It can be concluded from Tables III and IV that the antimicrobial activity of the normal series, in cannabichromene and cannabigerol homologs and isomers, is more pronounced than in case of the iso-series. Cannabichromene type compounds having a methyl or a pentyl group in the side chain show the highest antimicrobial activity. An intermediate type of activity is seen when R is a hydrogen. However, lengthening the side chain up to C_{15} leads to a tremendous decrease in activity. Total saturation of the two double bonds in the cannabichromene and cannabigerol type compounds having a methyl side chain in most of the cases leads to an increase in the antifungal and antibacterial activities as in the case of Compounds XII and V, respectively (Tables IV and V).

This is the first reported synthesis and spectral data of most of the compounds prepared in this investigation. In addition, the antimicrobial activities of these compounds show encouraging results. The activities of the compounds prepared in this report were compared qualitatively with those of some known cannabinoids, namely, cannabigerol, cannabidiol, cannabicyclol, Δ^8- and Δ^9-tetrahydrocannabinols and were found to be far superior in most instances (Tables II and III).

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Preparation of Hydrophilic Albumin Microspheres Using Polymeric Dispersing Agents

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Abstract □ A new method for preparing glutaraldehyde cross-linked human serum albumin microspheres has been developed. Important aspects of this method include addition of glutaraldehyde in the organic phase and use of concentrated solutions of hydrophobic polymers (polymethylmethacrylate) or hydrophilic polymers (polyoxyethylene–polyoxypropylene block copolymer) as dispersion media. Uniform, round, solid, 3–150-μm hydrophilic microspheres were readily prepared by this process. The average size of microspheres was a function of dispersion time and energy input. Surface properties were altered by chemical modification using either 2-aminoethanol or aminoacetic acid to quench residual aldehyde groups. Optical and scanning electron microscopy and electronic particle size characterization indicate that the process is versatile in producing solid microspheres in a wide size range. Albumin microspheres of this type are readily dispersed in aqueous media for injection, without the need for surfactants.

Keyphrases □ Microspheres—hydrophilic albumin, preparation using polymeric dispersing agents □ Polymeric dispersing agents—preparation of hydrophilic albumin microspheres □ Glutaraldehyde—cross-linked human serum albumin microspheres, preparation using polymeric dispersing agents

Insoluble drug carriers for prolonged and controlled delivery of therapeutic agents in biological systems recently have generated growing interest (1–3). Many different carrier systems have been studied, including synthetic liposomes, erythrocyte ghosts, permeable polymeric microcapsules, and solid microspheres (4–7). Each of these drug carriers has its own advantages and problems, and there are adequate reviews of the literature (8).

The use of albumin microspheres as drug carriers has been studied to an increasing extent (9, 10). Soluble human serum albumin in blood plasma is a natural circulatory drug carrier (11). Equilibrium binding to various drugs depends primarily on hydrophobic and electrostatic interactions (12). This type of drug binding eliminates the need for covalent attachment between drug and carrier and may facilitate drug release. Human serum albumin is also degraded *in vivo*. The stability of albumin microspheres is, therefore, a function of the degree of albumin cross-linking, porosity, and accessibility of microspheres to enzymatic and phagocytic processes in the body (13).

Current methods of albumin microsphere preparation involve either thermal denaturation at elevated temperatures (110–165°) or chemical cross-linking in vegetable oil or isooctane emulsions (14, 15). Because small amounts of surfactants are needed to disperse such microspheres in water, they appear to be somewhat hydrophobic because of the method of formation. Widder *et al.* (16) hypothesize that hydrophobicity is due to the polar regions of the albumin aligning at the oil–water interface to form a hydrophobic crust or mantle at room temperature. Since their process involves thermal denaturation for microsphere stabilization, a further increase in surface hydrophobicity may occur due to additional albumin conformational changes and surface binding of oil at elevated temperatures. Surface hydrophilicity is important, because a hy-

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drophilic albumin surface may enhance surface physical and chemical behavior *in vivo*, and the surfactants presently used to disperse human serum albumin microspheres may influence tissue interactions, drug release, and activity.

The use of insoluble drug carriers for localized or intratumor injection chemotherapy was reported previously (17–19). Therefore, methods have been explored for preparing hydrophilic albumin microspheres which readily form stable dispersions for injection without surfactants and which may be easily surface-modified to introduce functional groups (*e.g.*, —CHO) or ligands (*e.g.*, antibodies) for binding to tumor tissue (17, 19).

