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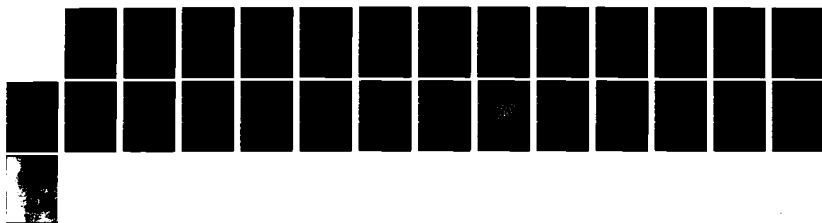   SRI INTERNATIONAL MENLO PARK CA   Z REYES APR 82
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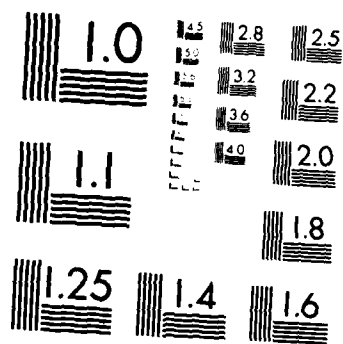
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PREPARATION OF HEMOGLOBIN-CONTAINING MICROCAPSULES

ANNUAL AND FINAL REPORT

ZOILA REYES, Ph.D.

APRIL 1982

Supported by
U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD17-80-C-0177

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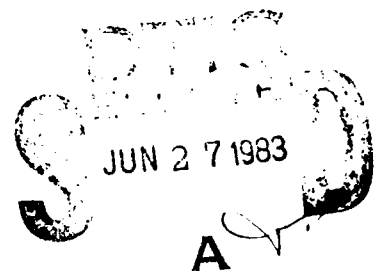
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REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO. AD A129801	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) PREPARATION OF HEMOGLOBIN-CONTAINING MICROCAPSULES		5. TYPE OF REPORT & PERIOD COVERED Annual (6/1/81 - 1/31/82) Final (10/1/80 - 1/31/82)
		6. PERFORMING ORG. REPORT NUMBER 2254-2
7. AUTHOR(s) Zoila Reyes		8. CONTRACT OR GRANT NUMBER(s) DAMD17-80-C-0177
9. PERFORMING ORGANIZATION NAME AND ADDRESS SRI International 333 Ravenswood Avenue Menlo Park, California 94025		10. PROGRAM ELEMENT. PROJECT, TASK AREA & WORK UNIT NUMBERS 62772A.3S162772A874.AC.135
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research and Development Cmd. Fort Detrick Frederick, Maryland 21701		12. REPORT DATE April 1982
		13. NUMBER OF PAGES 25
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)		15. SECURITY CLASS (of this report) Unclassified
		15a. DECLASSIFICATION. DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited.

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Department of the Army position unless so designated by other documents.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

hemoglobin, blood substitutes, microcapsules, red blood cell substitutes

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

    Microcapsules containing stroma-free hemoglobin (SFHb) were prepared
from ethyl cellulose (EC), lecithin-protein-polysaccharide complexes,
and protein-polysaccharide complexes.

    The EC microcapsules were prepared in small-scale experiments in sizes
ranging from 0.8 to 1.2 μm, with wall thickness of 0.02 to 0.03 μm. These
microcapsules had good stability and showed oxygen-carrying properties

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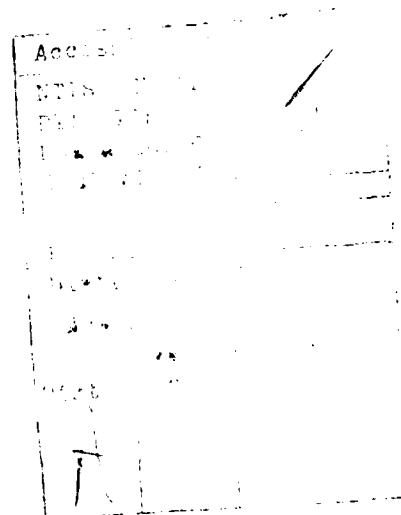
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comparable with those of STFHb. However, in scale-up experiments with EC, the Hb was denatured.

The microcapsules made from complexes of lecithin-gelatin-gum arabic, lecithin-gelatin-chondroitin sulfate, and lecithin-gelatin-carrageenan were generally large, 10 to 15 µm, and had irregular shapes and thick walls. In some of the experiments with these systems the Hb was crosslinked when the capsules were hardened.

The microcapsules made from gelatin-chondroitin sulfate complexes were prepared in aqueous media, and although some of the early capsules had irregular shapes, spherical capsules were made by improving the procedure. These capsules ranged from 3 to 12 µm and the Hb in the capsules retained its ability to reversibly combine with oxygen.

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I INTRODUCTION

The development of a suitable blood substitute has been under investigation for many years. An ideal blood substitute should contain substitutes for both the plasma protein and the red cells. Plasma substitutes, such as hydroxyethyl starch, dextran, and gelatin, have been used clinically for several years, but suitable substitutes for red blood cells have not been developed.

Stroma-free hemoglobin (SFHb) can function as a plasma and red cell substitute; however, it is rapidly removed from the circulatory system and has a high oxygen affinity that prevents release of oxygen to the tissues at normal oxygen tensions. Various chemical modifications of the hemoglobin (Hb) structure have been studied to slow the rapid elimination of Hb and lower its oxygen affinity. Some of these modifications improved vascular retention, but the Hb either lost its oxygen-carrying properties or increased its oxygen affinity.

Microencapsulation of SFHb is being investigated to develop Hb-containing microcapsules that show improved retention time in the circulatory systems and better oxygen-transporting properties.

II BACKGROUND AND APPROACH

In recent years, microcapsules containing Hb have been prepared and evaluated as models for red blood cells. The capsules were made from various modified natural and synthetic polymers and lipids. The Hb in these artificial cells reversibly combined with oxygen; however, when suspensions of some of these microcapsules were intravenously injected in animals, they were rapidly removed from the circulatory system. The failure of the artificial cells to survive for a longer period of time in the blood stream was attributed to the large size of the capsules, their lack of surface charge, or the rigidity and chemical nature of the capsule membrane.

