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ARTIFICIAL RED CELLS
WITH POLYHEMOGLOBIN MEMBRANES

FINAL REPORT

BY

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Artificial red cells were prepared with polyhemoglobin membranes. Red-cell-size microdroplets containing 30% of hemoglobin were held in liquid membrane capsules and treated with glutaraldehyde that cross linked the hemoglobin at the surface of each microdroplet. A water-soluble surfactant was used to eject the cells from the oil and suspend them in saline. The hemoglobin was retained by the artificial red-cell membrane, but biotonometry revealed that it held oxygen more tenaceously after encapsulation.			

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I. SUMMARY

In support of the Army's program to improve the intravascular persistence of hemoglobin solution used as a blood substitute, artificial red cells with membranes of cross-linked hemoglobin were prepared by a technique that utilized Exxon's Liquid Membrane technology in a different manner than in previous applications of this technology to biomedical programs. Hemoglobin solution was emulsified in an isoparaffinic oil phase with a surfactant to form red-cell-size microdroplets containing 30% of hemoglobin, and this emulsion was injected into a stirred aqueous solution of glutaraldehyde to form a double emulsion. Within 3 minutes a polyhemoglobin film formed around each microdroplet. After the emulsion was washed to remove unreacted glutaraldehyde, a water-soluble surfactant, Triton X-100, was added to eject the cells from the oil.

Research efforts were directed toward selecting surfactants, establishing preparative conditions, and evaluating the physical characteristics of the cells. A Waring Blendor was found to be effective for making emulsions with most of the microdroplets 1-4 μ m in diameter, and the resulting artificial red cells had a similar size distribution. A surfactant was required to keep the cells dispersed in saline solution.

Biotometry revealed that less than half of the encapsulated hemoglobin released oxygen. The release occurred at low oxygen tension (P_{50} of 5.3 to 7.7 mm of Hg), and the dissociation curve was no longer sigmoidal. When pyridoxalated hemoglobin was used, the P_{50} was 8.9 mm of Hg. Poor oxygen release was attributed to excessive penetration of glutaraldehyde into the interior of the cells. Investigation of cross-linking agents with greater reactivity or lower water solubility is recommended as an approach to solving the problem of excessive penetration.

FOREWORD

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II. INTRODUCTION

The research reported herein was carried out as part of the Army's program to utilize hemoglobin solution as a blood substitute. A major emphasis in that program is the modification of hemoglobin to improve its oxygen-release characteristics and to increase the time that it remains in circulation. Encapsulation in an artificial red-cell membrane would prevent the loss of hemoglobin, and it might allow retention of the phosphate compounds that promote oxygen release. Minimum criteria for artificial red cells are: compatibility with the blood and the circulatory system, particles small enough to pass through the capillaries, and adequate oxygen transport properties. Since biocompatibility was considered the most difficult criterion to establish and satisfy, it was deferred so that the other two criteria could be addressed.

The approach for this study was to encapsulate hemoglobin solution in a membrane of polymerized, cross-linked hemoglobin. Glutaraldehyde was chosen as the cross-linking agent because it has been studied extensively by other investigators. Exxon's liquid membrane technology was utilized in the preparative procedure because it offers a unique method of treating preformed microdroplets of hemoglobin with a water-soluble agent under quiescent conditions that do not disturb the droplet surface as the polyhemoglobin membrane is being formed. The technology is used quite differently in this program than in its previous application for the oxygenation of blood⁽¹⁾ and the removal of uremic toxins via the gastrointestinal tract.⁽²⁾

III. LITERATURE REVIEW

Stroma-free hemoglobin solution (SFHS) has been evaluated extensively as an oxygen carrier and oncotic agent. Problems of intravascular coagulation and renal damage that were reported in early studies with intravenously injected hemolysate were shown by Palani et al. to be caused by stroma, not the hemoglobin molecule.⁽³⁾ Procedures for preparation of SFHS generally involve hemolysis, centrifugation, filtration, and dialysis. The method of Rabiner is often employed.⁽⁴⁾ De Venuto et al. described a technique for crystallizing hemoglobin to improve the purity.⁽⁵⁾ The major contaminant in their product was carbonic anhydrase. A commercial process for preparation of SFHS was developed by Warner Lambert Corp., but the product is not being marketed.⁽⁶⁾ Their product was used in clinical safety trials by Savitsky

et al.⁽⁷⁾ The only adverse effects were mild hypertension, decreased urine output, and mild prolongation of activated partial thromboplastin time. SFHS has been administered in Russia to patients after thoracic surgery with no ill effects reported.⁽⁴⁾ Thus, SFHS appears to be safe for human use.

The most severe shortcomings of SFHS as a whole-blood substitute are its high oxygen affinity and poor intravascular persistence. Kaplan and Murthy studied the oxygen-carrying capacity of SFHS at various levels of red-cell replacement. They found that extraerythrocyte hemoglobin did not contribute substantially to tissue oxygenation until the erythrocytes had unloaded about 50% of their oxygen capacity.⁽⁸⁾ 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).⁽⁹⁾ Teisseire et al. also investigated these effects.⁽¹⁰⁾ They found a P_{50} of 27 torr for 30% hemoglobin versus 13.56 torr for 7.3% hemoglobin. Reducing the pH from 7.4 to 7.2 increased P_{50} from 13.56 to 16.5. DPG depletion of stored red cells reduced their P_{50} from 29.9 to 17.52 torr. Benesch et al. found that deoxy - but not oxyhemoglobin - binds DPG, and the binding levels off at 1 mole of DPG per mole of hemoglobin tetramer.⁽¹¹⁾ Messmer et al. observed significantly higher P_{50} in vitro with DPG addition to SFHS, but P_{50} was not enhanced in vivo because DPG is eliminated almost instantaneously from plasma into urine and thoracic duct lymph.⁽⁹⁾ Incorporation of DPG or another active phosphate compound into artificial red cells containing human hemoglobin could alleviate the problem of poor oxygen release in SFHS.

The effect of DPG on hemoglobin is not universal. Benesch et al. found that DPG scarcely altered the oxygen affinity of sheep hemoglobin.⁽¹¹⁾ Bunn et al. studied DPG content and hemoglobin - oxygen dissociation in 71 mammalian species.⁽¹²⁾ They found that cats and ruminants had low DPG content in their red cells and hemoglobin with low oxygen affinity that was not affected greatly by DPG. The other mammals required DPG in their red cells to achieve low oxygen affinity. This suggests that bovine hemoglobin would be useful in artificial red cells.

Benesch et al. showed that pyridoxal phosphate is analogous to DPG in decreasing the oxygen affinity of hemoglobin.⁽¹³⁾ In the studies of Messmer et al., attachment of pyridoxal phosphate to hemoglobin increased

P₅₀ from 24 to 34 torr but did not improve intravascular persistence.⁽⁹⁾ Jesch *et al.* found that pyridoxalation of hemoglobin reduced its half time for intravascular persistence ($t_{1/2}$) from 140 to 100 minutes.⁽¹⁴⁾ Therefore, pyridoxalation provides only a partial solution to the shortcomings that limit the use of SFHS, but it may be useful in artificial red cells.

The short $t_{1/2}$ of free hemoglobin in the blood is due to leakage through vascular walls, urinary excretion, and haptoglobin binding. Nagel and Gibson found that hemoglobin tetramer is incapable of binding haptoglobin, and its dissociation to dimers is a prerequisite for the binding reaction.⁽¹⁵⁾ Dissociation is also necessary for urinary excretion. Therefore, considerable effort has gone into cross linking hemoglobin to prevent dissociation of the tetramer. The use of imidoesters for protein modification was introduced by Hunter and Ludwig in 1962.⁽¹⁶⁾ They pointed out that imidoesters do not neutralize the charge of primary amine groups with which they react. Therefore, the cross-linked protein remains water soluble. Waterman *et al.* found dimethyl adipimidate to be a powerful anti-sickling agent due to its ability to react with hemoglobin S inside sickle red cells and inhibit the aggregation process.⁽¹⁷⁾ Mazur patented a process for intermolecular cross linking of hemoglobin with diimidoesters to prevent dissociation of the tetramer.⁽¹⁸⁾ He found that longer chains (e.g. sebacimidate) between reactive groups were associated with more intramolecular cross linking, while short chains (e.g. malonimidate) produced none. Mok, Chen and Mazur reported high intermolecular cross linking in 10% hemoglobin solution with diethyl malonimidate.⁽¹⁹⁾ They reported $t_{1/2}$ of 27 hours for this hemoglobin polymer in rabbits compared to $t_{1/2}$ of 2 hours for normal SFHS. Haptoglobin binding of the cross-linked hemoglobin was also reduced.

Morris *et al.* disclosed a method for intramolecular cross linking of hemoglobin.⁽²⁰⁾ They observed that the P₅₀ was substantially higher with deoxyhemoglobin than with oxyhemoglobin. Bonson *et al.* disclosed the use of bifunctional cross-linking agents to achieve soluble macromolecules containing about six hemoglobin tetramers.⁽²¹⁾ Their P₅₀ values ranged from 2.5 to 120 torr, but the oxygen dissociation curves were hyperbolic (like myoglobin) rather than sigmoidal. These studies, though limited in their success toward improving the characteristics of SFHS, have provided considerable insight into the reactivity of hemoglobin with cross-linking agents, and much of this information is applicable to the formation of artificial red-cell membranes of polyhemoglobin.