The present report is concerned with the preparation of human serum albumin microspheres by a new procedure which yields hydrophilic, solid spheres. Glutaraldehyde cross-linking of the albumin phase also produces free aldehyde groups on the surface of the microspheres. Because of aldehyde–protein amino group coupling, these aldehyde handles may enhance tissue immobilization. The aldehyde functionality also facilitates surface chemical coupling of 2-aminoethanol or aminoacetic acid for increased hydrophilicity. Attachment of drugs, immunoglobulins, lectins, enzymes, and anionic or cationic ligands is also feasible.

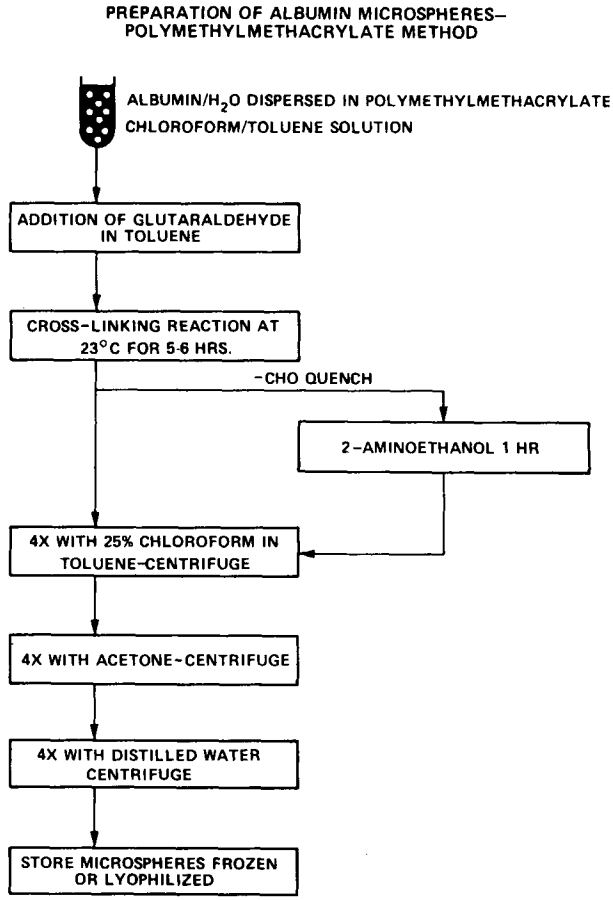
EXPERIMENTAL

Preparation of Microspheres—*Polymethylmethacrylate–Chloroform/Toluene Dispersion*—Human serum albumin[1] (150 mg) was dissolved in 0.5 ml of distilled water (pH 7.0) at 23° and added dropwise to a 25–30% solution of polymethylmethacrylate[2] (intrinsic viscosity 1.4) in a mixture of 1.5 ml of chloroform[3] and 1.5 ml of toluene[3] in a 16- × 125-mm screw-cap test tube. The mixture was dispersed with a vortex mixer[4] for various time periods (1–10 min) until the desired microsphere size was achieved. Microsphere particle size was determined by optical microscopy[5] using aliquots removed at various times.

Glutaraldehyde in toluene was used for cross-linking. Aqueous glutaraldehyde (25% biological grade)[2], 1.0 ml, and 1.0 ml of toluene were combined in a 13- × 100-mm test tube. The two phases were dispersed by sonication[6] with a microtip power head attachment (20 sec at 50 W). The resulting toluene solution of glutaraldehyde (0.14 mole was determined with 3-methyl-2-benzothiazolinone hydrazone)[1] (20) was allowed to phase separate, pipeted off, and added to the albumin dispersion. After addition of the glutaraldehyde-saturated toluene, the dispersion was mixed with a rotary mixer[7] at room temperature until albumin cross-linking was complete (5–6 hr). One milliliter of either 2-aminoethanol[8] or aminoacetic acid[1] then was added to cap any free aldehyde groups. After a 1-hr reaction time, the suspension was centrifuged (2000 ×*g*, 2 min) and the supernatant discarded. Microspheres were then washed four times each with 25% chloroform in toluene, acetone, and distilled water. Between each washing, microspheres were centrifuged (2000×*g*, 2 min), the supernatant discarded, and the pellet resuspended. The washed microspheres were either stored frozen or lyophilized. Yields were 75–85% (Scheme I).

Polyoxyethylene–Polyoxypropylene Copolymer–Chloroform Dispersion—Human serum albumin (150 mg) was dissolved in 0.5 ml of distilled water (pH 7.0) at 23° and added dropwise to a 25–30% solution of polyoxyethylene–polyoxypropylene copolymer[9] in 4.0 ml of chloroform in a 16- × 125-mm screw-cap test tube. The mixture was emulsified with a vortex mixer for various time periods (1–10 min) until the desired size of microsphere was achieved.

