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Artificial Red Cells with Crosslinked Hemoglobin Membranes

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ABSTRACT

Artificial cells containing concentrated hemoglobin (Hb) solution were prepared by interfacial polymerization of Hb with glutaraldehyde (GA) in liquid membrane capsules (LMC). A solution containing 30% of Hb was emulsified in mineral oil as red cell-size microdroplets, and this emulsion was dispersed in an aqueous phase containing glutaraldehyde to form LMC. The LMC were semipermeable templates that held the microdroplets of Hb in suspension while GA diffused through the oil to the microdroplet surfaces. The GA crosslinked Hb at the surface of each microdroplet to form an artificial red-cell membrane encapsulating Hb solution. A water-soluble surfactant was used to eject the cells from the LMC and suspend them in saline.

Several surfactants were evaluated. Cell size was controlled by agitation speed during preparation of the original emulsion. Cells of 2.52 = ±1.69 μm were prepared. The encapsulated Hb retained capacity to bind and release O_2. The cells had a P_{50} of 8.9 torr (1200 Pa) and a capacity of 0.55 cc O_2/g of total Hb, indicating that the crosslinked portion of the Hb did not contribute to O_2 transport.

Index Entries: Artificial cells, with crosslinked hemoglobin membranes; artificial red cells, with crosslinked hemoglobin membranes; ar-

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tificial blood; hemoglobin, crosslinked membranes of; polyhomoglobin, crosslinked membranes of; liquid membranes, and artificial cells; surfactant; glutaraldehyde; crosslinked hemoglobin, in artificial red cells; red cells, crosslinked hemoglobin membranes of; blood, crosslinked hemoglobin membranes in artificial; membranes, crosslinked hemoglobin.

INTRODUCTION

Encapsulation of stroma-free hemoglobin solution (SFHS) in a membrane of glutaraldehyde-crosslinked hemoglobin offers a new approach to the preparation of oxygen-carrying blood substitutes. The need for blood substitutes arises in situations in which fresh whole blood of the proper type is not readily available, e.g., in emergency vehicles or during war or natural disasters. Collecting and storing whole blood in anticipation of such situations is impractical or impossible. Even under normal situations, storage and distribution can be wasteful. The Canadian Red Cross found in 1973 that 24% of stored blood was returned unused and 5.5% was lost (*1*).

Part of the problem of wasted blood is attributable to the short storage life of the red blood cell. Hemoglobin remains viable for long periods under proper storage conditions (*2*), but the red cell membrane tends to leak or rupture after a few weeks. The membrane is also the site of the antigens that determine blood type and endanger the patient in the event of a mismatched transfusion. The hemoglobin is only mildly antigenic (*3–5*). Almost all of the red cell's antigenic sites are on the membrane (*6*). Therefore, hemoglobin with membrane components (stroma) carefully removed is considered a viable blood substitute (*7*).

The need to encapsulate SFHS arises because of its short intravascular persistence. The half life of SFHS in vivo is only about 2 h because of dissociation of the hemoglobin tetramer into dimers that are excreted by the kidneys, leak through vascular walls, or are bound by haptoglobin (*8*). Most of the technology for crosslinking hemoglobin was gained in efforts to prevent the dissociation of the tetramer or to form hemoglobin polymers that would remain in the circulation for extended periods (*9,10*).

Encapsulation is also viewed as a potential solution to the problem of high oxygen affinity of SFHS that results in poor release of oxygen to the tissues. Kaplan and Murthy found that SFHS, added as a partial replacement for red cells, did not contribute substantially to tissue oxygenation until the erythrocytes had released about 50% of their oxygen capacity (*11*). Messmer et al. attributed the high oxygen affinity of SFHS to lack of 2,3-diphosphoglycerate (DPG) and of the intraerythrocyte ionic milieu, low hemoglobin concentration in comparison to the red-cell contents,

and the difference between plasma and intracellular pH (7.4 vs 7.25) (*12*). Restoration of the intracellular environment of the hemoglobin is a major goal in the development of artificial red cells. Other methods might be used to increase the availability of oxygen. Since hemoglobin is only mildly antigenic across some species, bovine SFHS, which does not require DPG, might be an alternative if the artificial membrane is permeable to DPG (*13*). The attachment of pyridoxal phosphate to hemoglobin to reduce oxygen affinity is also effective (*14*).