Encapsulation of hemoglobin in solutions has been reported by several investigators. 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.⁽²²⁾ He also reported the formation of membranes in the absence of HMDA, which meant that the membrane must have been composed of polymerized hemoglobin.⁽²³⁾ Chang utilized an anionic reagent 4,4'-diaminobiphenyl-2,2'-disulfonic acid to improve the dispersability of his nylon cells, but their intravascular persistence was short.⁽²⁴⁾

Kondo et al. microencapsulated SFHS in polymers such as polystyrene, polyamide, and polyisobutylene but did not discuss the fate of the polymers.⁽²⁵⁾ Miller and Djordjevich encapsulated hemoglobin in liposomes with film composed of cholesterol and phospholipids.⁽²⁶⁾ A P₅₀ of 32 torr was measured for liposomes containing 22% of hemoglobin. Miller made no mention of the tendency of liposomes to invade other cells and what effect this phenomenon might have on the intravascular persistence and ultimate fate of the encapsulated hemoglobin. Lim and Moss encapsulated hemoglobin in a semipermeable membrane prepared by interphase polymerization of HMDA and terephthaloylchloride.⁽²⁷⁾ This nylon membrane would likely be resistant to biodegradation. Buchs et al. mentioned that they are investigating the use of liquid-membrane capsules to prevent the loss of hemoglobin solution in organ preservation.⁽²⁸⁾

IV. PREPARATIVE METHOD

The method employed for preparing artificial red cells is illustrated in Figure 1. The starting material is hemoglobin solution taken from natural red cells in Step 1. The red cell contents are injected into a rapidly stirred oil phase in Step 2 to form a hemoglobin-in-oil emulsion containing equal volumes of aqueous and oil phases. The isoparaffinic mineral oil contains a special surfactant that prevents coalescence of the red-cell-size microdroplets of hemoglobin solution.

Liquid Membrane Capsules (LMC), illustrated in Step 3, are formed when the hemoglobin-in-oil emulsion is injected into an aqueous suspending phase under conditions of low-shear agitation. The special oil-soluble surfactant used to make the hemoglobin-in-oil emulsion effectively prevents leakage of hemoglobin from the microdroplets into the aqueous suspending phase. Glutaraldehyde, a water-soluble cross-linking

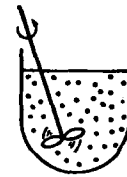
Figure 1

PROCESS STEPS IN PREPARATION OF ARTIFICIAL RED CELLS

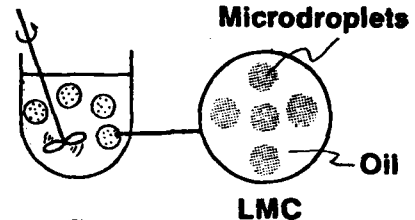
1 Prepare Stroma-Free Hemolysate



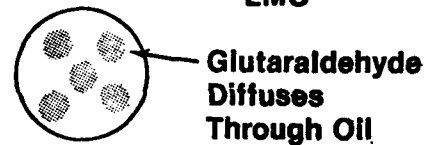
2 Emulsify Red-Cell Contents in Special Oil



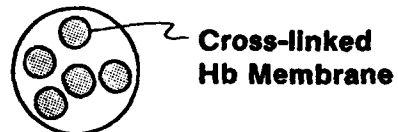
3 Disperse Oil in Aqueous Solution to Form Liquid Membrane Capsules (LMC)



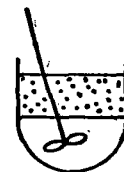
4 Add Glutaraldehyde to Aqueous Phase



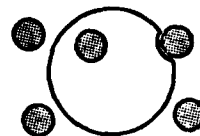
5 Cross Link Hemoglobin at Surface of Each Microdroplet



6 Quench Reaction, Rinse, and Separate Phases

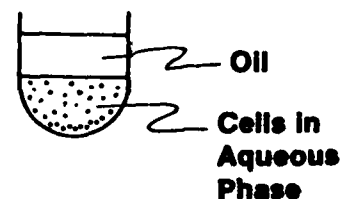


7 Add Aqueous Surfactant to Recover Cells From LMC



8 Modify Membrane to Improve Biocompatibility

9 Separate Phases and Wash Cells



agent for hemoglobin, is added to the suspending phase in Step 4. Since it is somewhat soluble in the oil phase, the glutaraldehyde diffuses through the oil and cross links the hemoglobin at the surface of each microdroplet in Step 5.

The LMC provide a quiescent environment for the formation of a uniform shell of polyhemoglobin that serves as the artificial red-cell membrane. Membranes of suitable strength are formed in about 3 min. The reaction is quenched in Step 6 by addition of a water-soluble amine that reacts with residual glutaraldehyde. Then the emulsion is rinsed with isotonic saline. The artificial red cells are still suspended in the oil, and the aqueous suspending phase falls to the bottom whenever agitation is stopped. The rapid phase separation facilitates the quenching and rinsing operations.

The cells are recovered from the oil phase in Step 7 by addition of an aqueous solution containing a special water-soluble surfactant. This surfactant counteracts the effects of the oil-soluble surfactant and causes the artificial red cells to be ejected from the LMC into the aqueous suspending phase. When stirring is stopped and the phases separate in Step 9, the cells are suspended in the aqueous phase at the bottom of the reaction vessel. The oil is aspirated off, and the cells are washed by repeated centrifugation and resuspension in isotonic saline to remove residual oil and surfactants.

V. STUDIES OF CELL-SIZE DISTRIBUTION

The ultimate size of the artificial red cells is determined by the size of the microdroplets formed when the hemoglobin solution is emulsified in oil (Step 2 of Figure 1). While some adjustment in cell-size distribution could be made after Step 9, such an adjustment would reduce the yield of cells. The objective was to make all of the cells with diameters in the range of 1-4 μm . Cells larger than 4 μm would not pass through the smallest capillaries in the human body, and cells smaller than 1 μm would contain a large fraction of cross-linked hemoglobin, which does not release oxygen readily.

A. Methods of Cell-Size Determination

A light microscope was used routinely to examine samples of emulsions, LMC, and artificial red cells. The sizes of droplets and cells were determined by measurements on photomicrographs of the samples. This technique was simple and often provided valuable information not revealed by automatic devices, but it was time-

consuming and potentially misleading for quantitative determination of size distributions.

The Sedigraph® was useful for analysis of droplet-size distribution of the hemoglobin-in-oil emulsion, but it was ineffective for suspensions of artificial red cells in saline. The Sedigraph scans a sample of suspended particles with x-rays and relies on a difference between the x-ray transmission of the phases as well as the difference in their densities to determine sedimentation rates that are used to calculate droplet-size distribution. Although the artificial red cells would sediment in saline, there was not enough contrast for x-ray measurements. Sufficient contrast might have been obtained by suspending the cells in distilled water, but diffusion of salts through the artificial membrane might have changed the relative densities of the cells and the suspending phase.

The Coulter Counter® measured the conductivity of an electrolyte solution flowing through an orifice and detected minute changes as suspended particles passed through the orifice. The magnitude of each change was interpreted electronically as a particle of known diameter. The Coulter Counter was useful for measuring the size distribution of particles or droplets suspended in saline, but it would not be useful for studying a hemoglobin-in-oil emulsion, because the continuous phase must be an electrolyte.

A Model ZBI Coulter Counter was used in initial attempts to determine cell-size distribution. The ZBI had a Channelyzer® that divided the particles into 100 size channels and gave particle populations in each channel. When a suspension of artificial red cells was tested in the ZBI, the channels for sub-micron particles filled so rapidly that no useful data could be obtained in the range above 1μ m. Even though these sub-micron particles contained only a small fraction of the total mass of hemoglobin, their presence made this instrument useless for this application.

A Model TA II Coulter Counter was subsequently found to be appropriate for cell-size-distribution studies. The TA II had only 16 channels, but it presented the data as either volume or population. The small particles that upset the ZBI were insignificant in the volume output mode of the TA II.

B. Devices for Preparing Emulsions

The purpose of this phase of the study was to select the best available device for preparing emulsions of hemoglobin in oil and to determine the operating conditions that produced the greatest fraction of droplets in the 1-4 μ m size range. Three small-scale stirring devices were employed to make emulsions with equal parts of oil and hemoglobin solution.

The stirred-jar device was a custom made apparatus used in early experiments to make the artificial red cells. Its components included a 120-ml glass jar, a shaft-mounted, 3-blade, 3.75-cm-diameter, propeller-type stirrer, and a 0.32-cm-OD dip tube protruding through the screw cap which covered the jar. The positions of the dip tube and the propeller could be adjusted relative to one another and relative to the bottom of the jar to achieve some control of the shear rate. The maximum speed for smooth operation was 6,000 rpm. A 50-ml batch of hemoglobin solution was injected through the dip tube over a 2-min period into 45 g of stirred oil, and stirring was continued for an additional 3 min to prepare the emulsion for this study.

A Waring Blendor® with a single-speed, 20,000-rpm motor and a semi-micro, stainless-steel container with 25-ml minimum capacity was assembled from components in the laboratory. The hemoglobin was injected into the vortex of stirred oil through a hypodermic needle protruding through the cover. The hemoglobin solution was injected from a syringe over a 2-min period, and stirring was continued for an additional 3 min.

The Ultra-Turrax® sold by Tekmar Company was a siren-type disperser that operated with a maximum speed of 10,000 rpm. It was fitted with a 0.32-cm-OD tube to inject the hemoglobin solution inside the siren. The siren was inserted into a 300-ml glass beaker containing 75 g of oil, and 75 ml of hemoglobin solution was injected over a 2-min period while the oil was stirred at 60% of maximum speed. Stirring was continued for an additional 3 min.

C. Results of Cell-Size-Distribution Study

Emulsions were made in all three of the stirring devices under the conditions described above. Samples of emulsions made with the Waring Blendor and the Ultra-Turrax were analyzed on the Sedigraph. These Sedigraph

curves are presented in Figures 2 and 3. Artificial red cells were prepared from 25-ml batches of emulsions made with all three stirrers. The cell-size distribution of samples prepared from emulsions made with the Waring Blendor and the stirred jar were determined on the Model TA II Coulter Counter with a 50 μ m aperture. The graphs are presented in Figures 4 & 5, and the numerical data are presented in Table I.

The Sedigraph analyses shown in Figures 2 & 3 indicate that the Waring Blendor and the Ultra-Turrax produced hemoglobin-in-oil emulsions with virtually the same drop-size distribution under the conditions employed for these experiments. The data indicate that more than 80% of the hemoglobin was in droplets with diameters in the 1-4 μ m range. Moreover, with slight increases in the time or speed of mixing, it is reasonable to expect that all particles above 4 μ m could be eliminated with either type of stirrer.