Our approach was to microencapsulate Hb with biocompatible materials to produce capsule membranes having mechanical properties similar to those of the natural erythrocytes. The materials selected for evaluation include complexes of lecithin with low-molecular-weight polysaccharides or protein, such as albumin-lecithin, gum arabic-lecithin, and collagen-mucopolysaccharides, as well as blood-compatible synthetic polymers such as poly(2-hydroxyethyl methacrylate). In addition, we planned to make capsules with diameters of 1 to 2 μm, which is theoretically small enough to be able to easily traverse the capillary networks and large enough to reduce problems of extravasation, even when the apertures shrink to 3 μm or less during trauma.

III   RESULTS AND DISCUSSION

The materials evaluated as encapsulating agents for aqueous solutions of SFHb included ethyl cellulose (EC), hydroxyethyl starch acetate (HESA), lecithin-protein-polysaccharide complexes, and protein-polysaccharide complexes. Poly(2-hydroxyethyl methacrylate) was only briefly tested.

The SFHb used in this work was supplied by the Letterman Army Institute of Research (LAIR) as a 28% or 30% aqueous isotonic saline solution in frozen state. The Hb solution samples were thawed, filtered through a Millipore filter (0.22 μm pore size), divided into 10-mL samples, and stored in a freezer for use in the microencapsulation tests. Some of the samples contained small amounts of solid particles, which were removed by centrifugation before filtration.

The double emulsion microencapsulation technique was used in early work with the lecithin complexes, and in all the tests with EC and HESA. This procedure consists of emulsifying the aqueous Hb solution in an organic solvent solution of the encapsulating polymer system or of one of its components to form a water-in-oil (W/O) emulsion. This emulsion is then emulsified in an aqueous solution of a suitable emulsifier or suspending agent to form a W/O/W emulsion. The organic solvent is then evaporated to produce the microcapsules. These are separated by centrifugation at a low speed, washed several times with 0.9% aqueous NaCl solution, and stored in a refrigerator in some of the saline solution. All the steps of the microencapsulation procedure are conducted under a nitrogen or argon atmosphere. Specific details of the procedure are given in Report No. 1 and in some of the experiments described below.

A.   Ethyl Cellulose

Initial tests with EC were conducted with 0.5 and 1.0-g samples of the SFHb and solutions of EC (Hercules N 100) in Freon TMC (an azeotrope of Freon TF and methylene chloride). These tests showed that the Hb in the capsules reversibly combined with oxygen.

A larger sample of microcapsules was prepared for evaluation at LAIR. This sample was prepared in four batches, using the following materials per batch:

Solution 1

    5.0 g 28% SFHb solution
    2.5 g 2% gum arabic aqueous solution, pH 7
    2.5 g 3% glucose in water

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Solution 2

    2.0 g EC (Hercules N 100)
    98.0 g Freon TMC

Solution 3

    160 g 10% gum arabic, pH 7.

The three solutions were cooled to 10°C before use. Solution 1 was emulsified in solution 2 by sonication in an ice bath for 45 s. The fine particle emulsion obtained was added slowly to solution 3, which was stirred with a magnetic stirrer in an ice-water bath. The resulting dispersion was stirred in a VirTis homogenizer at a moderate speed for 10 min in an ice-water bath, and then it was transferred to a 1-L beaker (previously weighed) using 50 mL deionized water to rinse the homogenizer container. The beaker and contents were weighed, and the dispersion was stirred rapidly with a magnetic stirrer under a stream of argon and nitrogen to evaporate the Freon TMC at room temperature. After 2 h, approximately 60% of the solvent had evaporated. To ensure complete removal of the solvent, the batches were stirred overnight at room temperature under blanket of nitrogen.

The microcapsule suspension was diluted with 50 mL saline and centrifuged. The liquid layer was decanted, and the microcapsules were washed five times with 0.9% NaCl (previously filtered through a 0.22-μm filter) and dispersed in the saline solution. A few capsule aggregates that formed during the solvent evaporation were separated by centrifuging the saline suspension for 30 s at a low speed.

From the combined microcapsule batches, a 95-g sample of the suspension, estimated to contain 16% microcapsules, and labeled S-1186017, was sent to LAIR for evaluation.

The size, size distribution, and shape of the microcapsules were determined by microscopic examination at 1,260X magnification. The microcapsules were spherical and ranged from 0.6 μm to 2.8 μm in diameter; the major fraction had 0.8-μm diameter.

The microcapsule wall thickness was determined from electron photomicrographs of cross sections of the capsule imbedded in an epoxy resin. The wall thickness ranged from 0.02 to 0.03 μm.

For determining the absorption spectrum of the microencapsulated Hb, 1.0-mL sample of the microcapsule suspension was diluted with 10 mL 0.9% NaCl. The absorption spectrum was taken immediately after dilution (Curve 1, Fig. 1) by using an opaque cell in the reference beam. The spectrum was then determined after the sample had been stirred under argon in a bell jar for 30 min at room temperature while passing argon at the rate of 10 mL/min (Curve 2, Fig. 1). Curves 3 and 4 show the changes in the spectrum of the microencapsulated Hb after passing oxygen at the rate of 10 mL/min for 15 and 30 min, respectively. No further change in the spectrum of Curve 4 was observed by passing oxygen for 14 min longer. The spectrum of Curve 2 was obtained again by passing argon for 30 min. This test shows that the microencapsulated Hb retained its ability to combine reversibly with oxygen.
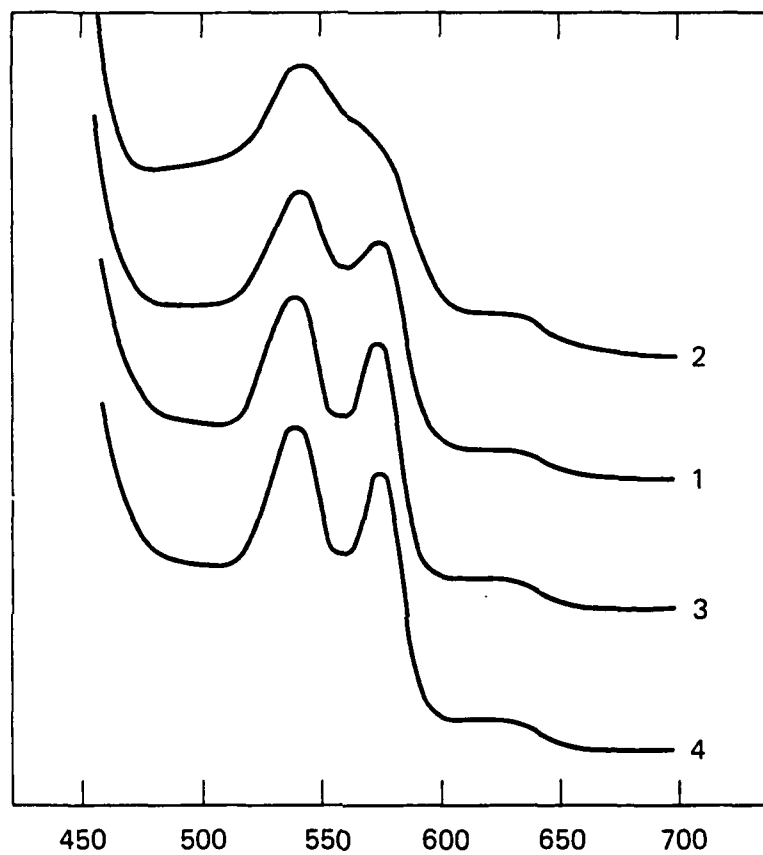
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FIGURE 1 CHANGE OF ABSORPTION SPECTRUM BY OXYGENATION
OF ENCAPSULATED Hb