In view of the limitations of SFHS and the well-publicized success of fluorochemical emulsions for blood substitutes, one might question the importance of efforts to develop artificial red cells containing SFHS. Indeed, the fluorochemical emulsion marketed by Green Cross appears to be well-tolerated by patients, but adequate tissue oxygenation is only achieved if the patients breathe oxygen at very high concentrations (*15*). Fluorochemical emulsions and blood have about the same oxygen carrying capacity at pO_2 of 760 torr, but the fluorochemical emulsions have only 15% of this capacity when equilibrated with alveolar air (pO_2 = 100 torr). Moreover, the studies of Wesslar et al. indicate poor prospects for developing fluorochemicals with substantially higher oxygen capacity (*16*). Therefore, fluorochemical emulsions are not likely to become widely accepted blood substitutes.

The objective of the research described herein was to develop a biocompatible membrane to encapsulate SFHS in red cell-size packages that would remain in the circulation long enough to support a patient through a crisis and allow replenishment with natural red cells. Then the artificial red cells, including the membranes, would be metabolized. Other researchers have worked toward the same objective. Kondo et al. microencapsulated hemoglobin in a variety of polymers (*17*). Earlier, Chang utilized organic phase separation to coat droplets of hemoglobin solution with cellulose nitrate, and he used interfacial polymerization of sebacoyl chloride and 1,6-hexamethylenediamine (HMDA) to form a nylon membrane around droplets of hemoglobin solution (*18*). He also reported the formation of membranes in the absence of HMDA, which meant that the membranes must have been composed of polymerized hemoglobin (*19*). Chang utilized an anionic reagent 4,4-diaminobiphenyl-2,2-disulfonic acid to improve the dispersability of his nylon-encapsulated cells, but their intravascular persistence was short (*20*). Lim and Moss encapsulated hemoglobin in a semipermeable membrane prepared by interphase polymerization of HMDA and terephthaloyl chloride (*21*).

Liposomes have been used to encapsulate hemoglobin. Miller and Djordjevich used films of cholesterol and phospholipids (*22*). Hunt used similar materials to form micron-size liposomes for animal studies (*23*). He found that a low dosage was cleared rapidly by the reticuloendothelial system, but that mode of removal was overwhelmed by large doses.

APPROACH

The approach for this study was to use polymerized hemoglobin as the encapsulating membrane for cells containing SFHS to minimize the amount of foreign material in the cells. Glutaraldehyde was selected as the crosslinking agent, because it has been reported to cause a minimum of denaturation of proteins (24). Moreover, hemoglobin crosslinked with glutaraldehyde was reported by Bonsen et al. to retain its capacity for oxygen transport (*10*).

The technique for forming the artificial red cells was adapted from the liquid membrane formulations developed by Asher for removing urea *via* the gastro intestinal tract (*25*). By selecting the proper surfactants, he was able to encapsulate urease in liquid membranes that released or ejected their contents when they reached the small intestine. Another surfactant formulation produced stable liquid membranes encapsulating citric acid, the sorbent for the ammonia formed when urease decomposed urea. These sorbent liquid membranes remained intact for their entire passage through the gastrointestinal tract.

The preparative technique for artificial red cells is illustrated in Fig. 1. The hemoglobin solution was prepared and purified by the crystallization technique developed by DeVenute et al. (*26*). The SFHS was emulsified in an isoparaffinic mineral oil containing an oil-soluble surfactant. Liquid Membrane Capsules (LMC) were formed when the SFHS-in-oil emulsion was injected into the aqueous suspending phase under conditions of low shear. When glutaraldehyde, the water-soluble crosslinking agent, was added to the aqueous suspending phase, it began to diffuse through the oil and react with the hemoglobin at the surface of each microdroplet. After about 3 min the reaction was quenched by addition of an amine to the suspending phase to react with the residual glutaraldehyde. Then the LMC were rinsed repeatedly with saline. The artificial cells remained suspended in the oil during the rinsing operation.

To recover the cells from the oil, a saline solution containing a water-soluble surfactant was added to the system with agitation. This surfactant counteracted the effects of the oil-soluble surfactant and caused the artificial cells to be ejected from the LMC into the aqueous suspending phase. The oil, which still contained the oil-soluble surfactant and some of the smallest cells, was aspirated off. The cells were washed by repeated centrifugation and resuspension in saline to remove residual oil and surfactants.