Figure 4 shows the size distribution of artificial red cells made from the emulsion prepared in the Waring Blendor. These data indicate that 88% of the hemoglobin was in cells in the 1-4 μ m range. Comparison of the data in Figures 2 and 4 indicates that the droplets of the emulsion were slightly smaller than the cells that were made from them. This difference was probably due to clumping of the cells that were evaluated in the Coulter Counter. However, the close agreement of the data from the two instruments indicates that clumping was not severe.

Figure 5 shows the size distribution for artificial red cells made from the hemoglobin-in-oil emulsion prepared in the stirred jar. With 92% of the particles in the 5-20 μ m range, these cells were much too large to flow through capillaries. The scanning electron micrograph in Figure 6 shows some of the cells made from an emulsion prepared in the stirred jar. The membrane of the fractured cell appears to be about 0.5 μ m thick. Subsequent studies showed that this degree of penetration of glutaraldehyde had an adverse effect on the oxygen release from small cells.

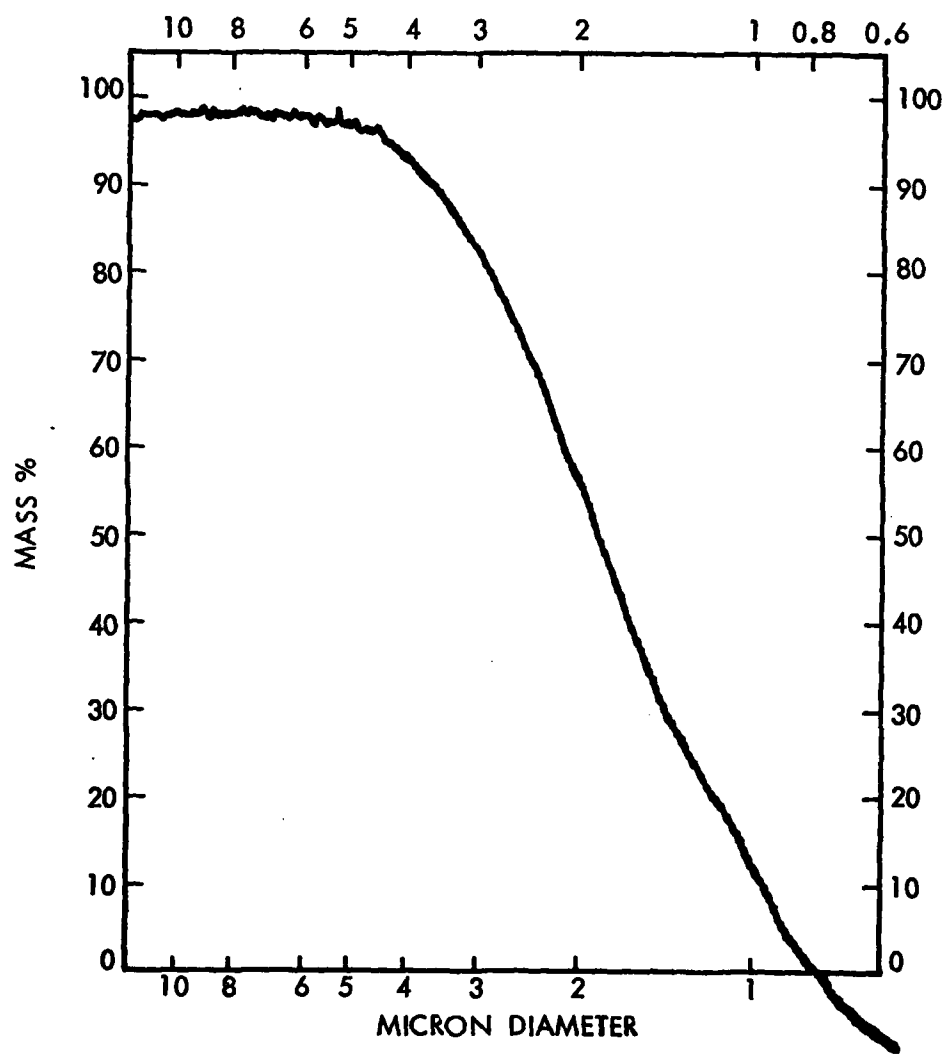


Figure 2. Sedigraph analysis of hemoglobin-in-oil emulsion made with Waring Blendor.

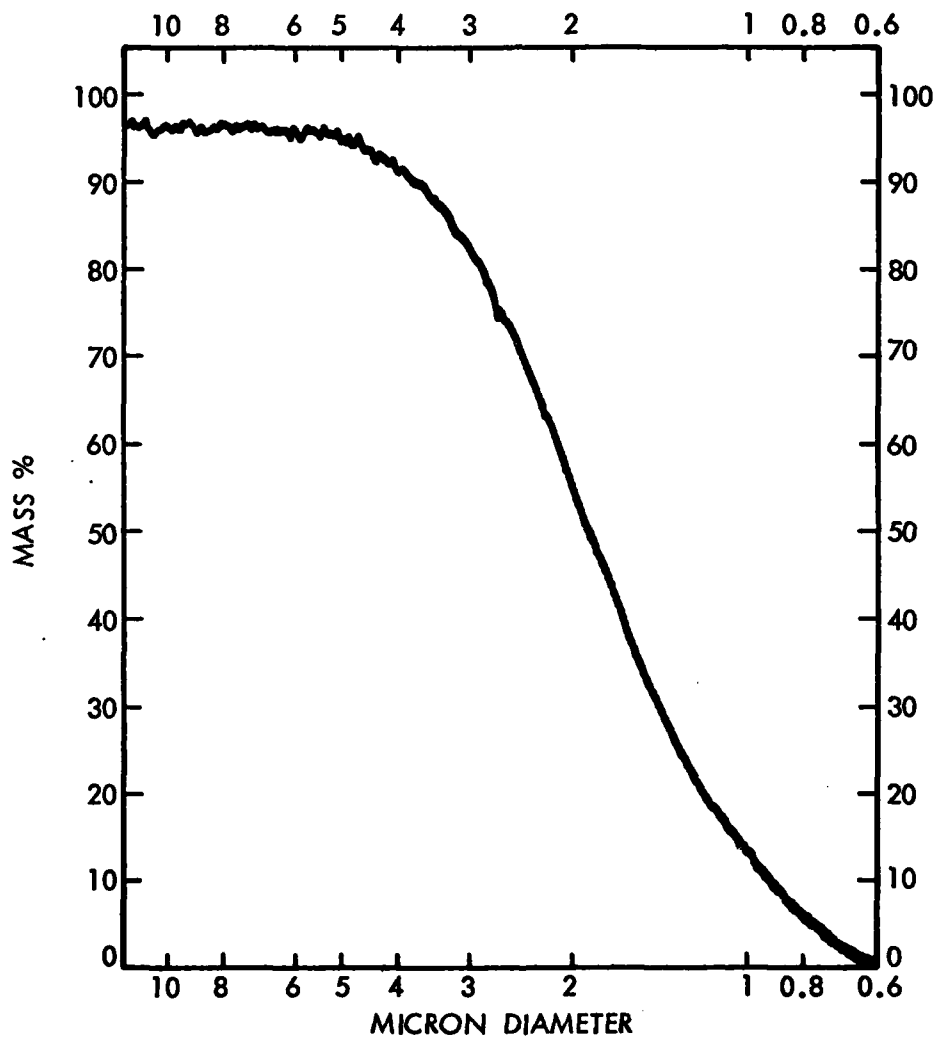


Figure 3. Sedigraph analysis of hemoglobin-in-oil emulsion made with Ultra-Turrax.

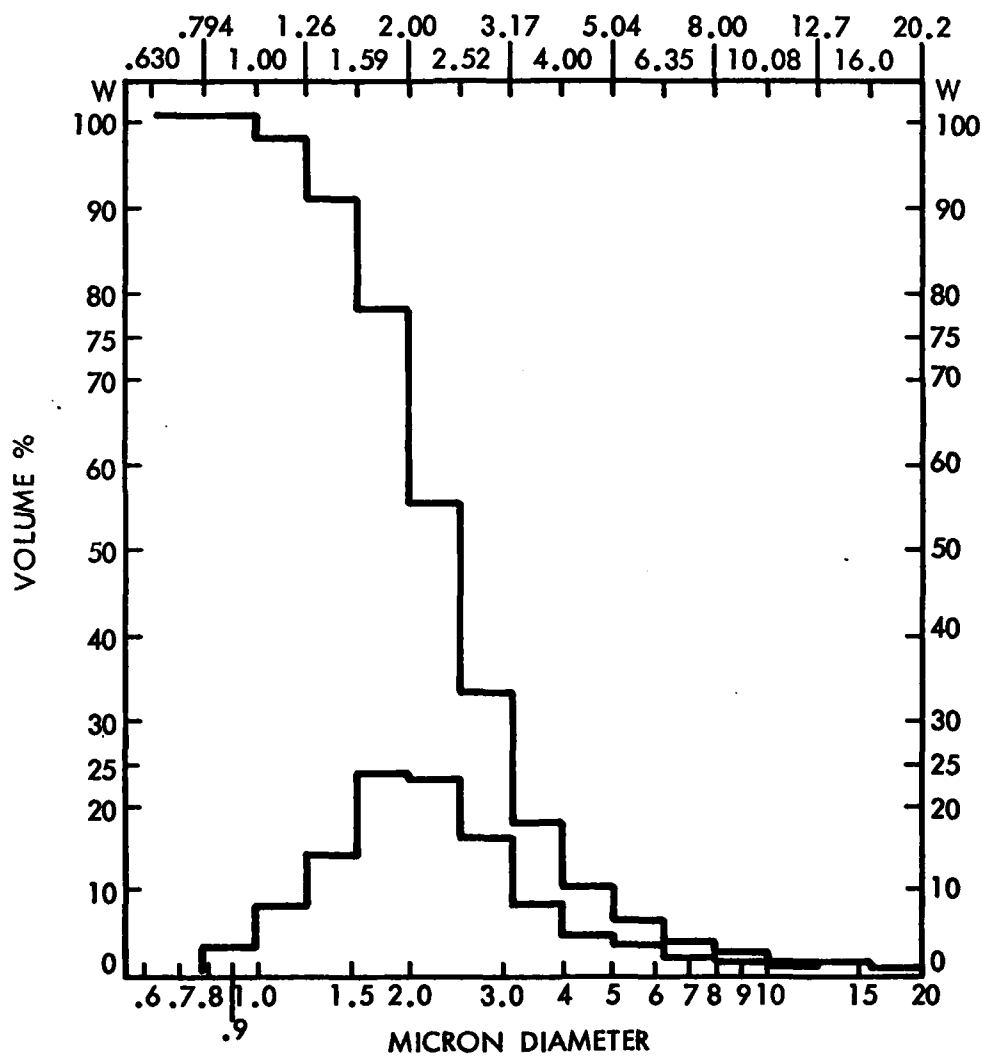


Figure 4. Coulter size distribution of artificial red cells made with Waring Blendor.