1. Immediately after diluting the saline suspension.
2. After passing argon for 30 min at the rate of 10 ml/min
3. After passing oxygen for 15 min at the rate of 10 ml/min
4. After passing oxygen for 30 min at the rate of 10 ml/min

The oxygen affinity of the microencapsulated Hb was measured by the biotonometric method.[*] The oxygen dissociation curve showed a P_{50} of 16 mm Hg.

The Hb content of the capsules was low, less than 6%. Because the HB/EC ratio used in these experiment was 0.7/1, we evaluated higher ratios, 2.5/1 and 3/1 in small-scale tests conducted with 1 or 2.0 g of the Hb solution. Good microcapsules were produced in these tests and no significant changes in the encapsulation method were required. However, when the procedure was scaled up to 10 and 15 g, the Hb was denatured during the transfer of the capsules from the organic-liquid phase to the aqueous phase. This was caused by the high stirring speed used to emulsify the organic phase in the aqueous solution.

A series of encapsulation tests was then conducted with EC to s. the effect of some of the process variables on the structure of Hb. In all these tests the solutions and emulsions were maintained at 8 10°C in an atmosphere of nitrogen or argon during the encapsulation procedure. We evaluated the effect of the following variables:

1. Emulsifier

In the first step of the procedure the aqueous solution of SFHb is emulsified in a solution of EC in Freon TMC. The Hb solution can be emulsified in the EC solutions with or without the aid of an emulsifying agent. Generally we used a magnetic stirrer or a Vortex mixer for the initial emulsification. To produce an emulsion of uniform droplets, a brief sonication of 2 to 3 s or a 1 to 2 min stirring in a VirTis homogenizer was used after the first emulsification. Under identical stirring conditions, the use of an emulsifier, Span 85 (sorbitan trioleate), at a 1% concentration in the EC solution yielded emulsions with 0.1 to 2-μm diameter particles, whereas emulsions prepared without an emulsifier had slightly higher particle size, 1 to 5 μm. The Hb was not affected by the emulsification step, because on breaking the emulsion by adding solvent, we could separate the Hb in the top aqueous layer by centrifugation.

2. Buffer System

The aqueous solution of SFHb and that of the gum arabic used were adjusted to pH 7 or 7.2 with dilute NaOH. A 0.001 M tris-HCl buffer of pH 7 was also used with the Hb solution. No appreciable effect on the Hb or the microcapsules produced was observed.

3. Stirring Conditions

As indicated in Section III.A.1, the short sonication period or the stirring required in the VirTis homogenizer to produce fine

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[*] J. R. Neville, J. Appl. Physiol. 37, 967-971 (1974).

particle emulsions of the Hb solution in the EC solution did not appear to affect the Hb-structure. The effect of using a moderate stirring speed setting of 30 in the VirTis homogenizer was studied with a 5-g sample of a tris-buffered Hb solution and a 2% EC solution containing 1% Span 85. A 3-min stirring was needed to produce a fine particle emulsion of the Hb solution in the EC solution. No effect on the Hb was observed during this step (an ice-water bath was always used for cooling the solutions during emulsification). To form the capsules, the first Hb emulsion is emulsified in an aqueous gum arabic solution where the EC solvent is evaporated. For the second emulsification in this experiment we used a mixture of 10% gum arabic (20 g) and 75 g of a 1:1 mixture of polyethylene glycol (PEG) 200 and water. The emulsification of the Hb emulsion into this aqueous phase took 2.5 min in the VirTis homogenizer. Because PEG 200 is a good plasticizer for EC, no encapsulation takes place in this medium; when the water immiscible EC solvent is evaporated, the Hb is gradually released. The Hb was not affected by the VirTis stirring.

In the small-scale experiments conducted with 1 to 2 g of the Hb solution, the emulsification steps and the evaporation of the organic solvent required a short period of time, during which little damage is done to the Hb. For larger Hb solution samples (10 to 20 g) longer periods of time are required for all these steps and, in spite of the controlled conditions used, some of the Hb denatured. Usually at the Hb-EC interface some denatured Hb formed, but the main fraction of encapsulated Hb can be released from good capsules by treating an aqueous suspension of the capsules with ethyl ether or chloroform at 10°C and centrifuging to separate the organic solvent solution of EC from the Hb aqueous solution. A small amount of solid particles appearing at the interface of the solutions corresponds to the amount of denatured Hb.

To minimize denaturation of Hb during the EC encapsulation process, the large samples should be prepared in a series of small batches. No further work was conducted with EC, because at that time promising results had been obtained with another encapsulating system (a protein-polysaccharide complex), which required little stirring and only water as encapsulating medium.

B. Hydroxyethyl Starch Acetate

Hydroxyethyl starch acetate (HESA) was prepared by acetylation of hydroxyethyl starch (Sigma Chemical Company) with acetic anhydride.[*] Microcapsules containing Hb were produced in small-scale experiments conducted with solutions of HESA in chloroform or methylene chloride and 1.0-g samples of the SFHb solution. No crosslinking agents were used, but a small amount of Span 85, a water-in-oil emulsifier, was

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[*] R. L. Lohman, Jr., J. W. Sloan, and C. E. Rist, J. Am. Chem. Soc., 72, 5717 (1950).

needed to stabilize the initial W/O emulsion.