Glutaraldehyde in chloroform for cross-linking was prepared from 25% aqueous glutaraldehyde and chloroform by sonication as previously described. Gluturaldehyde, 0.40 mole in 1.0 ml of chloroform, was added and the dispersion was mixed with a rotary mixer at room temperature until albumin cross-linking was complete (5–6 hr). Then, 1.0 ml of either 2-aminoethanol or aminoacetic acid was added to cap any free aldehyde groups. After a 1-hr reaction time, 10 ml of acetone was added, the suspension briefly shaken, then centrifuged (2000×*g*, 2 min) and the supernatant discarded. Microspheres were then washed 10 times each with acetone and distilled water. Between each washing, microspheres were centrifuged (2000×*g*, 2 min), the supernatant decanted, and the pellet resuspended. As before, washed microspheres were either stored frozen or lyophilized. Typical yields were 75–85% (Scheme II).



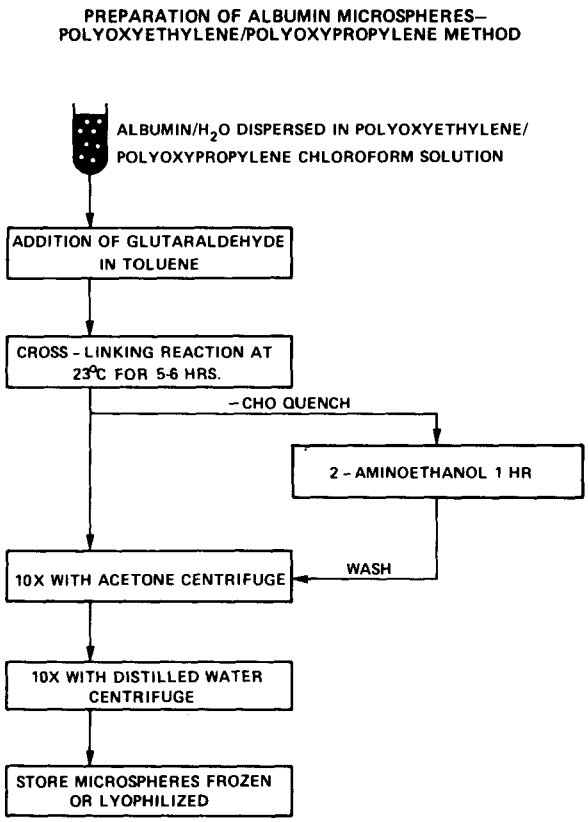
Scheme I—Albumin microsphere preparation using polymethylmethacrylate solution for dispersion.

Microscopic Characterization of Microspheres—Size and morphology were characterized by scanning electron microscopy (SEM)[10] at 20 kV. SEM samples were prepared by applying a 200 Å coating of gold–palladium on samples using a sputter coater[11] in an argon atmosphere with a digital thickness monitor. Internal structures were determined by transmission electron microscopy[12]. Microspheres mounted in epoxy were sliced with an ultramicrotome[13]. Microsphere compositions and porosity were verified with 0.5 ml of dye added to 10-mg spheres in 2 ml of distilled water[14] using optical microscopy. Protein stained microspheres were also mounted in epoxy, sliced with the ultramicrotome, and examined by optical microscopy.

Effect of Polymer Dispersant Concentration—Human serum albumin microspheres were prepared by the two methods, and the concentrations of polymer solutions (polymethylmethacrylate and polyoxyethylene–polyoxypropylene) were varied from 0 to 25% in 2% intervals. After cross-linking with glutaraldehyde, the dispersions were evaluated for microsphere stability against coagulation.

Measurement of Microsphere Size Distribution as a Function of Dispersion Time—Human serum albumin microspheres were prepared

[1] Sigma Chemical Co.
[2] Polyscience.
[3] Fisher Scientific Co.
[4] Vortex Genie Scientific Industries, Inc.
[5] Nikon Biphot.
[6] Heat Systems-Ultrasonics, Model W-375.
[7] Labquake Labindustries.
[8] Mallinckrodt.
[9] BASF W-jandotte Corp.
[10] JEOL model 35C.
[11] Hummer V.
[12] Phillips model 310.
[13] Sorvall model MT-2.
[14] Coomassie blue G-250, BIO-RAD Laboratories.



