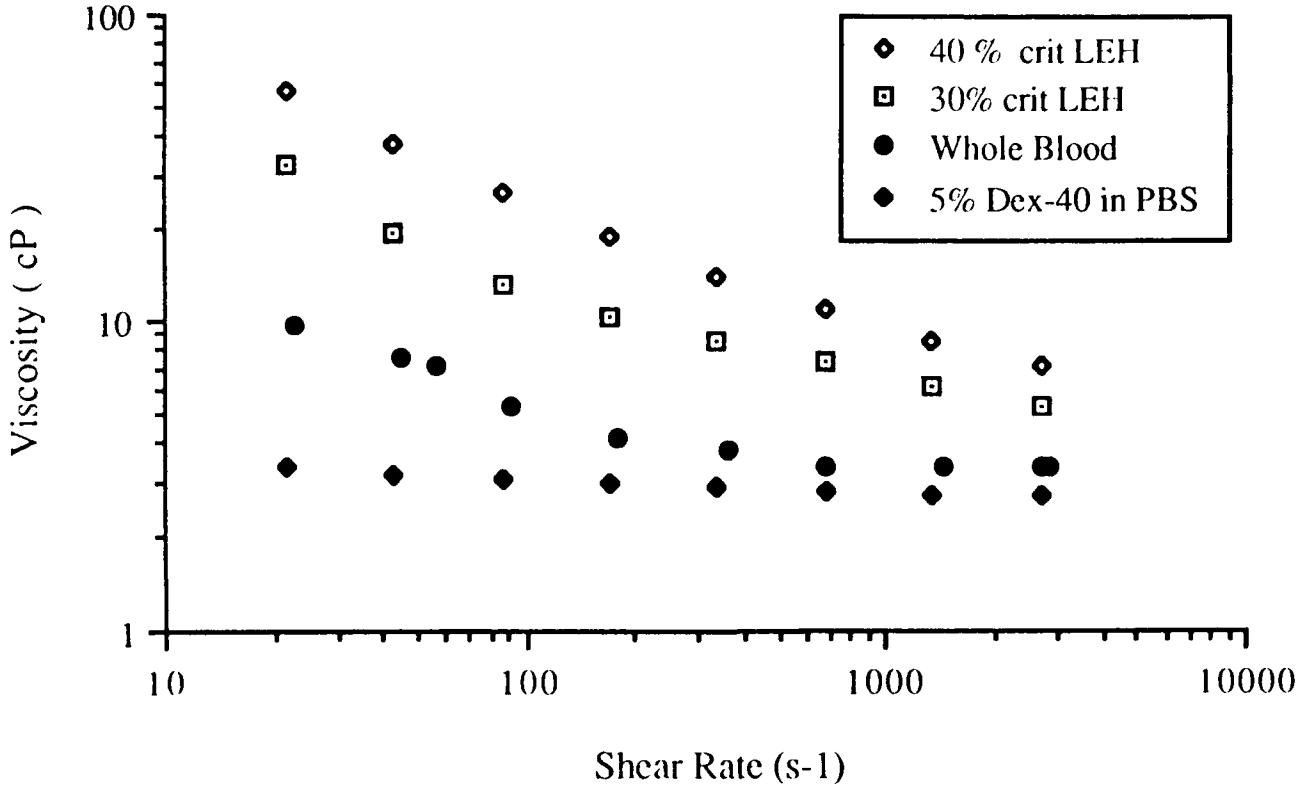
**Note:** All the above results are for 200 μmol/ml lipid loading batches.