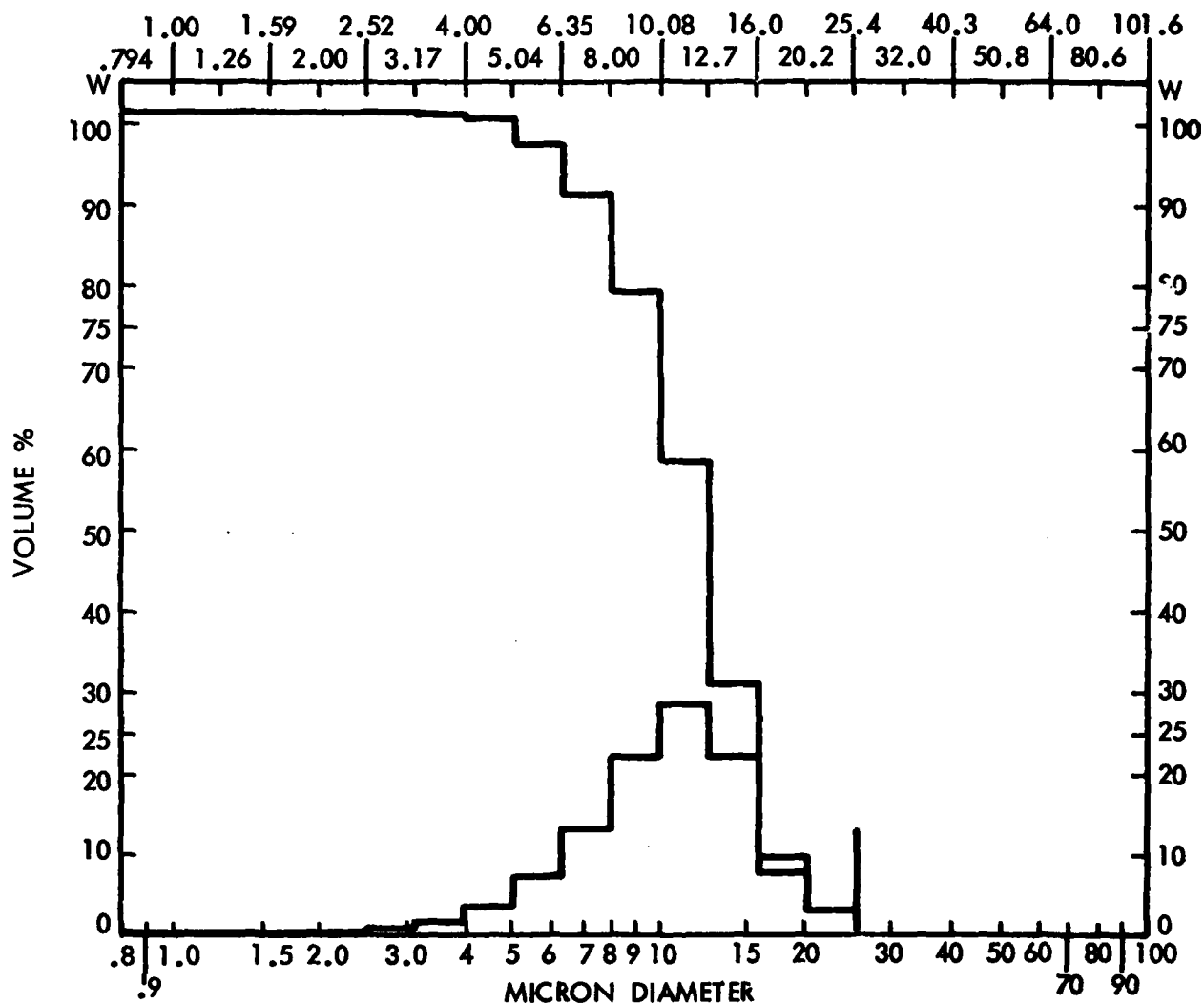


Figure 5. Coulter size distribution of artificial red cells made with stirred jar.

Table 1. Numerical Data from Model TA II Coulter Counter

Channel	<u>d μm</u>	<u>Stirred Jar</u>		<u>Waring Blendor</u>	
		<u>Cumulative</u>	<u>Differential</u>	<u>Cumulative</u>	<u>Differential</u>
1	0.630	100	0	100	0
2	0.794	100	0	100	2.5
3	1.00	100	0	97.5	7.1
4	1.26	100	0	90.4	13.0
5	1.59	100	0	77.4	22.4
6	2.00	100	0	55.0	22.2
7	2.52	100	0	32.8	15.5
8	3.17	100	0.8	17.3	7.5
9	4.00	99.2	2.7	9.8	3.9
10	5.04	96.5	6.3	5.9	2.8
11	6.35	90.2	12.1	3.1	1.4
12	8.00	78.1	20.7	1.7	0.8
13	10.08	57.4	27.4	0.9	0.1
14	12.7	30.0	21.2	0.8	0.8
15	16.0	8.8	6.8	0	0
16	20.2	2.0	2.0	0	0



Figure 6. The fracture of this sample reveals a thin film of cross-linked hemoglobin that forms the artificial red-cell membrane. These cells were prepared by a 5-min glutaraldehyde treatment of liquid membrane capsules containing hemoglobin solution.

VI. SURFACTANTS

Surfactants play a vital role in the preparation of artificial red cells. An oil-soluble surfactant is used to stabilize the emulsion of hemoglobin solution in oil in Step 2 of the preparative procedure. Without the surfactant, rapid stirring is needed to keep the hemoglobin solution dispersed as microdroplets, and such high shear rates disrupt the formation of the polyhemoglobin membranes at the surfaces of the microdroplets. The use of LMC allows the microdroplets to remain in a quiescent environment while the membranes form.

After the polyhemoglobin membranes are formed, the artificial red cells must be removed from the LMC. A second surfactant is used to counteract the stabilizing effect of the first surfactant and eject the cells from the LMC. Addition of an aqueous solution of this second surfactant causes the oil phase to coalesce and the cells to become suspended in the aqueous phase. The presence of this surfactant in the aqueous phase helps to keep the cells suspended therein.

A. Studies with Special LM Surfactant

A proprietary oil-soluble surfactant (hereinafter called the LM surfactant) was utilized in the initial experiments to prepare artificial red cells and in the studies of cell-size distribution. The LM surfactant was completely miscible in the isoparaffinic mineral oil used for the oil phase in all of the experiments. Emulsions with 1% or 4% of the LM surfactant produced cells that appeared to be of comparable size, but cells made with 0.5% of the LM surfactant at the same stirring rate appeared to be slightly larger.

The process of ejecting the cells from LMC made with 1% of the LM surfactant required agitation at 2000 rpm for 1 min to mix the water-soluble surfactant with the LMC. The ineffectiveness of slower agitation suggested that a film of cross-linked hemoglobin might have formed on the outside of the LMC. Such a film of hemoglobin could form if microdroplets near the surface of the LMC migrated into the suspending phase and the released hemoglobin immediately reacted with glutaraldehyde. To test for this leakage of microdroplets, we prepared emulsions of hemoglobin solution in mineral oil containing 1% and 4% of the LM surfactant. These emulsions were injected into a stirred saline suspending phase to form LMC. After 5 min of stirring, the emulsion was separated from the suspending phase and the hemoglobin content of the suspending phase was determined by the cyanomethemoglobin method. (29) The hemoglobin concentration was found to

be 0.5 mg/dl in the suspending phase in contact with the LMC containing 1% of the LM surfactant (0.25% of the hemoglobin in the LMC had escaped), but there was no detectable hemoglobin leakage with 4% of the LM surfactant. When the two emulsions were injected into suspending phases containing glutaraldehyde, no hemoglobin was detected in the suspending phase. Subsequent microscopic examination of the emulsion separated from the suspending phase in each experiment revealed that LMC containing 1% of the LM surfactant had not coalesced. The LMC in the other three experiments did coalesce.

The results of these experiments support a conclusion that some of the microdroplets escaped into the suspending phase when the oil contained only 1% of the LM surfactant, but 4% was sufficient to suppress this effect to undetectable levels. When the glutaraldehyde was present in the suspending phase, the hemoglobin that escaped from the LMC formed a cross-linked film on the outside of the LMC and prevented coalescence of the oil droplets when the phases separated. Apparently the strong agitation used in the ejection step ruptured the polyhemoglobin film on the outside of the LMC and allowed the water-soluble surfactant to interact with the contents of the LMC and eject the cells.

B. Other Oil-Soluble Surfactants

The special LM surfactant that was used in the studies described above was a proprietary industrial surfactant that is not commercially available. Moreover, the materials used to prepare the special surfactant are not easily purified, so preparation of a well characterized product that would satisfy the criteria of the Food and Drug Administration for intravenous use was perceived to be a last resort. Searching for a commercial surfactant that might meet FDA criteria was considered a more attractive alternative.

The following specifications were used in the selection of candidate surfactants.

- Oil solubility - The surfactant must be virtually insoluble in the hemoglobin solution to prevent its inclusion in the cells.
- Inert towards hemoglobin - Reaction or association with hemoglobin would result in inclusion of some of the surfactant in the membrane. (Such inclusion might be useful for modifying the characteristics of the membrane in subsequent refinements of the cells.)