Surfactant Studies

Surfactants play a vital role in the preparation of artificial red cells. An oil-soluble surfactant is needed to make the SFHS-in-oil emulsion. Without the surfactant, rapid stirring would be needed to keep the SFHS

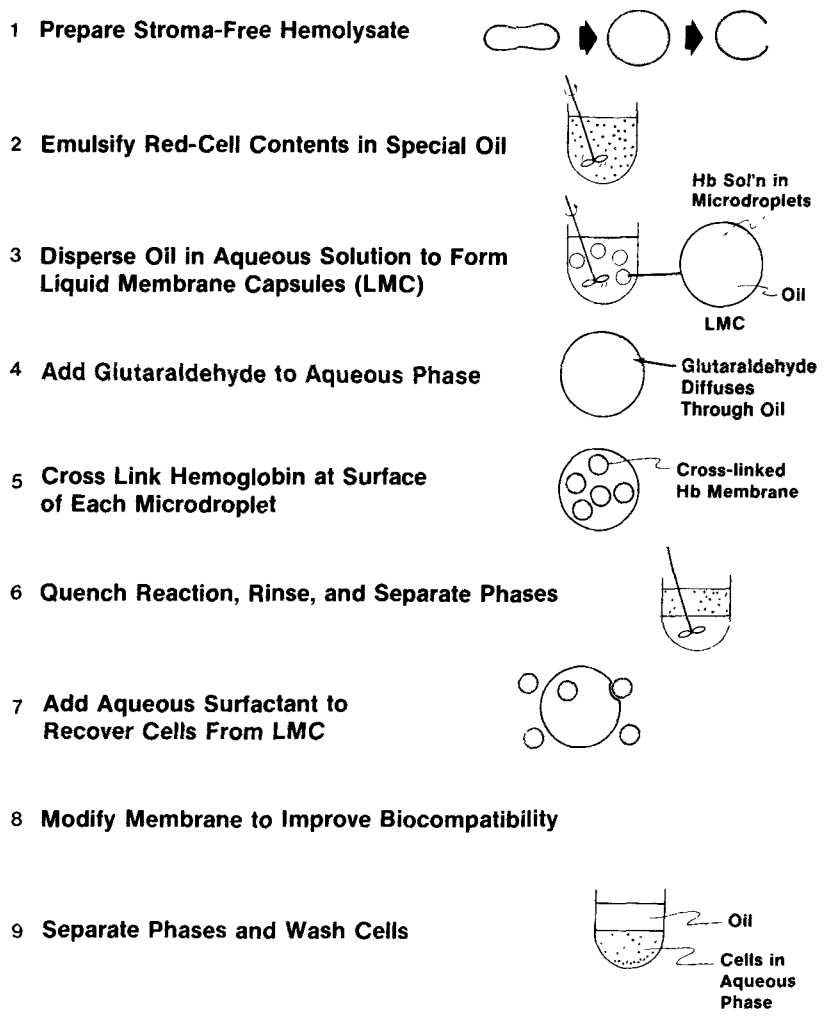

Fig. 1. Process steps in preparation of artificial red cells.

dispersed as red cell-size microdroplets. Attempts to form cells by addition of glutaraldehyde solution to a stirred suspension of SFHS and oil led to massive gellation of the SFHS and no distinguishable cells. With a suitable surfactant, the microdroplets remained suspended in a quiescent environment within the LMC while their surfaces were being crosslinked with glutaraldehyde.

The oil-soluble surfactant found to be best for LMC formation has the approximate structure:

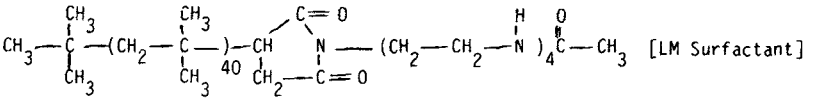

TABLE 1
Surfactants Tested for Preparing Artificial Red Cells

Designation	HLB	Supplier	Effectiveness: Emulsifying SFHS in oil	Effectiveness: Ejecting cells
LM surfactant	—	Exxon	Best	—
Span 80	4.3	ICI Americas Inc.	Poor	—
Santone 10-10-0	2.0	Durkee Industrial Food Group	Fair	—
Cholesterol	2.0	Aldrich Chemical Co.	Poor	—
Alcolec PG	—	American Lecithin Co.	Fair	—
Triton X-100	13.5	Rohm and Haas Co.	—	Best
Renex 30	14.5	Witco Chemical Co.	—	Good
Tween 21	13.3	ICI Americas Inc.	—	Poor
Tween 20	16.7	ICI Americas Inc.	—	Poor

The long polyisobutylene chain makes the LM surfactant particularly compatible with the isoparaffinic mineral oil used to make the emulsion. However, it was not FDA approved, so other surfactants that are commonly used in food and pharmaceuticals were tested. Their source and properties are shown in Table 1.