A larger batch was prepared with the following materials:

Solution 1

   15.0 g 28% SFHb solution
    7.5 g   2% GA solution, pH 7
    7.5 g   3% glucose

Solution 2

    6.0 g HESA in 94.0 g chloroform
    1.2 g 2% Span 85 in chloroform

Solution 3

   160 g 10% GA solution, pH 7.

The procedure was the same as that used for EC.

The microcapsules produced were very small, 1-2 µm, but they were easily damaged during centrifugation and washing with saline. This was caused by the brittleness of the HESA membranes, which could not with-stand the pressure during centrifugation. Thus, the Hb was gradually extracted from some of the capsules during the washing operation.

C.   Poly(2-Hydroxyethyl Methacrylate)

A suspension polymerization procedure similar to that designed by Dahlquist et al.[*] for immobilization of enzymes was evaluated for the microencapsulation of Hb with poly(2-hydroxyethyl Methacrylate), [poly HEMA].

An experiment was conducted using 0.5 g of 28% SFHb solution, 0.2-g bovine albumin dissolved in 1-mL 0.1 M phosphate buffer of pH 7.2, and 5 mg of N,N,N',N'-tetramethylenediamine in 0.5 ml of the buffer as the aqueous phase. The organic phase consisted of 0.3 g of freshly distilled HEMA in a mixture of 3.0-g chloroform and 7.0-g cyclohexane containing 0.1-g Sorbitan sesquioleate (Atlas Chemicals) as suspension stabilizer. Both the aqueous and the organic solutions were purged with nitrogen, cooled to 5°C, mixed in a capped tube, and emulsified in a Vortex homogenizer; then a solution of 6-mg ammonium persulfate in 0.5 mL of the buffer was added and the mixture was stirred in an ice-water bath for 1 h.

Because the distilled HEMA contained a small amount of a crosslinker (ethyleneglycol dimethacrylate) as impurity, we expected that the poly HEMA formed would be insoluble in the suspension medium. Large spherical

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[*]A. Dahlquist, B. Mattiasson, and K. Mosbach, Biotechnol. Bioeng. 15, 395 (1973).

beads (10 to 20 μm diameter), and small irregular particles were formed and were separated by centrifugation and washed with buffer and 0.9% aqueous NaCl solution. Some of the beads contained Hb, but most of the Hb was not encapsulated. The beads were very tacky and formed large aggregates on standing undisturbed. No further work was done with HEMA.

D. Lecithin Complexes

Lecithin, or phosphatidyl choline, is found in the cells of all living organisms and has been used to model membranes, either alone or in combination with cholesterol and long-chain anions or cations.

Because lecithin is the salt of a strong acid and strong base, it has little buffering capacity at physiological pH ranges. Its iso-electric point is expected to be close to pH 7; however, the experimental values vary because of contamination. At pH values of 6.7 to 7.5, lecithin can form complexes with negatively charged colloids, such as gum arabic, and also with positively charged proteins and inorganic salts. Complexed lecithins, especially in combination with proteins, are more stable toward oxidation.

Two grades of lecithin were used in the microencapsulation tests: refined soybean (ICN Nutritional Biochemicals) and egg lecithin (L-α-phosphatidylcholine, Sigma). Both types of lecithin were crystallized twice from petroleum ether (30-60°) and acetone and stored under acetone in an argon atmosphere in a refrigerator.

In the microencapsulation tests conducted with lecithin-gelatin and 2.0 g of the Hb solution, the procedure was as follows.

- Mix the Hb solution with 2.0 g of 5% aqueous gelatin solution at 25°C and pH 7.

- Emulsify the resulting aqueous solution in 8.0-g mineral oil containing 0.4% lecithin (soybean) and 0.4% Span 85 (sorbitan trioleate, Atlas Chemicals) a W/O emulsifier.

- Cool the emulsion in an ice bath to 10°C, dilute it with 2-mL petroleum ether (PE), 30-60°C, and stir it while 2-mg tolylene diisocyanate (TDI) dissolved in 2-mL PE was dropped in.

- Stir for 10 min longer in the ice bath and then dilute the emulsion with an equal volume of PE at 10°C and allow the capsules to settle.

- Decant oil layer and repeat treatment with PE to remove the mineral oil.

- Disperse the capsules in 4 mL PE and emulsify in
  10 mL of a 2% aqueous Tween 80 (polyoxyethylene
  sortitan monooleate, Atlas Chemicals) solution.

- Evaporate residual PE under nitrogen, dilute the
  emulsion with 3-mL deionized water (10°C), and
  separate the capsules by centrifugation in an
  argon atmosphere.

- Wash the capsules once with deionized water and
  four times with 0.9% NaCl solution, and then
  suspend them in saline for storage in a refrigerator.
  The capsules were 5 to 10-μm in diameter.

The visible absorption spectrum of a dilute sample of the capsule
suspension was determined in the presence of air and after saturation
with argon, using a frosted cell in the reference beam of the spectro-
photometer. These spectra were similar to those of an oxygenated and
deoxygenated sample of the original Hb solution diluted with saline.

On storage in the refrigerator under argon, the capsule suspension
showed considerable discoloration within 4 days.

A similar experiment was conducted with a mixture of 2.0 g of the
Hb solution, 0.2 g of 10% gum arabic (GA) solution, pH 7, and 2.0 g of
the 5% gelatin at 25°C and pH 7. The amounts of the other materials
were the same as those used with the Hb-gelatin mixture.

The visible absorption spectrum of the microcapsules obtained was
almost identical to that of the lecithin-gelatin microcapsules; however,
the lecithin-gelatin-GA microcapsules showed greater resistance to
discoloration during storage in a refrigerator.

Attempts were made to prepare a larger sample of microcapsules for
evaluation at LAIR. However, in all the scale-up experiments the Hb
was denatured during the encapsulation process. In subsequent work
we found that denaturation was caused by the longer stirring periods
and the higher stirring speed required to produce capsules with 2 to
3 μm diameters.