## LIST OF FIGURES


B-50 @ 70 Psig(Large chamber)
B-50 @ 40 Psig (Large)
F20Y @ 40 Psig (smallest)
H230Z @ 40 Psig (largest)
% Enc
Lipid Loading (µmol/ml)
0
5
10
15
20
25
30
0
50
100
150
200
250
300
350


100
10
1
Viscosity (cP)
10
100
1000
10000
Shear Rate (s-1)
42% LEH in Alb
28% LEH in Alb
Whole Blood
7.5% Alb in PBS

(26)


100
10
1
Viscosity ( cP )
10
100
1000
10000
Shear Rate (s-1)
40 % crit LEH
30% crit LEH
Whole Blood
5% Dex-40 in PBS

% of Particles Counted
0
10
20
30
40
50
80
100
120
140
160
180
200
220
>240
Particle Size (nm)

(4)

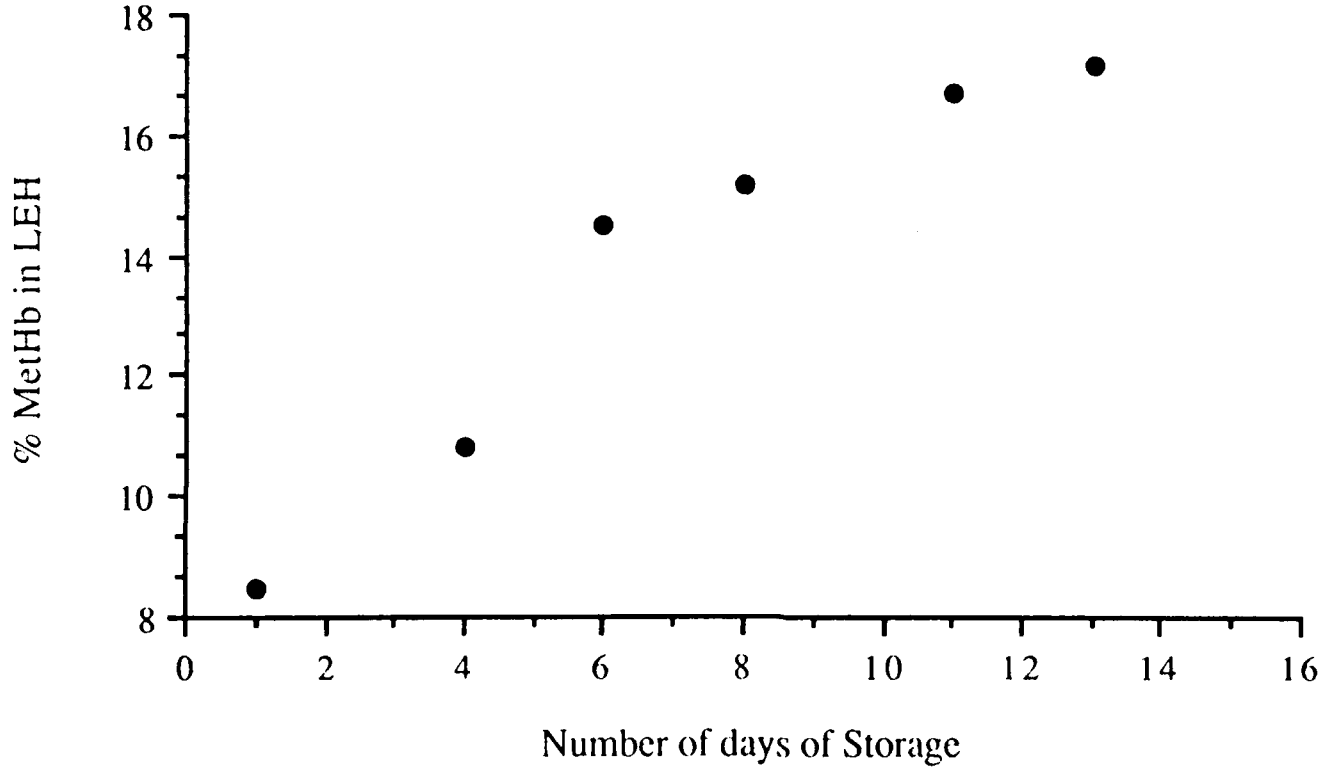

% MetHb in LEH
18
16
14
12
10
8
0
2
4
6
8
10
12
14
16
Number of days of Storage

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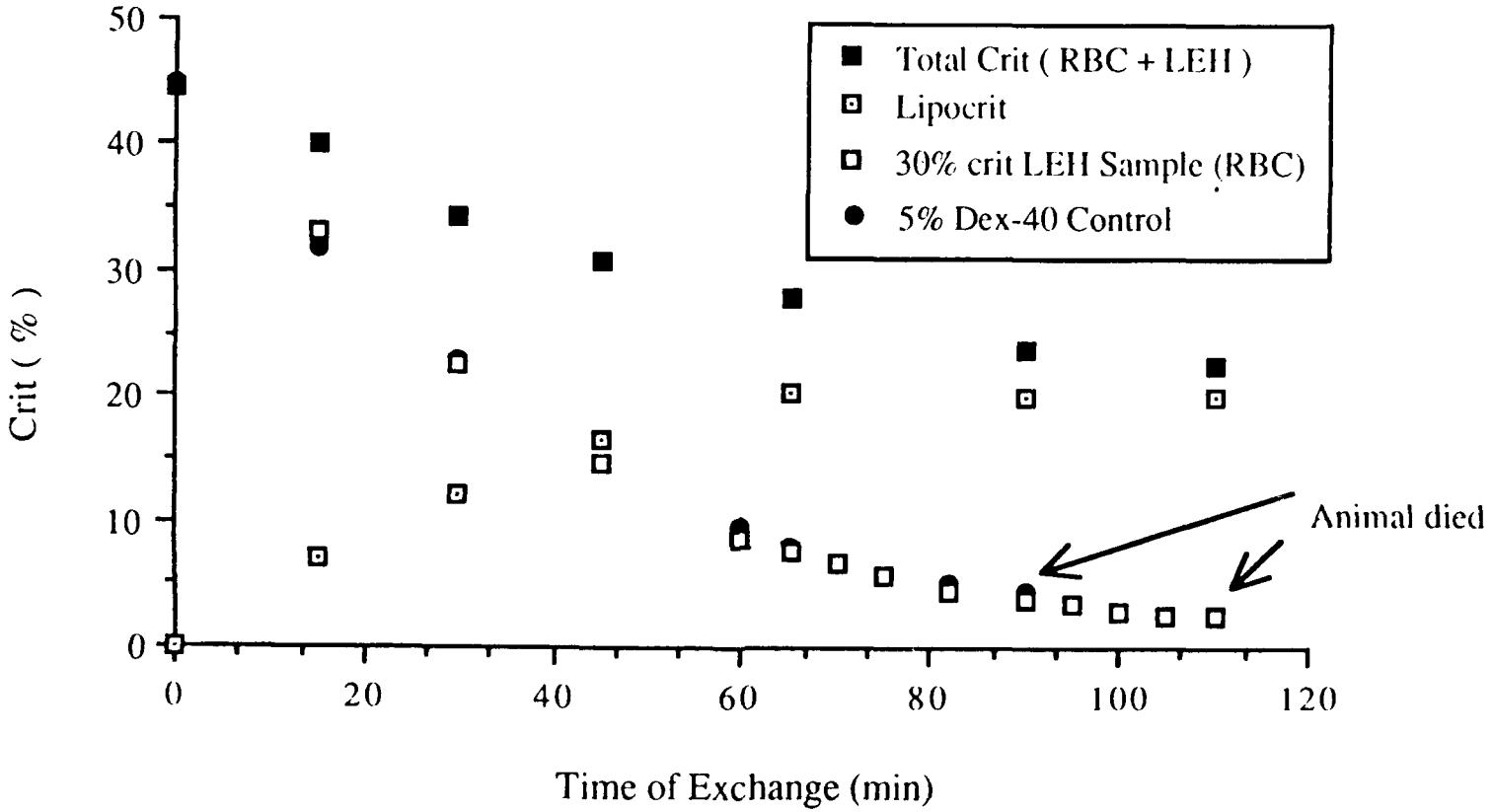
Figure 5 missing

(7)


Total Crit ( RBC + LEH )
Lipocrit
30% crit LEH Sample (RBC)
5% Dex-40 Control
Animal died
Crit ( % )
Time of Exchange (min)
0
10
20
30
40
50
0
20
40
60
80
100
120