The surfactants were dissolved in oil and stirred with SFHS in a Waring Blendor. Emulsions made with Span 80 and with cholesterol were too unstable to allow cell preparation. Useful emulsions were produced with the Santone and Alcolec PG (lecithin), but the artificial cells produced with those emulsions appeared to clump more than those made with the LM surfactant. The lecithin used in this test was a purified mixture of soy phosphatides. Further purification and evaluation of lecithin would be expected to yield fractions that would be more effective for cell formation.

The ejection of cells from the LMC is accomplished by adding a hydrophilic (high HLB) surfactant. Four commercial surfactants were tested to determine their effectiveness in ejecting cells from LMC made with the LM surfactant. The results are shown in Table 1. Triton X-100 (octylphenoxy polyethoxy ethanol) was the most reliable ejecting surfactant tested. Renex 30 (polyoxyethylene ether alcohol) was sometimes as effective as Triton X-100, but the product was not consistent. However, Triton X-100 is not considered the ideal ejecting surfactant because it is reported to be incompatible with blood (*27*).

During the course of these studies cells were made from both a concentrated (30%) SFHS prepared by DeVenuto and a dilute (~10%) bovine hemolyzate that was extracted with toluene and centrifuged after the method of Bonsen (*10*). Cells containing dilute hemolysate were easily dispersed in saline while those containing concentrated SFHS tended to

clump when they were rinsed repeatedly with saline to remove the last traces of surfactant. Evidently the membranes of the latter were less hydrophilic, and a surfactant was needed to keep them dispersed in saline.

Cell-Size Distribution

In early developmental work the artificial red cells were made in a 120 mL glass jar fitted with a variable-speed stirrer. SFHS-in-oil emulsions were prepared with a high-speed stirring (6000 rpm) and LMC were made with low-speed stirring (400 rpm). The SEM of cells made with this technique is shown in Fig. 2. Particle-size studies with a Coulter Counter (*see* Fig. 3) revealed that these cells had a mean diameter of 11.05 μm (±3.8 μm SD) that would be too large to pass through human capillaries. When a Waring Blendor was used to make the SFHTS-in-oil emulsion, the cells had a mean diameter of 2.52 μm (±1.69 μm SD), which should pass through the smallest human capillaries. Smaller diameters were obtained with higher Blendor speeds.

Oxygen Transport Characteristics

Concentrated (30%) SFHS that had been treated with pyridoxal phosphate was used to make artificial red cells for evaluation of their oxygen transport characteristics. The biotonometry apparatus developed by Neville was used to study oxygen-carrying capacity and oxyhemoglo-

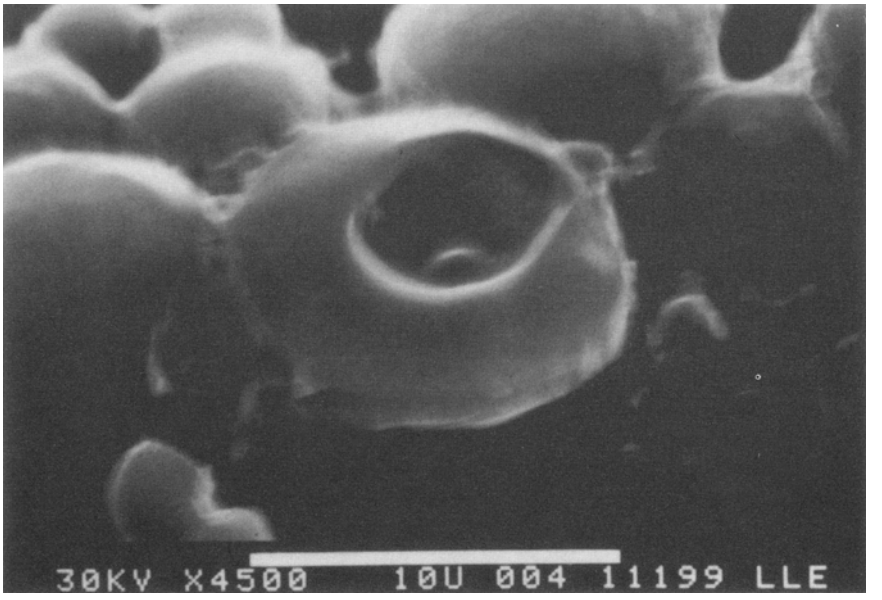

Fig. 2. Shell of crosslinked hemoglobin in a scanning electron micrograph.