- Non-reactive with the cross-linking agent - Reactive functional groups on the surfactant would result in its inclusion in the surface of the cell membrane. Chemical reaction of the surfactant could also alter the stability of the emulsion and cause the cells to clump.
- Non-toxic - If the above specifications apply, very little surfactant would be retained by the cells. However, complete removal cannot be presumed.

The four candidate surfactants chosen for evaluation were selected from lists of materials that are commonly used in the food and pharmaceutical industries:

- Span 80, ICI Americas Inc., sorbitan monooleate, HLB 4.3
- Santone 10-10-0, Durkee Industrial Food Group, decaglycerol decaoleate, HLB 2.0
- Cholesterol, Aldrich Chemical Co., HLB 2.0
- Alcolec PG, American Lecithin Co., purified soy phosphatides

Emulsions were prepared in the Waring Blendor by the method described in Section V-B. Useful emulsions were produced with the Santone and the lecithin, but the artificial cells produced with those emulsions appeared to clump more than those made with the LM surfactant. The emulsions made with the Span 80 and the cholesterol were not sufficiently stable to allow cell preparation.

Lecithin is an injectable surfactant and would likely be considered "safe" for this application. Therefore, further experiments were conducted to ascertain its utility in the preparation of artificial red cells. Emulsions prepared in the Waring Blendor with 4% and 15% of lecithin as the surfactant were used to make artificial red cells. No remarkable effects of lecithin concentration were noted. In both cases the coalescence of the LMC during the rinsing operation (Step 6) was slower than with the LM surfactant. There was more spontaneous ejection of the cells from the LMC during rinsing, and there was more clumping of cells ejected with the water-soluble surfactant (Triton X-100).

The observation of some spontaneous ejection of cells during this rinsing of the LMC prompted an experiment

to see if this could be enhanced to the point that a water-soluble surfactant would not be needed. Cells were made with an emulsion prepared with 15% of lecithin in the oil phase. After the cross linking, quenching, and rinsing, the LMC were suspended in saline solution and stirred at 4000 rpm for 5 min. Then the phases were allowed to separate. Most of the cells remained in the oil phase, but some cells were ejected into the aqueous phase. Since the yield was low and the ejected cells clumped, this approach was not pursued.

C. Water-Soluble Surfactants for Ejecting Cells from LMC

In Step 7 of the procedure an aqueous solution containing a water-soluble surfactant is mixed with the emulsion containing the artificial red cells. After a few seconds of stirring there is a noticeable change in the appearance of the mixture. When the stirring is stopped and the phases are allowed to separate, most of the cells are in the aqueous phase. The ejection of the cells is the result of the antagonistic effect of mixing two surfactants that are quite different in character. Oil-soluble surfactants tend to form water-in-oil emulsions, and water-soluble surfactants tend to form oil-in-water emulsions. Having both types of surfactants present in a system reduces the stability of either type of emulsion and causes a separation of the oil and water phases. The mechanism of the ejection step was elucidated by Asher, et al. who observed this phenomenon when LMC were exposed to bile and pancreatin. (30,31)

In the early stages of the program, Renex 30 (Witco Chemical Co.) was found to be a useful surfactant for ejecting the cells. However, a new batch of Renex 30 was found to be less effective than the earlier sample. This batch-to-batch variation was unacceptable, so a more reliable surfactant was sought. Since the HLB (Hydrophile-Lipophile Balance) of Renex 30 was 14.5, available surfactants with HLB close to this value, Triton X-100 (HLB 13.5), Tween 21 (HLB 13.3), and Tween 20 (HLB 16.7) were evaluated. Artificial cells were prepared by the usual procedure, and aqueous solutions of these surfactants at 6% concentration were used in the ejection step. Neither of the Tweens was effective, but Triton X-100 ejected the cells. In subsequent experiments a 3% solution of Triton X-100 was found to be adequate, and it was more effective than the Renex 30.

Although Triton X-100 was effective for ejecting the cells so that they could be evaluated in vitro in an aqueous suspension, a better surfactant is needed

for in vivo evaluations. Triton X-100 is not considered safe for injection because it is reported to extract lipids from red-cell membranes and thus promote hemolysis. (32) Ideally the surfactant for ejecting the cells should be safe for intravenous injection so that it need not be removed from the cells.

D. Dispersion of Cells

In the early stages of the program when relatively large ($10\ \mu\text{m}$) artificial red cells were being prepared, the cells ejected from the LMC were easily dispersed in the aqueous phase that contained the water-soluble surfactant. Later, when smaller cells ($< 3\ \mu\text{m}$) were being prepared for injection into rats, the cells agglomerated during repeated washing with saline to remove the surfactant. The clumping may have been due to hydrophobicity of the membrane surfaces or to the presence of unreacted aldehydes on the membranes.

To investigate the possibility that unreacted aldehydes caused agglomeration of the cells, samples of cells were treated with reagents containing reactive amine groups that were expected to react with residual aldehydes. Lysine, hexylamine and glucosamine were used both as quenching agents (Step 6) and as membrane modifying agents (Step 8). None of these agents in either mode of application affected the agglomeration of the cells, a result suggesting that there were few reactive aldehydes on the outer surfaces of the cell membranes.

When ejected cells were suspended in albumin or plasma, their tendency to agglomerate was substantially reduced. However, when the cells were subsequently washed repeatedly with saline, they agglomerated just as those that had not been treated with the protein solutions. While the albumin solution and plasma aided in the dispersion of the cells, perhaps by adsorbed protein on the cells, there was evidently no permanent binding of the proteins to the cell membranes.

VII. OXYGEN-HEMOGLOBIN DISSOCIATION

The ability of hemoglobin to pick up and release oxygen can be altered by changes either in the structure of the globin (denaturation) or in the oxidation state of the iron in the heme (methemoglobin formation). These changes might be brought about by physical damage to the hemoglobin molecules due to shear or contact with the oil and surfactants or by chemical damage due to reaction with the glutaraldehyde.

A. Experimental Methods

Biotonometry was the technique chosen for evaluating the function of hemoglobin in the artificial red cells. The fact that biotonometry would be used in the Army's evaluation of these cells was a major consideration in its selection for use in the research program. Biotonometry, as reported by Neville,⁽³³⁾ utilized yeast to remove oxygen at a constant rate from a sample of hemoglobin solution or a suspension of cells. The oxygen tension was measured by a Clark electrode in an Instrumentation Laboratory pH/Blood Gas Analyzer Model 213, and the data were recorded on a Heath Model SR-206 Dual Pen Chart Recorder.

The yeast in TRIS buffer was incubated at 37°C for 10 min in a closed 10 ml test tube. The head space was purged with oxygen (containing 5% of CO₂), and the tube was inverted several times. The sample was added immediately, and the oxygenation step was repeated. A small portion of the liquid was drawn into the pH cell, and the remainder was poured into a flat-bottom vial that contained a magnetic stir bar and was maintained at 37°C in a water bath. The oxygen electrode was inserted into the vial, and the oxygen tension was recorded until it reached zero. The disappearance curve was linear with time until the oxygen tension dropped to the level where the hemoglobin began to release oxygen. Thereafter, the deviation from linearity indicated the amount of oxygen released at a given oxygen tension.

The interpretation of the biotonometry data is illustrated in Figure. 7. The first curve is a plot of oxygen-tension decrease with a full-scale value of 500 mm Hg, and the second curve portrays the same data on an expanded scale of 0-100 mm Hg. The tangents to these curves would intersect at the zero value (point C) if the recorder pens were not offset by 4 mm. The value AC, measured in mm on the bottom scale is proportional to the time required for the yeast to completely remove the amount of oxygen dissolved in the solution at a partial pressure of 500 mm Hg if no Hb were present. A second tangent drawn from the expanded curve to point E corrects for non-linearity that is experienced as the oxygen tension approaches zero. The value CE represents the total amount of oxygen released by the Hb. Values of 30, 50 and 70% of CE are calculated and plotted on the graph. (The oxygen tension at which the line CD and the curve DE are separated by the distance $0.3 \times CE$ is the uncorrected value of P₇₀.)