Scheme II—Albumin microsphere preparation using polyoxyethylene–polyoxypropylene solution for dispersion.

using both described procedures. Dispersion times of 1, 3, 5, and 8 min were used at constant energy input (speed setting 8 on vortex mixer). The washed microspheres were suspended in 10 ml of distilled water. Aliquots were withdrawn from a well-shaken sample and placed on an SEM sample holder. One hundred random microspheres for each dispersion time were counted and measured. Aliquots (10 μl) were also added to 150 ml of continuously stirred 0.14 *M* potassium chloride solution. A 1.0-ml sample was withdrawn over a 4-sec time span by an electronic particle size counter[15] equipped with a 100-μm sampling aperture. The counter was calibrated with 9.76 μm of polystyrene microspheres. This enabled accurate size measurements from 2 to 40 μm. The total number of microspheres counted ranged from 7000 to 23,000/sample.

Microsphere Size Distribution as a Function of Energy Input—Human serum albumin microspheres were prepared using the polymethylmethacrylate procedure. Dispersion time was held constant (10 min), and energy input was varied by adjusting the speed setting on the vortex mixer, using speeds of 2, 4, 6, and 8. The washed microspheres were suspended in 10 ml of distilled water and size distributions were measured by the SEM technique.

Measurement of Reactive Aldehyde Groups—Human serum albumin microspheres (10 μm average diameter) were prepared by the polymethylmethacrylate method. The cross-linked microspheres were divided into two samples. One was quenched with 0.5 ml of 2-aminoethanol while the other sample was left unquenched. [^{3}H]Leucine[16], specific activity 134.2 Ci/mmoles/ml, was diluted with a carrier (L-leucine)[17] to a final activity of 5 μCi/50 mmoles/ml. One milliliter of isotope solution was added to each of three samples of 7.4 mg/ml unquenched and three samples of 7.99 mg/ml quenched microspheres in 13- × 100-mm test tubes. The samples were incubated for 40 min in a tabletop sonicator[18], then washed four times with distilled water (pH 7.0) by centrifugation (1000×*g*, 2 min). Microsphere pellets were resuspended in 2 ml of a scintillation cocktail[16] and slightly shaken until microspheres were completely dissolved. From each of these solutions, 1.0-, 0.5-, and 0.25-ml aliquots were removed and added to scintillation counter containers. The

[15] Coulter Counter model TA11.
[16] Aquasol New England Nuclear Corp.
[17] Pierce Chemical Co.
[18] E/MC RA Research.

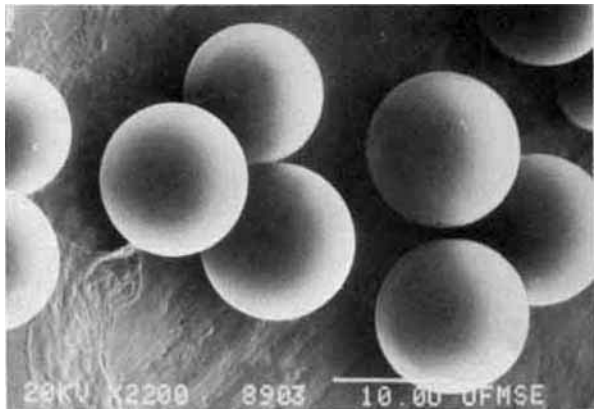


Figure 1—*Scanning electron micrograph (2200×) of albumin microspheres. Polymethylmethacrylate dispersant, 10-μm average diameter.*

final volumes were adjusted to 15 ml with additional cocktail. Activity was determined using a scintillation counter[19] and values plotted against prepared standards.

RESULTS

Figure 1 shows the uniform, smooth spherical geometry of albumin microspheres typically prepared by the procedure (SEM photomicrograph at 2200×). The human serum albumin microspheres in Fig. 1 averaged 10 μm in diameter and were made using polymethylmethacrylate as the polymer dispersant.

The SEM photomicrograph in Fig. 2 clearly shows the solid internal structure of the human serum albumin microspheres. Figure 2 is consistent with the transmission electron microscopic results (not shown) of sectioned microspheres obtained from both dispersant systems. Microtomed sections of the protein stained microspheres showed the blue dye color throughout the albumin spheres. This complete dye penetration is indicative of the porous internal structure of the albumin spheres.

Washed, lyophilized, or air-dried microspheres were readily resuspended in a variety of aqueous media such as distilled water, physiological saline, phosphate buffer, and acetate buffer. The microspheres were easily wetted and dispersed without the need for surfactants.