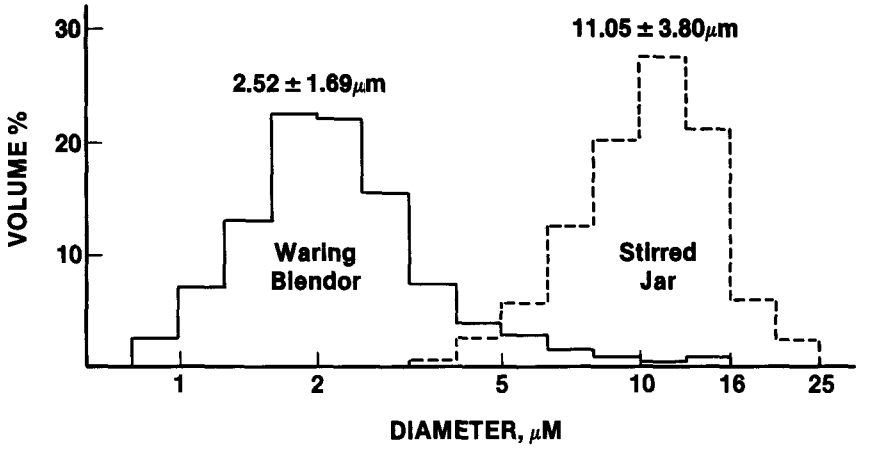

Fig. 3. Cell size distribution.

bin dissociation (*28*). The oxyhemoglobin dissociation curves at various stages in the preparative procedure are shown in Fig. 4, and the values of P_{50} and O_2 capacity are shown in Table 2. The process of hemolysis, crystallization, and pyridoxalation reduced both the P_{50} and O_2 capacity. Agitation and contact with the surfactants (without glutaraldehyde)

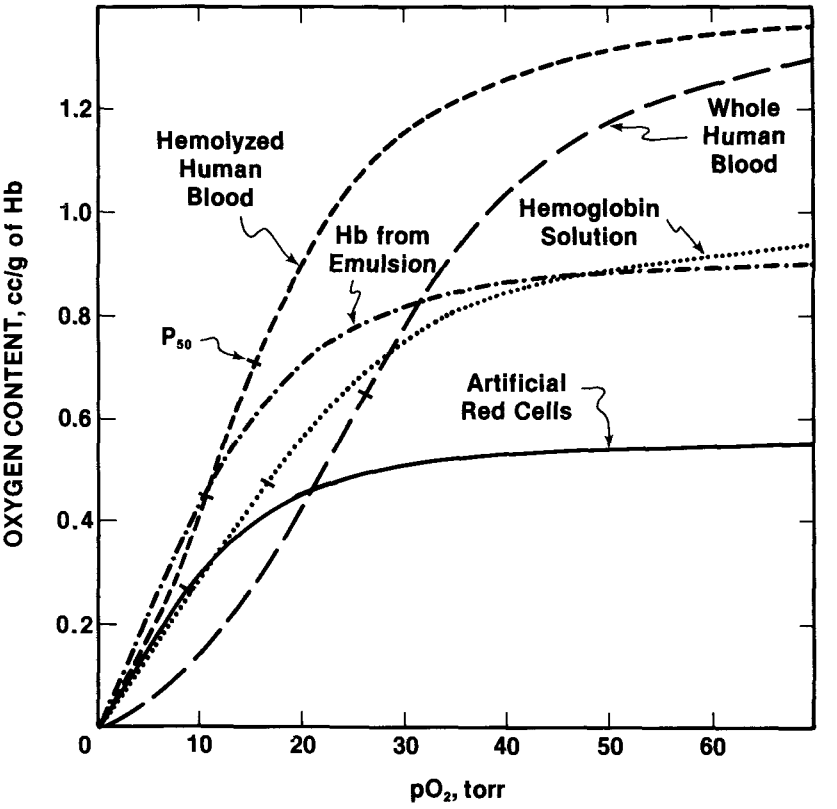

Fig. 4. Oxyhemoglobin dissociation at various stages of cell preparation.

TABLE 2
Hemoglobin Activity at Various Stages of Cell Preparation

	P_{50}, torr	O_2 capacity, mL/g
Human blood	25	1.34
Hb solution	16	0.96
Hb removed from emulsion	10	0.90
Artificial red cells	8.9	0.55

lowered the P_{50} substantially with only a minor reduction in O_2 capacity. Addition of the glutaraldehyde caused a minor reduction in P_{50} and a major loss of O_2 capacity.