To simplify the microencapsulation of Hb, we investigated several
modifications of the procedure. In a series of tests, the interaction
of lecithin with GA and HB was allowed to take place before they were
added to a gelatin coacervate for encapsulation.

These tests were conducted with 50 mg of lecithin (either egg or
soybean lecithin) dissolved in 10 g of solvent (a mixture of equal
weights of PE, 30-60°, and Freon TF was used); 1.0 g of 1% aqueous GA
solution, pH 7; and 1.0-g 30% Hb solution, pH 7 mixed with 0.1-mL
Ringer Locke solution. The GA solution was either emulsified by
sonication in the lecithin solution and the emulsion mixed with the Hb

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solution, or the GA was combined with the Hb first and then the mixture emulsified by sonication (10 s) in the lecithin solution in an ice-water bath. A finer particle dispersion was obtained by the latter procedure. Both types of dispersion were stirred for 10 to 15 min in the ice-water bath, then diluted with 5-mL PE and allowed to stand in the cold for 5 min longer. Most of the solvent and free lecithin were removed by decantation or centrifugation at a low speed. In the absence of the organic solvent the Hb droplets aggregate rapidly; therefore 1.5-mL solvent is added to keep the emulsion particles suspended. In some of the experiments a small amount of Span 85 (0.5 g of 2% Span 85 in PE) was incorporated in the lecithin solution to improve emulsification.

For encapsulation, the fine particle suspension was added quickly to a gelatin coacervate. The coacervates were formed by interaction of gelatin with anionic polysaccharides at pH values of 6.8 to 7.2, depending on the relative concentrations of the colloids used. Chondroitin sulfate (CS), a mucopolysaccharide (Vega Chemicals), and carrageenan, a sulfate polysaccharide (Gelcarin HWG carrageenan, Marine Colloids), were used.

A gelatin coacervate was obtained by mixing the following solutions: 10.0 g of 1% aqueous gelatin, pH 7 at 25°C; 0.5-g 2% aqueous CS, sodium salt, pH 7 at 25°C; and 2 drops of 2% calcium chloride. The coacervate was maintained at 25° to 30°C in a water bath and stirred continuously while a suspension of the lecithin-Hb droplets in 1.5 mL pentane was added. The pentane evaporated quickly during stirring and the Hb droplets were coated by the gelatin-CS coacervate droplets. After cooling to 10°C, solid microcapsules formed.

Microscopic examination showed that the capsules had clear, thick gelatin walls, irregular shapes, and ranged in size from 5 to 15 μm. The Hb droplets were encapsulated as clusters or aggregates of various sizes. These microcapsules can be destroyed by simply warming the dispersion and either changing the pH or adding a small amount of saline. Because coacervation takes place at a definite pH and is inhibited by salts, only very small amounts of salts are tolerated. The microcapsules were hardened by adding 0.25 mL of 12.5% glutaraldehyde, and after 2 h of continuous stirring in the ice-water bath, the capsules were separated by centrifugation, washed with saline several times, and suspended in saline for storage in a refrigerator. A small amount of Hb was not encapsulated.

Similar results were obtained with a gelatin-GA-CS coacervate formed at pH 7 from 5 g of 2% gelatin, pH 7, at 25°C; 1.0-g 0.5% CS, pH 7; 5.0-g 2% GA, pH 7, and 4.0-g water.

A gelatin-carrageenan complex coacervate was formed by adding 2.0 g of 0.5% aqueous carrageenan solution, pH 7, to 12.0 g of 1% aqueous gelatin at 25°C, pH 7. For encapsulation of the Hb, the small particle suspension obtained from 1.0 Hb solution as described above, was dispersed in the gelatin solution and the carrageenan solution at 35°C was added

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dropwise. The coacervate formed rapidly and the lecithin-coated Hb was encapsulated by the gelatin-carrageenan complex coacervate droplets. A significant amount of Hb was not encapsulated. The hardened capsules had very thick, clear membranes, contained aggregates of Hb droplets, and, like those described above, were large (>10 μm) and irregularly shaped.

Attempts were made to prepare smaller, uniform capsules by increasing the viscosity of the coacervated system. A nonionic polysaccharide, hydroxyethyl starch HES, was incorporated in the gelatin-chondroitin sulfate-gum arabic coacervate.

The following materials were used:

(1) 0.5 g 28% SFHb mixed with 0.1 g 10% GA, pH 7.

(2) 50 mg egg lecithin, 0.5 g 2% Span 85 in a mixture of 10 g PE and 10 g Freon TF.

(3) 2.0 g 2% HES, 0.5 g 3% glucose, 0.5 g 10% gelatin, 0.2 g 10% GA, and 0.5 g 2% CS. This solution was maintained in a water bath at 25-30°C. One drop of 3% aqueous acetic acid was added to adjust the pH of this solution to 6.28 and to induce coacervation of the gelatin.

Solution (1) was emulsified in (2) and the lecithin-coated Hb droplets were separated as described earlier and suspended in 1.5-mL pentane. The suspension was added to the coacervated mixture (3) with continuous stirring. A small amount of the Hb dissolved in the mixture, causing decoacervation of the solution. However, when the pH was adjusted to 6.3 with a drop of 1% aqueous acetic acid, coacervation was restored. After evaporating the pentane, the mixture was allowed to slowly cool to room temperature and then it was chilled to 10°C. Glutaraldehyde, 0.2 g of 12.5 % aqueous solution, was added to harden the capsules.

One hour after adding the glutaraldehyde, the capsules were separated by centrifugation and washed four times with saline. These capsules were more uniform, but relatively large, 7 to 10-μm diameter. A sample of the capsules in saline showed the absorption spectrum of oxyhemoglobin, but the oxyhemoglobin could not be deoxygenated. The Hb was crosslinked during the glutaraldehyde treatment, because the Hb could be released from the unhardened capsules by warming a saline suspension of them.

Similar results were obtained in an experiment with a gelatin-chondroitin sulfate coacervate prepared in the presence of HES.

Aqueous bovine albumin solutions form complex coacervates with various anionic polysaccharides such as carrageenan and gum arabic. However, CS does not form a complex coacervate with albumin.

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The complex coacervate obtained by adding 0.5% aqueous carrageenan solution to a 2.5% aqueous albumin solution at pH 6.2 consisted of very fine droplets. This coacervate gradually flocculated when a lecithin-treated Hb was added, and no encapsulation took place.