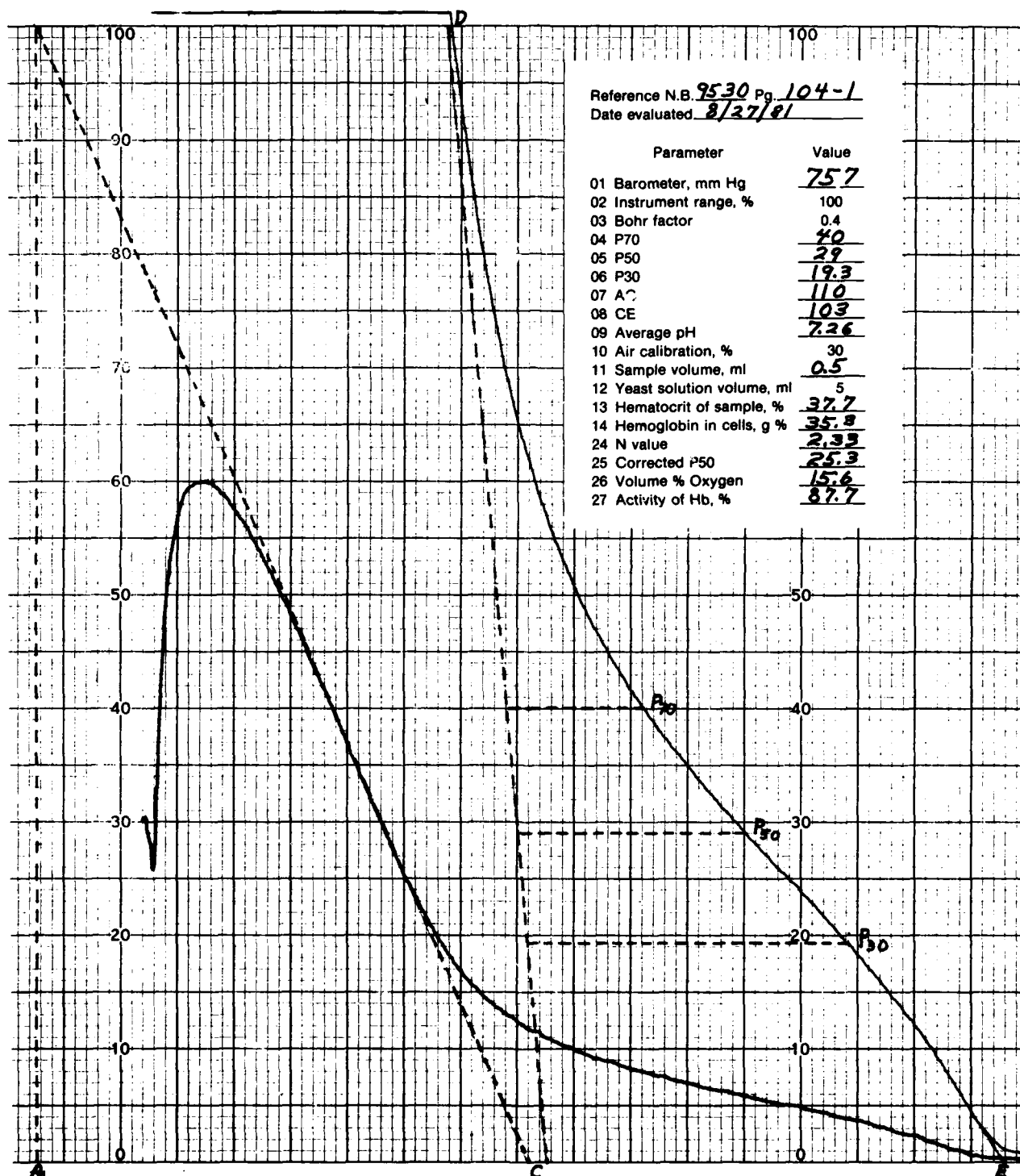


Figure 7. Bionometry data for human blood showing graphical projections.

The hemoglobin concentration of the sample was determined by the cyanomethemoglobin method.⁽²⁹⁾ A TI Programmable 59 calculator was used to correct the P₅₀ for the Bohr effect and to calculate the activity of the Hb. The calculator program, which is presented in the Appendix, was based on a program used at the Letterman Army Institute of Research.

B. Experimental Results

The first evaluations of hemoglobin function in the artificial red cells was conducted at the Letterman Army Institute of Research. A sample of cells smaller than 2 μ m had been prepared for injection into rats. When these cells were tested by biotometry, there was no measurable release of oxygen. The investigators concluded that excessive penetration of glutaraldehyde had occurred in these very small cells, and a protocol was devised to reduce the penetration.

The measures taken to reduce penetration included preparation of the emulsion at a slower stirring speed in Step 2, addition of smaller amounts of glutaraldehyde in Step 4, rinsing with hexylamine to quench the reaction in Step 6, and shortening the reaction times. Samples made under a variety of conditions in the Letterman laboratory were tested by biotometry. The results are shown in Table II. First an emulsion was made in a 7-speed Waring Blendor at a speed setting of 5, and two batches of cells were made with different amounts of glutaraldehyde. Neither of these batches of cells released oxygen, but the hemoglobin recovered from the emulsion did release oxygen (Test 3). Subsequent emulsions were made in the stirred jar in an attempt to increase the size of the cells so a smaller fraction of the hemoglobin would be affected by the glutaraldehyde. Also hexylamine was added to the first rinse solution in Step 6 to react with any residual glutaraldehyde and quench the reaction after short periods of cross linking.

With a reaction time of 3 min, at least 200 mg of glutaraldehyde was needed for a good yield of cells. With larger quantities of glutaraldehyde there seemed to be no particular pattern in the oxygen-release data. The P₅₀ values were in the 5.31 to 7.67 range compared to 12.8 for the broken emulsion (Test 6), and 27.5 to 41% of the initial hemoglobin activity remained. Both the P₅₀ and the activity of the hemoglobin were too low to be useful, and the cell size (not measured) was probably too large. However, the encapsulated hemoglobin did carry some oxygen, and the preparative conditions could be adjusted to reduce damage to the hemoglobin.

Table II: Data from Experiments Conducted at Letterman
Army Institute of Research

Test	Hb Conc %	Emulsion Preparation	Glutaraldehyde in 75 ml of saline, mg	Reaction time, min	Hexyl- amine, ml	Hemato- crit, %	P50 mm Hg	"N" value	Hemoglobin activity, %
1	28	Waring Blendor	192	5	0	9.5	same curve as yeast		
2	28	"	120	5	0	10	same curve as yeast		
3	28	"	0	emulsion broken for test			9.68	1.77	52.3
4	29	Stirred Jar	300*	5	0.4	25	7.67	1.34	34.9
5	29	"	150*	2	0.2	no cells	9.36	1.46	--
6	29	"	0	emulsion broken for test			12.0	1.82	48
7	29	"	225*	3	0.3	41	7.4	1.73	27.6
8	29	"	216	3	0.3	33	6.7	1.60	30
evaluated same batch next day									
9	29	"	160	3	0.3	9	5.54	1.61	35.5
10	28	"	200	3	0.3	insufficient quantity of cells			
11	28	"	240	3.5	0.3	36	6.8	1.60	27.5
12	29	"	216	3	0.3	22	5.31	1.61	41

*Aldrich 25% glutaraldehyde aqueous solution. An 8% EM-grade glutaraldehyde solution was used in all other tests.

The "hemoglobin activity" in Table II was calculated from the biotometry data on the basis of an assumed value of the hemoglobin content of the sample. For a sample of artificial red cells, the product of the hematocrit and the hemoglobin content of the starting solution was used as the hemoglobin content of the sample. For broken emulsions it was assumed that all of the hemoglobin had been recovered from the emulsion. The values of hemoglobin activity in Tests 3 and 6 indicate that only about half of the hemoglobin was recovered, but the hemoglobin content of those samples was not measured.

Subsequent to the experiments at Letterman, instrumentation for biotometry was purchased and set up in the contractor's laboratory. The biotometry curves in this report were all prepared in the contractor's laboratory. The trends in the shapes of the curves as the hemoglobin is processed is shown sequentially in Figures 7 through 12. In Figure 7 the familiar sigmoidal shape of the dissociation curve of hemoglobin in the human red cell is evident. When the red cells in the blood were lysed by addition of an equal volume of distilled water just before biotometry, the curve (Figure 8) was still sigmoidal, but the P_{50} was substantially lower (14.9 vs 25.3 for blood).

Figure 9 shows the biotometry curve for pyridoxalated hemoglobin solution that was prepared at the Letterman laboratory. Although pyridoxalation increased the P_{50} somewhat, the activity was lower, and the curve was only slightly sigmoidal. This hemoglobin solution was deoxygenated by evacuation and purging with nitrogen, and its biotometry curve (Figure 10) showed a lower P_{50} . The deoxygenated pyridoxalated hemoglobin was emulsified in mineral oil with the LM surfactant, and a portion of this emulsion was broken by addition of Triton X-100. Its biotometry curve in Figure 11 showed a further decrease in P_{50} and activity. Finally artificial red cells were made by injection of the emulsion into a solution of glutaraldehyde. The biotometry curve in Figure 12 shows that cells had a P_{50} of 8.9 mm Hg and only 41% of the hemoglobin released oxygen.

C. DISCUSSION OF RESULTS

All of the operations carried out with the hemoglobin appeared to reduce the oxygen-carrying capacity and the P_{50} of the hemoglobin. Moreover, each operation caused an extension of the length of the "tail" of the biotometry curve so that the curve became hyperbolic rather than sigmoidal. The slope of the tangent that established point E on the curves was arbitrarily chosen to be

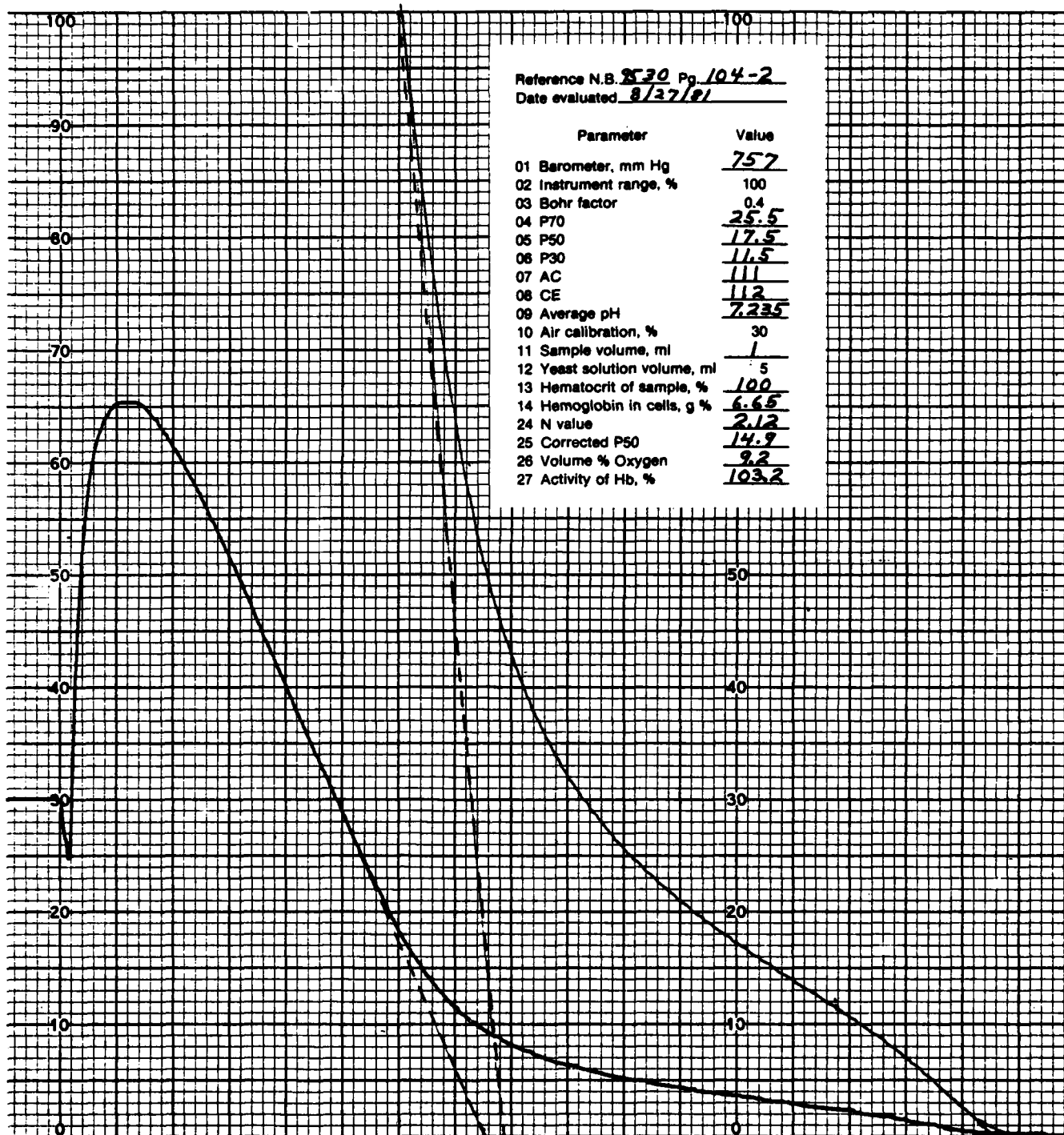


Figure 8. Biotometry data for hemolyzed human blood.