DISCUSSION

The results of these experiments indicate that the liquid membrane technique allows the use of a water-soluble crosslinking agent for preparing artificial red cells with polyhemoglobin membanes. The cells can be made small enough to pass through human capillaries, but their compatibility with the vascular system has yet to be proven. A study published elsewhere showed that the cells degraded in the peritoneal cavity of the rat, but the metabolism of the crosslinked hemoglobin is unknown (29).

The surfactants and the crosslinking agent, glutaraldehyde, appear to damage the hemoglobin. The surfactants likely cause denaturation of the hemoglobin where even subtle changes in the tertiary structure can reduce the P_{50}. Surfactants that are more compatible with hemoglobin are being sought. The reaction of glutaraldehyde with the hemoglobin is not sufficiently rapid or irreversible to prevent its penetration into the interior of the cell where it reacts with hemoglobin that is not needed for the membrane.

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REFERENCES

1. Doenicke, A., Grote, B., and Lorenz, W. (1977), *Br. J. Anaesth.* **49,** 681.
2. Rabiner, S. F. (1975), *Fed. Proc.* **34,** 1454.
3. Sasazuki, T. (1977), *Immunol.* **8,** 695.
4. Chernoff, A. I. (1953), *Blood* **8,** 399.

5. Garver, F. A. (1975), *J. Immunol. Methods* **1,** 271.
6. Cochin, A., Gupta, T. K. D., DeWoskin, R., and Moss, G. S. (1974), *J. Surg. Oncol.* **6,** 19.
7. Savitsky, J. P., Doczi, J., Black, J., and Arnold, J. D. (1978), *Clin. Pharmacol. Ther.* **23,** 73.
8. Nagel, R. L., and Gibson, Q. H. (1971), *J. Biol. Chem.* **246,** 69.
9. Mazur, A. (1975), US Patent 3,925,344.
10. Bonsen, P., Laver, M. B., and Morris, K. C. (1977), US Patent 4,001,401.
11. Kaplan, H. R., and Murthy, V. S. (1975), *Fed. Proc.* **34,** 1461.
12. Messmer, K., Jesch. F., Schaff, J., Schonberg, M., Pielsticker, K., and Bonhard, K. (1978), *Proc. Clin. Biol. Res. ISS Blood Substitutes and Plasma Expanders,* **19,** 175.
13. Bunn, H. F., Seal, U. S., and Scott, A. F. (1974), *Ann. NY Acad. Sci.* **241,** 498.
14. Benesch, R. E., Benesch, R., Renthal, R. D., and Maeda, N. (1972), *Biochemistry* **11,** 3576.
15. Sloviter, H. A. (1975), *Fed. Proc.* **34,** 1484.
16. Wesseler, E. P., Iltis, R., and Clark, L. C. (1977), *Fluorine Chem.* **9,** 137.
17. Kondo, A., Kitajima, M., and Miyano, S. (1970), Japan Patent 70 02,757.
18. Chang, T. M. S. (1972), *Artificial Cells,* Thomas, Springfield, IL.
19. Chang, T. M. S., MacIntosh, F. C., and Mason, S. C. (1966), *Can. J. Physiol. Pharmacol.* **44,** 115.
20. Chang, T. M. S. (1965), PhD Thesis, McGill University.
21. Lim, F., and Moss, R. D. (1978), Ger. Offen. 2,645,730.
22. Miller, I. F., and Djordjevich L. (1979), US Patent 4,133,874.
23. Hunt, C. A. (1980), Unpublished results presented at Current Concepts of Combat Casualty Resusitation Symposium, Bethesda MD, 15-19 Sept.
24. Hopwood, D. (1969), *Histochem. J.* **1,** 323.
25. Asher, W. J., Vogler, T. C., Bovee, K. C., Holtzapple, P. G., and Hamilton, R. W. (1977), *Trans. Am. Soc. Artif. Intern. Organs* **23,** 673.
26. DeVenuto, F. (1978), *J. Lab. Clin. Med.* **92,** 946.
27. Helenius, A., and Simons, K. (1975), *Biochem. Biophys. Acta* **415,** 29.
28. Neville, J. R. (1974), *J. Appl. Physiol.* **37,** 967.
29. Davis, T. A. Asher, W. J., and Wallace, H. W. (1982), *Trans. Amer. Soc. Artif. Int. Organs* **28,** 405.