Similarly, no encapsulation took place with albumin-GA coacervates formed at pH values of 4.7 and 5.3. The first one was obtained by mixing equal weights of 2.5% aqueous solutions of bovine albumin and GA; the second one was formed by using less GA, a ratio of 5:3 albumin:GA.

The formation of albumin and gelatin complexes with lecithin and polysaccharides in the absence of Hb was then studied to determine best conditions for encapsulation.

To prepare the lecithin-gelatin complexes, we first disperse purified soybean lecithin in 0.001 M tris-HCl buffer of pH 7.4 containing 0.001 M EDTA. The dispersions were made by sonication at lecithin concentrations of 10 mg/mL. Aliquots of the dispersion were mixed with 1% aqueous solutions of gelatin at the same pH. The lecithin/gelatin weight ratio was varied from 0.5:1 to 1:1. The mixtures were either incubated at 30°C under N_2 for 30 min or they were sonicated for 2 or 3 periods of 30 s. Then the mixtures were centrifuged to separate lipid aggregates. Sonication of the gelatin-lecithin mixtures yielded almost clear solutions, which became clear on addition of small amounts of anionic polysaccharides, such as chondroitin sulfate. These solutions can be coacervated by adjusting the pH to values of from 6 to 6.8, depending on the lecithin:gelatin ratio used and the amount of anionic polysaccharide added.

It was found that aqueous solutions of Hb are immiscible with these complex systems, thus, when the coacervate wets the surface of the Hb droplets, encapsulation can take place. In an experiment conducted with the complex obtained by sonication of a 1:1, lecithin:gelatin mixture by weight, 5 mL of the complex (a slightly hazy solution) was mixed with 0.25 mL of 5% chondroitin sulfate aqueous solution and the mixture was coacervated at 30°C by adjusting the pH to 6.8. Then, 0.2 g of 20% aqueous Hb at the same pH was added and the mixture was stirred briefly in a Vortex mixer. After the mixture was stirred for 1 min in a water bath at 25°C, it was allowed to cool with continuous stirring. Most of the Hb was encapsulated. The capsules appeared to have very thin walls, but when the mixture was chilled to 10°C in an ice-water bath, solid capsules were obtained. These capsules can be destroyed by warming the mixture and either changing the pH or adding a salt. There was no degradation of Hb.

In similar experiments with lecithin-gelatin-carrageenan and lecithin-albumin-carrageenan, it was not possible to encapsulate the Hb, because after addition of the Hb solution, the coacervates gradually flocculated and formed strings and particles of different sizes. The main problem with these complex coacervates was the presence of salts in the carrageenan and the tendency of lecithin to form a flocculent precipitate with carrageenan.

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Subsequent microencapsulation tests were conducted with complex coacervates formed by the interaction of protein with acidic polysaccharides in aqueous media.

E. Gelatin-Chondrointin Sulfate Complexes

The microencapsulation of aqueous Hb solutions with protein-polysaccharide complexes was studied with gelatin-CS either alone or slightly modified by incorporation of small amounts of GA. We had planned to evaluate collagen-CS complexes as capsule wall material; however, because of the instability of Hb at the pH range (2.5 to 4.5) required for preparation of good collagen solutions, no encapsulation tests were conducted with this system.

Numerous tests were conducted at different pH values above, near, or below the isoelectric point of Hb. The best results were obtained at pH 6.4 (isoelectric point) or slightly below it. Interpretation of the complexing reactions is very difficult because of the differences in molecular weights and structures of the interacting species, and also because nonelectrostatic interactions usually occurred. However, the complexes form most favorably at concentrations appropriate for electrical equivalence.

To promote rapid encapsulation of Hb by a gelatin-CS complex, we decided to form an Hb-CS complex coacervate containing a small amount of gelatin and encapsulate this coacervate with the gelatin complex. Examples of these tests follow.

For Sample No. S-11860-31 a complex coacervate of Hb-CS was formed at 25-30°C by mixing

    0.2 g 10% aqueous gelatin, pH 6
    0.5 g 30% Hb aqueous solution
    0.5 g 2.5% CS aqueous solution
    3.8 g deionized water
    2 to 3 drops of 1% aqueous acetic acid to adjust the pH to 6.4.

The coacervate formed was stirred under nitrogen in a closed vial for 5 min at 25-30°C and then it was allowed to cool to room temperature and chilled to 15°C.

The resulting mixture was then added to a coacervate produced by mixing 30°C the following materials:

    0.8 g 10% aqueous gelatin, pH 6
    0.2 g 2.5% CS aqueous solution
    0.4 g 10% GA aqueous solution, pH 6
    6.6 g deionized water
    1 or 2 drops of 1% aqueous acetic acid to induce coacervation at pH 6.3.

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Rapid encapsulation of the Hb-CS complex took place on mixing the two coacervates with continuous magnetic stirring under nitrogen. The mixture was stirred for 5 min at 30°C and then it was allowed to cool to room temperature and chilled to 10°C in an ice-water bath. The solid capsules obtained had very thick membranes, lemon shapes, and contained droplets of the Hb coacervate slightly vacuolized. The Hb could be released from these capsules by simply warming the mixture and adding a few drops of isotonic NaCl solution.

The capsules were hardened by adding 1.5 ml of 5% aqueous glutaraldehyde at pH 6.3 and stirring in the ice-water bath for 2 h. The capsules were separated by centrifugation and were washed four times with 0.9% NaCl solution and suspended in saline for storage in a refrigerator. Figure 2 shows the Hb-containing microcapsules obtained in this experiment. The size of capsules varied from 12 to 20 μm in length and 4 to 6 μm in width; the thickness of the membranes varied from 3 to 6 μm.