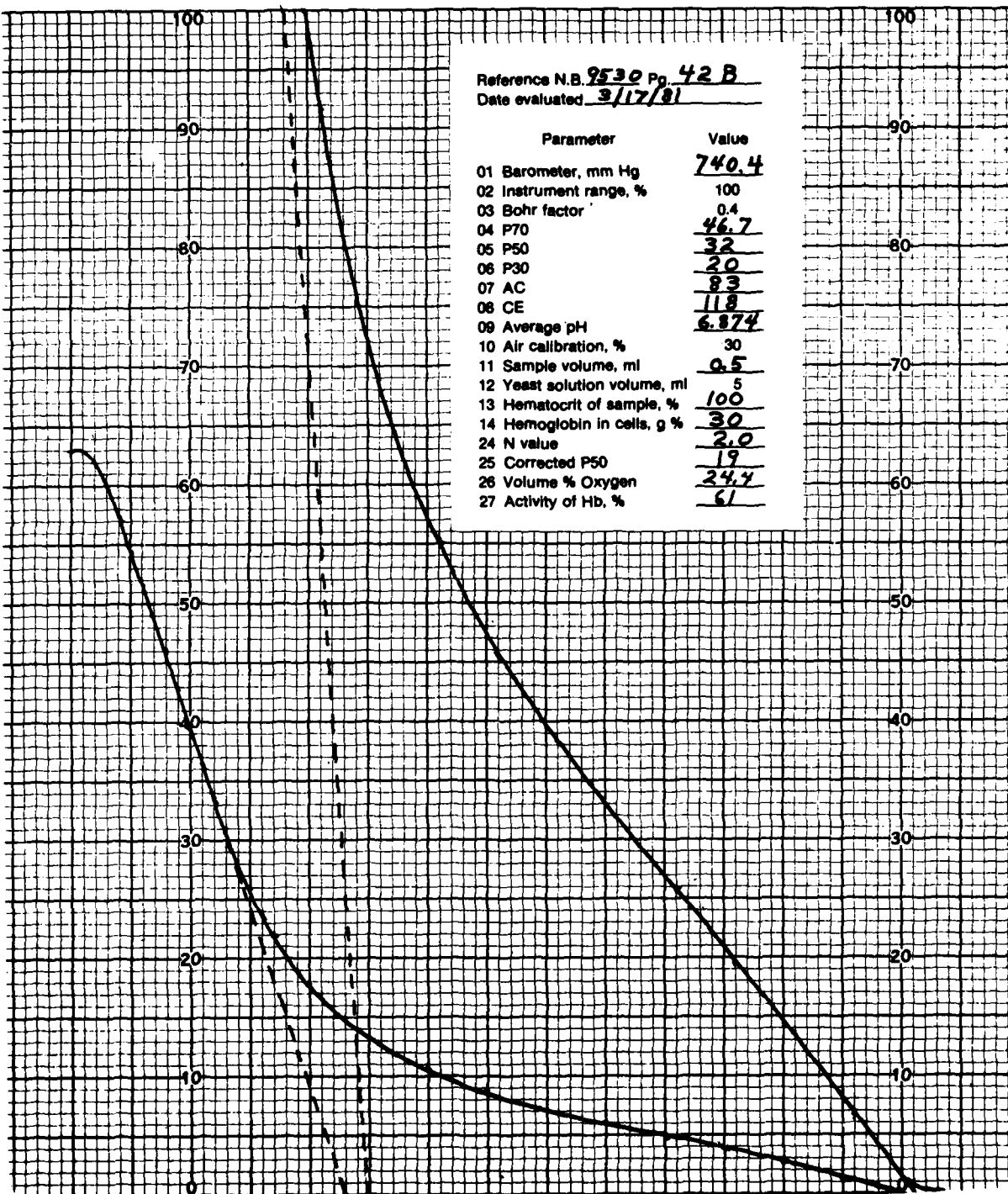


Figure 9. Biontonometry data for pyridoxalated hemoglobin solution prepared at Letterman.

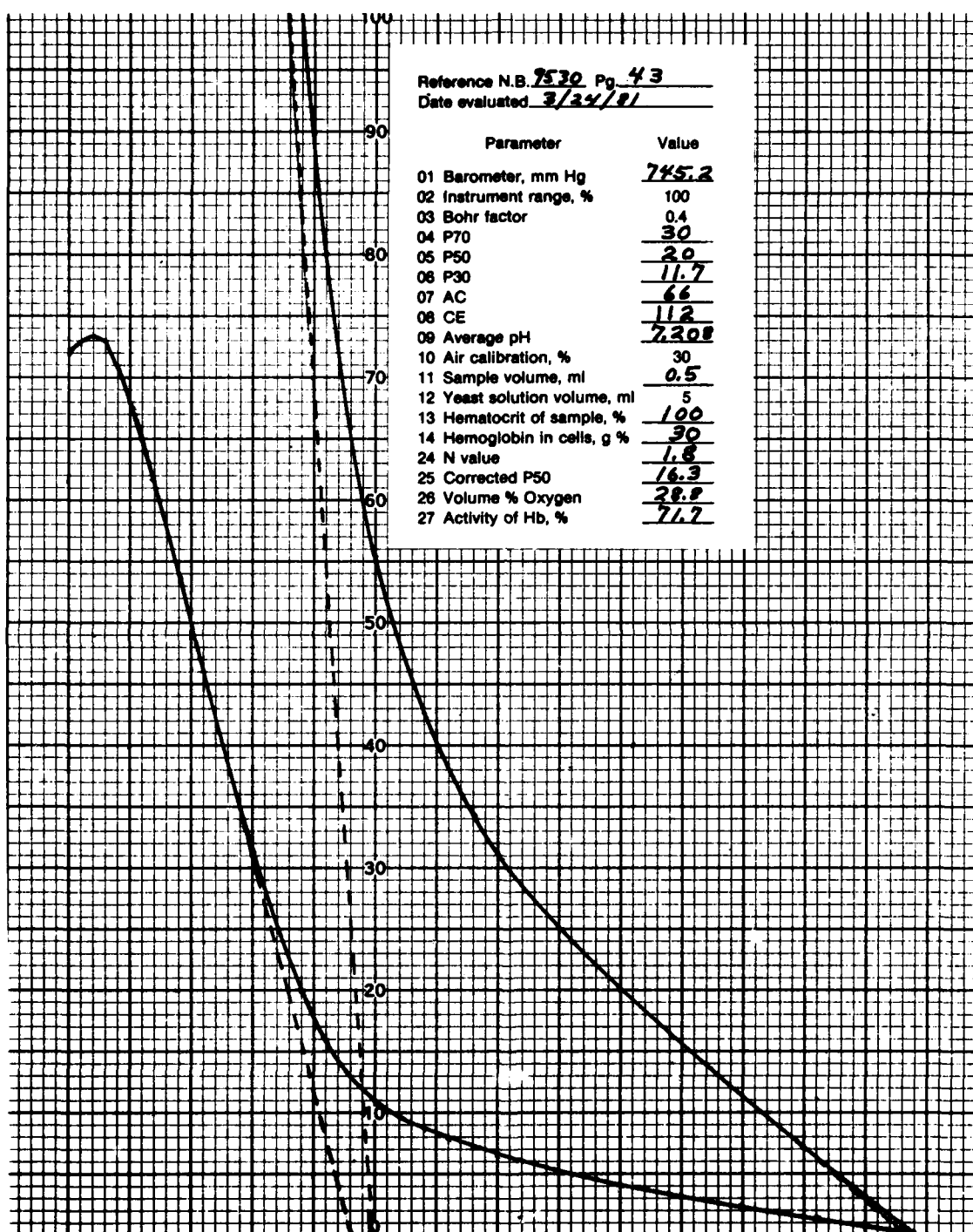


Figure 10. Biotometry data for deoxygenated pyridoxalated hemoglobin solution.