Figures 3 and 4 show the size distributions of the microspheres as a function of dispersion time using the vortex apparatus. Polymethylmethacrylate was used as a dispersant for the microspheres in Fig. 3 and polyoxyethylene–polyoxypropylene for those in Fig. 4. These data were obtained using an electronic size counter. Average diameters from these data were in close agreement with data obtained from SEM measurements. These results are presented in Table I. SEM size distribution as

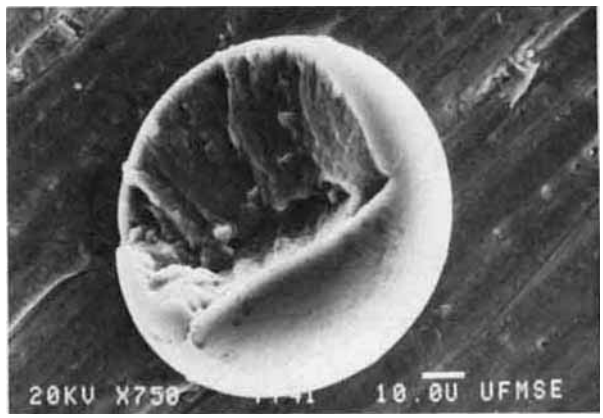


Figure 2—*Scanning electron micrograph (750×) of 100 μm albumin microsphere showing internal structure; polymethylmethacrylate dispersant.*

[19] Beckman model 230.

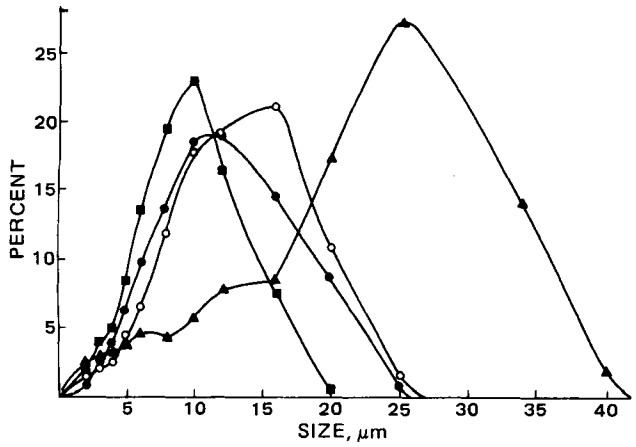


Figure 3—*Size distributions of albumin microspheres using polymethylmethacrylate dispersant. Constant power input, with time of dispersion varied. Key: (▲) 1 min; (○) 3 min; (●) 5 min; (■) 8 min (measurements by Coulter Counter).*

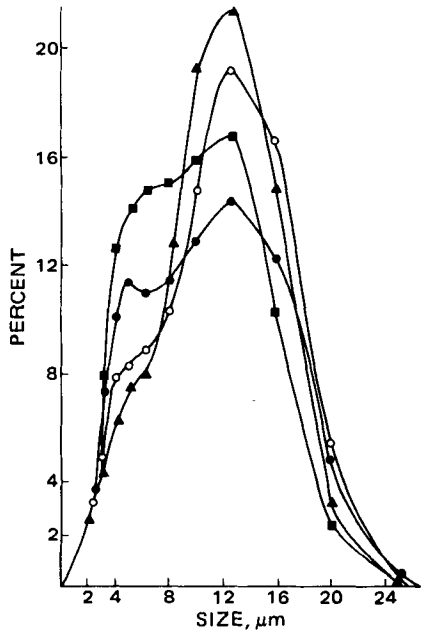


Figure 4—*Size distributions of albumin microspheres using polyoxyethylene-polyoxypropylene dispersant. Constant power input, with time of dispersion varied. Key: (▲) 1 min; (○) 3 min; (●) 5 min; and (■) 8 min (measurements by Coulter Counter).*

a function of power input is shown in Fig. 5 (polymethylmethacrylate dispersant) and summarized in Table II. Table III also indicates that a polymer concentration of at least 10% (w/w) was needed to stabilize the

Table I—Human Serum Albumin Microsphere Size *versus* Dispersion Time [a]

Dispersion Time, min	Diameter, μm (Measured by SEM) $\bar{X}$	*SD*	Variance	Measured with Coulter Counter Mean	Polymer Dispersant[b]
1	22	6.7	44	19	A
	12	5.3	28	11	B
3	12	3.7	13	12	A
	10	3.7	13	10	B
5	10	3.6	13	11	A
	8.5	2.7	7	9	B
8	8.5	3.0	9	9	A
	7.0	2.5	8	7	B

[a] Constant power input: speed setting 8 on vortex genie. [b] A, Polymethylmethacrylate; B, polyoxyethylene-polyoxypropylene.