In a second experiment, Sample No. S-11860-32, microcapsules containing Hb were prepared using double amounts of the materials used in the preceding experiment. The capsules obtained were spherical and because we allowed a longer period of time, 20 min instead of 5 min, at 25-30°C, for the encapsulation, the capsules show thinner, uniform membranes, and no vacuoles in the Hb droplets. The capsules were hardened and isolated as those of the preceding experiment. A photomicrograph of this capsules is shown in Fig. 3. The size of the capsules ranged from 3 μm to 12 μm; approximately 70% are in the 3 to 6 μm range, and 30% in the 10-12 μm range. The absorption spectrum of a suspension of the microcapsules in a phosphate buffer, pH 7.2 was determined in the range of 500 to 700 nm (Fig. 4) after deoxygenation, in the presence of oxygen, and after passing nitrogen for 2 h. For comparison, the spectrum of the original SFHb was determined under similar conditions, Fig. 5. Although it took a longer time to deoxygenate the microencapsulated Hb, the spectra show that Hb was not denatured and retained its ability to combine reversibly with oxygen.
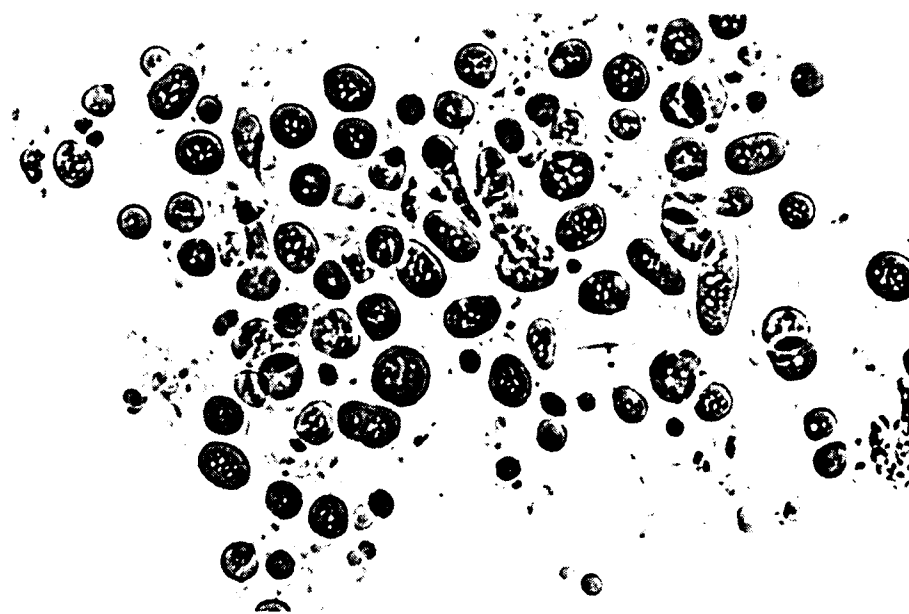
10 µm