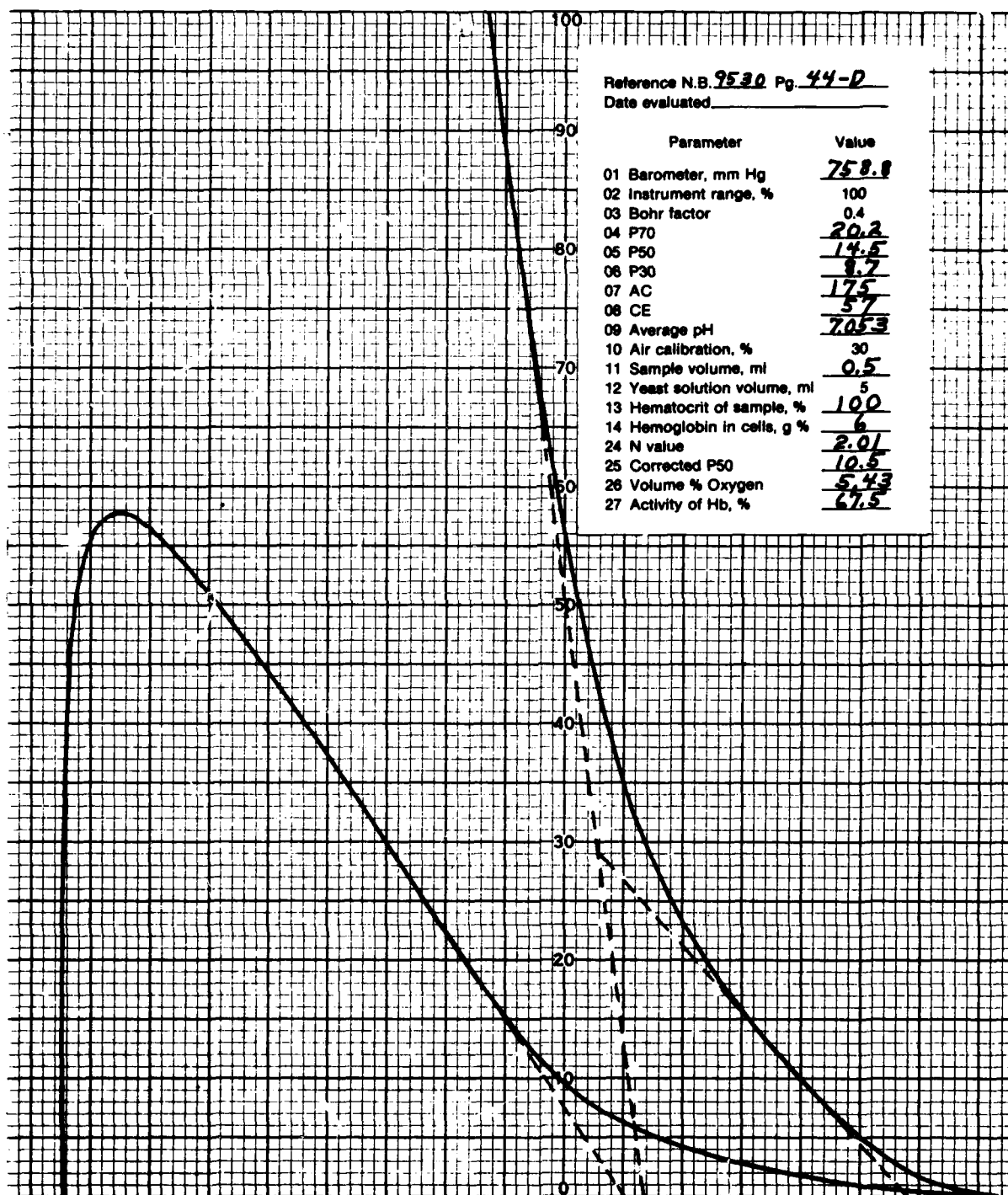


Figure 11. Biotometry data for pyridoxalated hemoglobin recovered from emulsions.

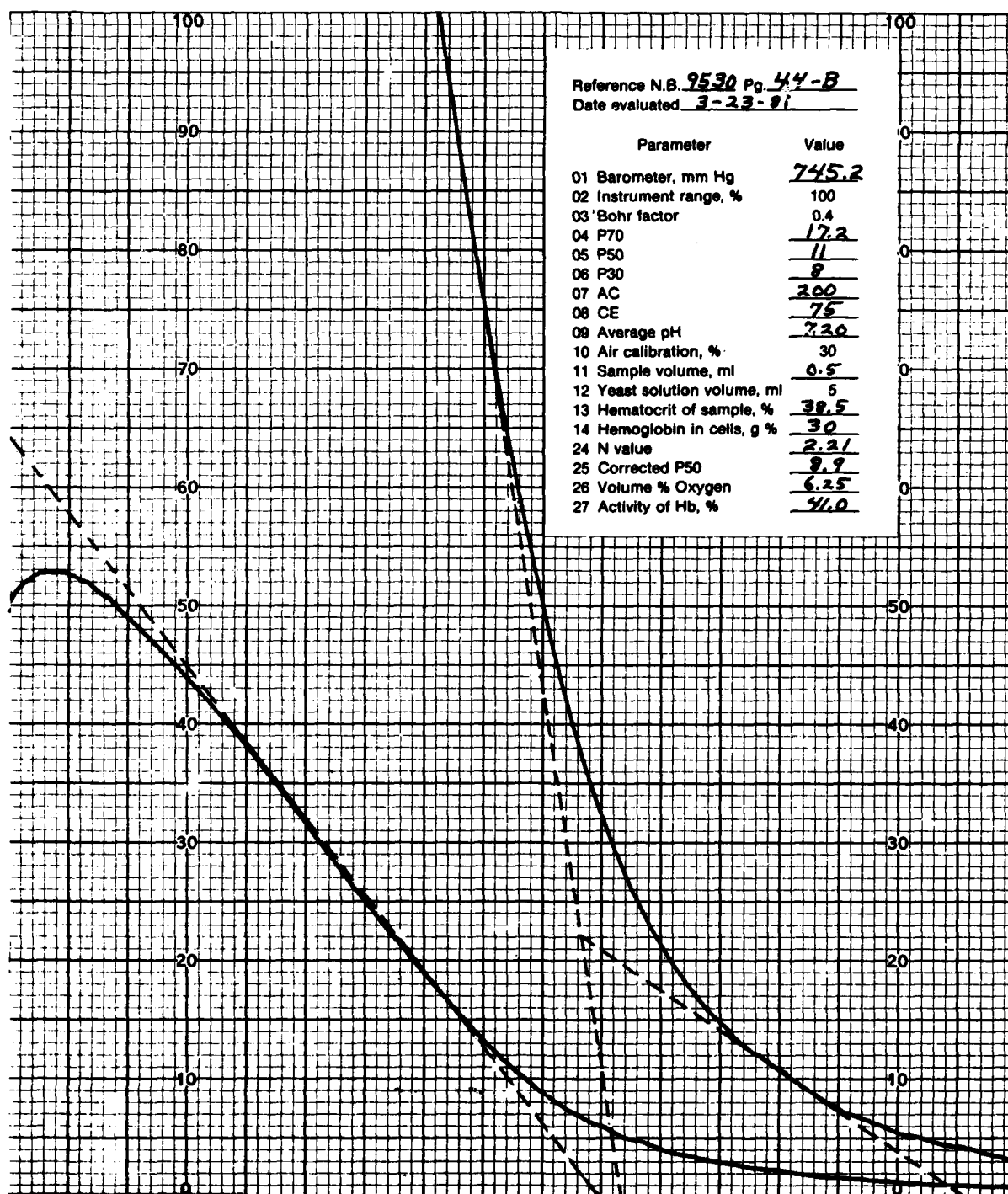


Figure 12. Biotometry data for artificial red cells containing pyridoxalated hemoglobin.

tangent to the flattest part of the curve. By reducing the slope in Figure 12, one could calculate that much higher fractions of the total hemoglobin in the cells released oxygen, but the P₅₀ values would be even lower.

By the time the hemoglobin from the red blood cells had been subjected to hemolysis, crystallization, deoxygenation, emulsification and cross linking, its affinity for oxygen had been substantially increased. Pyridoxalation apparently alleviated the problem to some extent, but the P₅₀ in the artificial red cells was too low for them to be useful for oxygenating human tissues. Clearly considerable effort must be devoted to reducing the damage to hemoglobin in each operation in order for the encapsulated hemoglobin to release adequate amounts of oxygen at useful partial pressures.

VIII. CONCLUSIONS AND RECOMMENDATIONS

The following conclusions are drawn from the results of the experimental program:

- Liquid Membrane Capsules (LMC) effectively maintained a dispersion of microdroplets of hemoglobin solution while the microdroplets were treated with the water-soluble cross-linking agent, glutaraldehyde.
- Solutions containing 30% of hemoglobin solution were readily encapsulated by this technique.
- A Waring Blendor was an effective device for emulsifying hemoglobin solution into microdroplets of appropriate size for artificial cells.
- The special LM surfactant was the most effective agent found for maintaining the stability of the LMC while the artificial red-cell membrane was being formed. But the special LM surfactant was not considered suitable for human use, and lecithin was the best substitute that was tested.
- Triton X-100 was the most effective water-soluble surfactant for ejecting the artificial red cells from the LMC.
- The cells agglomerated when the Triton X-100 was removed by repeated rinsing. A dispersing agent is apparently needed to prevent agglomeration.
- The cumulative effects of all of the operations to recover, concentrate, emulsify, and cross link the hemoglobin caused a substantial reduction

in the P₅₀ and oxygen-carrying capacity of the hemoglobin in the artificial red cells.

- Cells made with pyridoxalated hemoglobin had slightly higher P₅₀ (8.9 mm Hg) than those made with unmodified hemoglobin.
- The water-soluble glutaraldehyde apparently penetrated deeply into the interior of small microdroplets and destroyed the oxygen-release capacity of the hemoglobin with which it reacted.
- Artificial red cells that were small enough to pass through human capillaries did not have oxygen-carrying capacity and P₅₀ high enough to be useful in a blood substitute.

Although the preparative procedure was not refined to the point where useful artificial red cells were produced, the LMC technique showed considerable promise if the following improvements could be achieved:

- Reduce the penetration of the cross-linking agent into the microdroplets. This could be accomplished by changing the temperature, pH, or concentration to increase the reaction rate or by selecting a different cross-linking agent.
- Find biocompatible surfactants for preparing emulsions and ejecting cells from the oil phase.

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APPENDIX

TI-59 Program for Biotonometry Data

CLR	RCL 10	)
0.736	)	÷
÷	2nd Ln x	RCL 11
(	-	x
RCL 04	(	RCL 08
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)	RCL 01	RCL 14
x	x	÷
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x	RCL 11	=
RCL 05	+	STO 27
÷	RCL 12	R/S

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