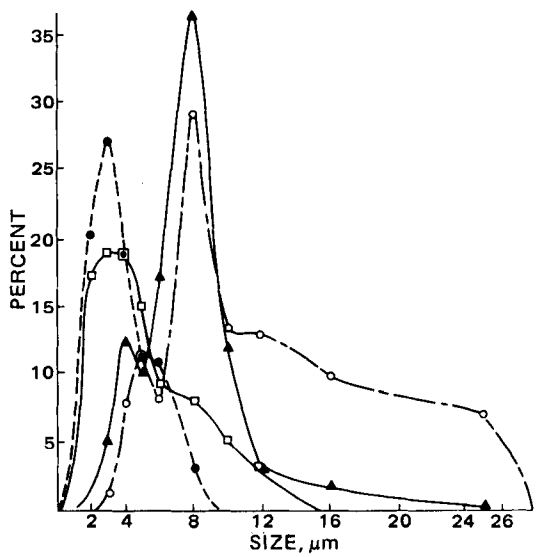


Figure 5—*Size distributions of albumin microspheres using polymethylmethacrylate dispersant. Constant dispersion time (10 min), with power input varied. Key: (○) speed setting 2; (▲) speed setting 4; (□) speed setting 6; (●) speed setting 8 (measurements by SEM).*

dispersion at short dispersion times (1.0 min). When smaller microspheres are desired (requiring longer dispersion times or higher energy input) higher concentrations of polymer are needed. This is also indicated in Table III.

[^{3}H]Leucine binds to the reactive aldehyde groups as shown in Table IV. By subtracting the amount of adsorbed [^{3}H]leucine (measured in aldehyde-quenched microspheres) from the amount adsorbed and covalently bound to the unquenched spheres, it was determined that 4.0 $\times 10^{-2}$ μmoles of leucine bind to reactive aldehyde groups in 1.0 ml of human serum albumin microspheres. Electronic particle analyses es-

Table II—Human Serum Albumin Microsphere Size *versus* Dispersion Energy [a]

Dispersion Energy (Speed Setting on Vortex Genie)	Diameter, μm Measured by SEM $\bar{X}$	*SD*
2	9	4.0
4	7	2.9
6	4.4	2.5
8	3.5	1.6

[a] Constant dispersion time: 10 min.

Table III—Effect Of Polymer Dispersant Concentration

Polymer Concentration, %	Dispersion Time[a], min	Dispersion Stability[b]
0 → 8	1	−
	5	−
10	1	+
	5	−
12	1	+
	5	−
14	1	+
	5	−
16	1	+
	5	−
18	1	+
	5	−
20	1	+
	5	−
25	1	+
	5	−
25 → 35	1	+
	5	+

[a] Power input constant (speed setting 8 on vortex genie). [b] −, indicates unstable dispersion; +, indicates stable dispersion.

Table IV—Concentration of Reactive Aldehyde Groups by Binding of [H^3]Leucine

Physical or Chemical Binding of Leucine with:	μMoles Leucine Bound/ml of Microspheres	Number of Leucines/10 μm Sphere	Number Reactive —CHO/ Sphere[a]
2-Aminoethanol Quenched Microspheres	8.9×10^{-2}	5.4×10^{6}	—
Unquenched Microspheres	1.3×10^{-1}	7.8×10^{6}	—
Chemically Reacted Leucine (Unquenched − Quenched)	4.1×10^{-1}	2.4×10^{6}	2.4×10^{6}

[a] Assumed one bound leucine equals one reactive aldehyde group.

tablished that there were ~1.0×10^{10} microspheres/ml in a 10-μm diameter preparation. This is equivalent to 2.42×10^{6} reactive aldehyde groups/sphere based on one leucine molecule covalently bound per reactive aldehyde group. From the data of Ikada *et al.* (21) 1.1 μg of a human serum albumin monolayer would cover a 1.0-cm^2 area, and if perfect molecular packing for albumin molecules were assumed, 3.02×10^{7} molecules would fill the surface area of a 10-μm microsphere. On this basis, the 2.42×10^{6} reactive aldehyde groups measured/10-μm sphere, therefore, would be equivalent to ~8 reactive aldehyde groups/100 human serum albumin molecules at the surface, which is clearly a maximum value, since a significant fraction of —CHO groups must also be present within the spheres.