JA-2254-5

FIGURE 2   PHOTOMICROGRAPH OF MICROENCAPSULATED Hb
SAMPLE NUMBER S-11860-31

10 μ
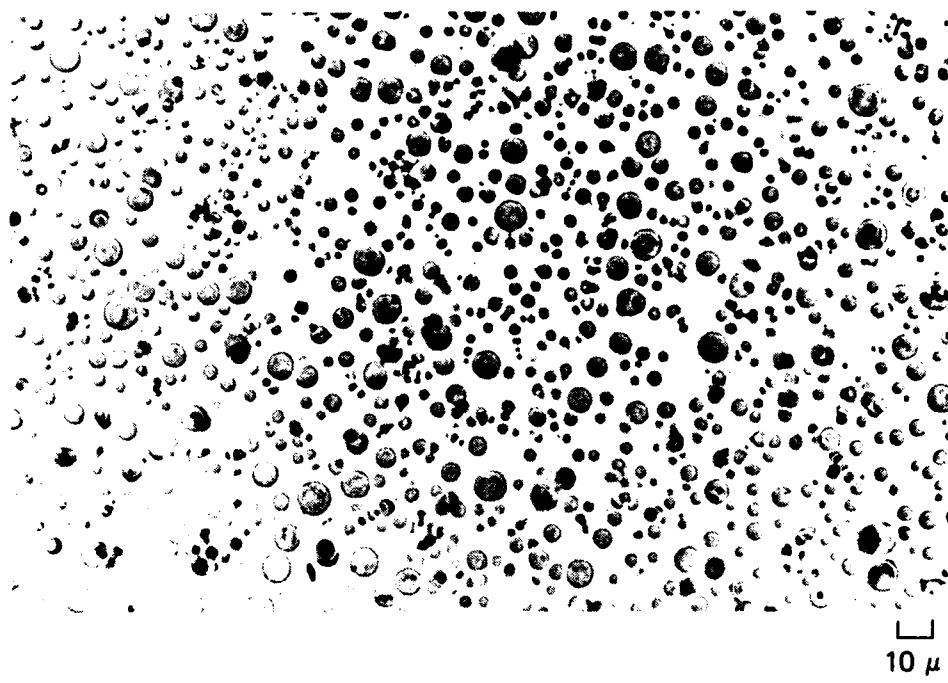
JA-2254-4

FIGURE 3   PHOTOMICROGRAPH OF MICROENCAPSULATED Hb
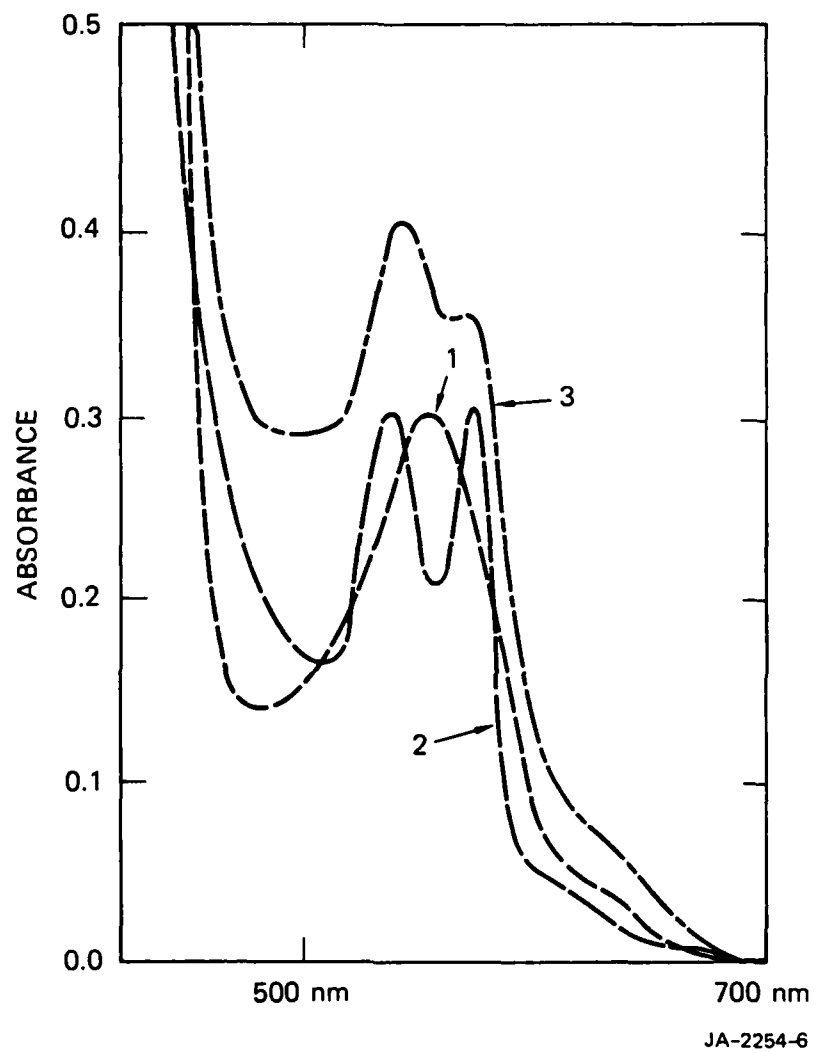SAMPLE NUMBER S-11860-32

JA-2254-6

FIGURE 4   VISIBLE SPECTRUM OF MICROENCAPSULATED Hb
SAMPLE NUMBER S-11860-32 IN PHOSPHATE BUFFER,
pH 7.2

1. Completely deoxygenated
2. After passing oxygen for 5 min at the rate of 10 ml/min
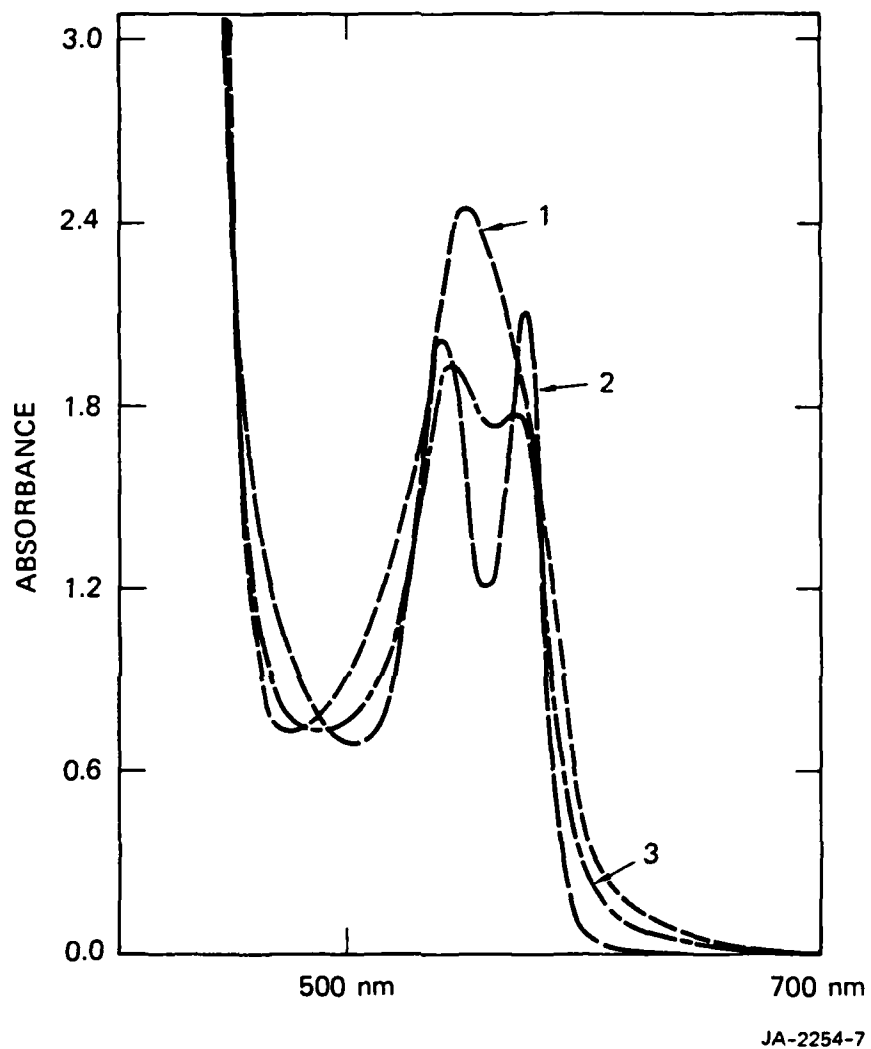3. After passing nitrogen for 2 hr at the rate of 10 ml/min

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JA-2254-7

FIGURE 5   VISIBLE SPECTRUM OF STROMA-FREE Hb IN
PHOSPHATE BUFFER, pH 7.2

1. Completely deoxygenated
2. After passing oxygen for 5 min at the rate of 10 ml/min
3. After passing nitrogen for 50 min at the rate of 10 ml/min

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IV CONCLUSIONS AND RECOMMENDATIONS

Small samples of microencapsulated Sr.Hb were prepared with most of the materials selected for this study; however, the presence of organic solvents in the encapsulation media and the long periods of mechanical emulsification required to produce large samples of uniform micro-capsules generally cause some denaturation of the Hb. Hb-containing EC microcapsules of uniform size, good stability, and with Hb having properties comparable to those of the original Hb solution were prepared in 5-g batches. When we increased the size of the batches and the Hb content of the capsules, the Hb was denatured.

The encapsulation of Hb in aqueous media was difficult and many experiments were required before we were able to produce Hb-containing microcapsules from a protein-polysaccharide complex, gelatin-CS, in which the Hb retained its ability to combine reversibly with oxygen. The system requires little stirring and yield microcapsules of fairly uniform size. It is possible that during hardening of the microcapsules some of the Hb was crosslinked. There was no time to determine the thickness of the capsule wall in the best sample made, and although oxygen diffuses rapidly through gelatin, nitrogen diffuses slowly, and argon could not displace oxygen from the capsules.

The studies should be pursued with this and other protein-acidic polysaccharide complexes and other crosslinking agents, because these systems will yield capsules similar to the red blood cells.

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