DISCUSSION AND CONCLUSIONS

Good dispersion and stabilization of aqueous human serum albumin was obtained in concentrated polymer solutions. No additional surfactants were required. Insoluble human serum albumin microspheres were produced with glutaraldehyde cross-linking in a wide particle size range as shown in the SEM photomicrographs. Optical photomicrographs (Figs. 6*a* and *b*) show the polymer coating surrounding the human serum albumin microsphere dispersion in the aqueous wash phase. This polymer coating or microcapsule apparently affords dispersion stability and prevents coagulation before and during glutaraldehyde cross-linking.

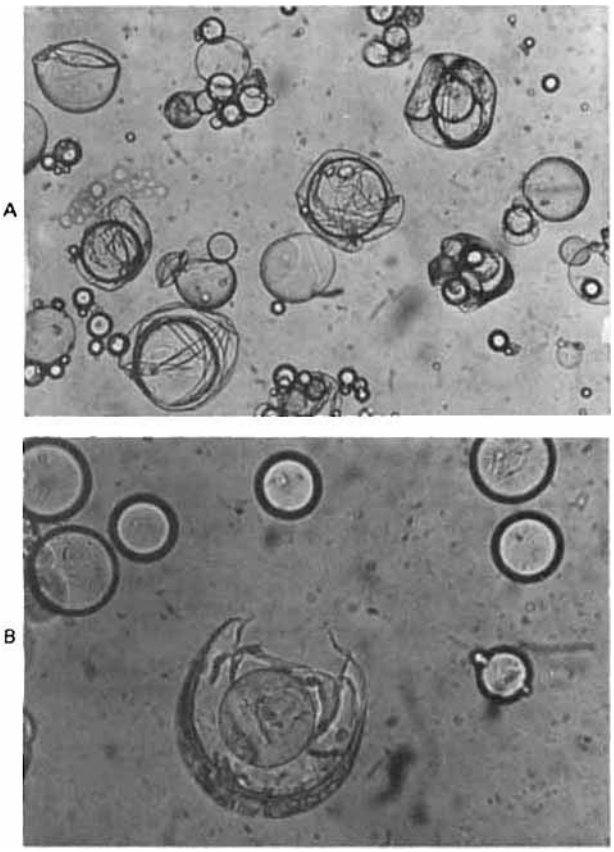


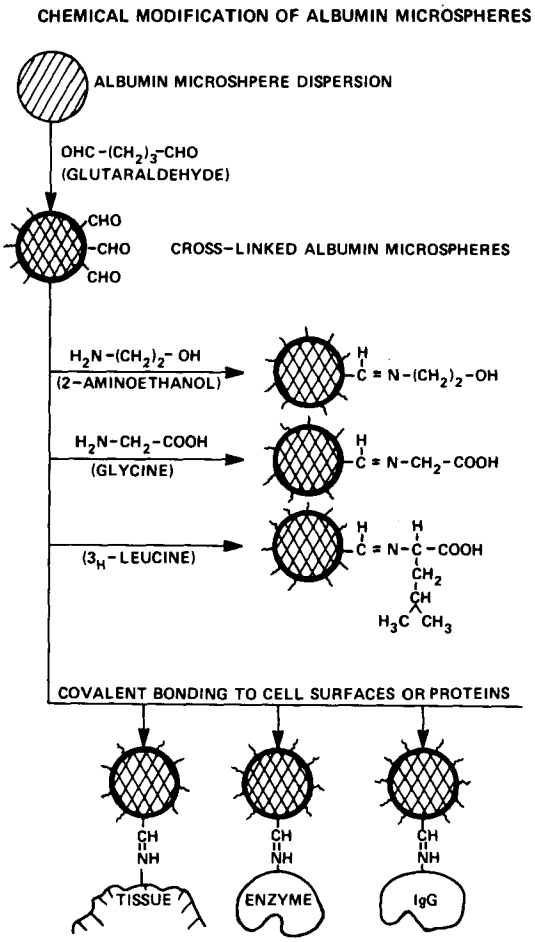
Figure 6—*(a) Optical micrograph of stabilized albumin microspheres showing the surrounding polymer coating. Microspheres in aqueous wash phase; average particle size 40 μm; polyoxyethylene–polyoxypropylene dispersant. (b) Polymer coated microspheres, as in (a), showing ruptured polymer coating during aqueous wash.*

The concentration of the polymer dispersant solutions was important; concentrations <25% (w/w) were not sufficient to prevent coagulation when small microspheres (≤10 μm) were desired, and concentrations >30% retarded complete removal of the polymer. Polymer molecular weight was also important. Low molecular weight polymethylmethacrylate (intrinsic viscosity 0.4) was unsatisfactory at concentrations of 25–30% (w/w), since coagulation occurred.

The size of the microspheres was directly related to power input and dispersion time (Figs. 3–5 and Tables I and II). Thus, by adjusting power input and time, particle size was readily controlled.

Two unique distinguishing features of this method for albumin microsphere preparation as compared with methods reported to date (9, 10, 14–16) are the addition of the glutaraldehyde cross-linking agent in an organic medium to the aqueous albumin dispersion and use of concentrated high polymer solutions as the organic phase for preparing dispersions. Human serum albumin microspheres prepared by thermal denaturation in oil or by glutaraldehyde cross-linking with aqueous aldehyde (sometimes incorporated at low temperature into the aqueous albumin phase) are hydrophobic to the extent that surfactants must be used to prepare microsphere dispersions in water (10, 22). This is probably a result of preferential hydrophobic organization of the albumin surface at a hydrophobic oil interface coupled with thermal denaturation or homogeneous cross-linking with glutaraldehyde.

Use of concentrated polymer solutions as the organic dispersing phase



Scheme III—Schematic representation of albumin microsphere surface chemistry.

affords excellent dispersion and control, wide particle size latitude, and smooth, uniform spheres without the need for surfactants. More importantly, because glutaraldehyde is presented to the aqueous albumin microsphere dispersion from the organic phase, there must be a strong preference for aldehyde-amino group reactions and cross-linking at or near the albumin surface. This probably produces a case-hardening effect with a higher cross-link density near the surface of the microspheres accompanied also by a much higher concentration of mono-reacted dialdehyde at the surface (Scheme III). The result of monofunctional glutaraldehyde capping of lysine amino groups at the albumin microsphere surface would be to increase surface anionicity and hydrophilicity, especially if a portion of the free aldehyde functions oxidize to carboxyl groups.

The availability of a relatively high surface concentration of aldehyde functionality also facilitates a variety of chemical modifications including: aminoalcohol capping to enhance hydrophilicity; enzyme, antibody, or other protein ligand binding; covalent attachment of amino-functional drugs. As suggested in Scheme III, such changes in surface functionality may be used to enhance tissue immobilization by covalent or physical binding, for specific tissue targeting using biospecific affinity ligands (*e.g.*, tumor-specific antibodies), or for improved diagnostic or immune assay reagents and procedures.

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Kinetics and Mechanism of the Equilibrium Reaction of Triazolam in Aqueous Solution

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Abstract □ The equilibrium kinetics of triazolam in aqueous solution was investigated in the pH range of 1–11 at body temperature. The quantitative study indicated that it forms equilibrium mixtures consisting of ring-opened and closed forms with the composition being dependent on pH. The equilibrium constants of the two species in the pH range studied were determined by GLC method. The apparent first-order rate constants were estimated from the decreasing or increasing absorbance of the mixture in solutions. The forward-reaction rate constant (k_f) showed a bell-shaped k_f–pH profile with a rate maximum at pH 4.59, which indicates not only that the carbinolamine intermediate forms during the equilibrium reaction, but that the rate-determining step of the reaction differs for the acidic and basic sides of the rate maximum. The reverse-reaction rate constant decreased with increasing pH and could not be estimated in the pH region >5.65. Theoretical curves for both forward and reverse reactions satisfactorily fit the observed data. The pKa values of triazolam and its opened-form amine were estimated to be 1.52 and 6.50, respectively.

Keyphrases □ Triazolam—kinetics, mechanism of equilibrium in aqueous solution □ Kinetics—triazolam, mechanism of equilibrium in aqueous solutions □ Equilibrium reaction—triazolam, kinetics, mechanism in aqueous solutions

1,4-Benzodiazepines, first synthesized in the early 1960s, are important minor tranquilizers. These tranquilizers are being extensively used as sedative, hypnotic, muscle relaxant, and anticonvulsant drugs. Their stability and reactivity in aqueous solutions have been under study extensively in the last 5 years. Reaction mechanisms for the hydrolysis of 1,4-benzodiazepines have been reported for chlordiazepoxide (1, 2), oxazepam (3), diazepam (4), nitrazepam (4, 5), desmethyldiazepam (6), and clonazepam (6). Furthermore, equilibrium reactions of these compounds have been reported for pyrazolodiazepinone (7), diazepam (8), nitrazepam (9), triazolobenzodiazepines (10), flunitrazepam (11), and desmethyldiazepam (12). It is now well known that hydrolysis of an azomethine bond

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