Adsorption of Fluorescein Dyes on Albumin Microspheres

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Received May 29, 1991; accepted November 11, 1991

The surface characteristics of bovine and egg albumin microspheres were examined using four anionic dyes; sodium fluorescein, eosin, erythrosin, rose bengal, and the cationic dye rhodamine B. The adsorption isotherms of the dyes on unloaded albumin microspheres exhibited Langmuir behavior for dilute solutions of rose bengal, erythrosin, and eosin, suggesting monolayer formation in the initial stages of the sorption process. The adsorption capacity of the microspheres for the dyes (k_2) and the affinity constants of the dyes for the microspheres (k_1) were found to depend on both the polarizability and the hydrophobic properties of the dye, presumably reflecting the heterogeneous character of the microsphere surface. Further, the extent of sorption at higher dye concentrations was found to depend on the ability of the dye to form stable aggregates inside the microspheres and on environmental long-range forces acting at these sites. At both low and high dye concentrations, the amount adsorbed to the microsphere surface increased with increasing hydrophobicity of the dyes. The lowest adsorption was observed for the nonsubstituted dye fluorescein, whereas the most hydrophobic dye used, rose bengal, was completely adsorbed onto the microsphere surface. The data suggest that the bovine albumin microsphere surfaces are highly hydrophobic and less porous than egg albumin microsphere surfaces.

KEY WORDS: bovine albumin; egg albumin; microspheres; adsorption; hydrophobicity; fluorescein dyes.

INTRODUCTION

A wide variety of drug carrier systems has been described in the literature. These include macromolecules such as DNA (1,2), liposomes (3), and other colloidal systems based on natural macromolecules, particularly proteins. Since many drugs specifically bind to proteins such as human, egg, and bovine albumin, albumin microspheres may be used as drug carriers (4–6). The morphological properties of the albumin microspheres have been studied in detail using scanning electron microscopy (SEM) (7,8). However, very little is known about the nature of interactions between a given drug and the microspheres.

The extent of incorporation of a drug in a carrier such as microspheres and its release from the matrix depend on several factors including the mode of interaction of drug with the albumin matrix. From this standpoint alone it is necessary to characterize the drug–microsphere association in terms of the physicochemical properties of the drug as well as the albumin employed. Further, in cases where drug degradation may occur under the conditions of microsphere preparation, it would be useful to examine if the drug would associate strongly with unloaded microspheres, so as to result in an effective drug delivery system.

In this paper, we describe the results of the adsorption behavior of a family of fluorescein dyes with varying hydrophobicities and polarizabilities on unloaded albumin microspheres. The objective of these studies was to determine if the physicochemical nature of the albumin microspheres can be described using simple adsorption behavior of the model dyes.

MATERIALS AND METHODS

Materials

Rhodamine B, eosin, erythrosin, sorbitan trioleate, sodium fluorescein, and egg albumin (grade II) were obtained from Sigma Chemical Co. (St. Louis, Mo.) and used without further purification. Bovine albumin powder (clinical reagent grade) was purchased from Armour Pharmaceutical Company, and rose bengal from BDH Chemicals Ltd., Poole, England. Cyclohexane was obtained from Reidel-Seelze, Hanover, and formaldehyde from Mallinckrodt Chemicals Works.

Preparations of Microspheres

Bovine and egg albumin microspheres were prepared by a modification of the method of Scheffel *et al.* (9). Five milliliters of albumin solution was added, with stirring at 1000 rpm, to 100 ml of cyclohexane containing 2% (v/v) sorbitan trioleate as an emulsifier in a 250-ml three-necked flask. Stirring was carried out using a stirrer assembly consisting of a glass rod with a Teflon propeller attached at one end and the other end attached to a motor mechanism with variable-speed control. After 10 min of continuous stirring to obtain a good dispersion, the flask was immersed in a water bath at 80°C for 90 min to denature the albumin. Alternatively, a 37% aqueous solution of formaldehyde, a cross-linking agent, was used to denature albumin. The duration of 90 min employed for denaturation of the native albumins was determined as follows. After denaturation, the suspension was centrifuged and the supernatant was assayed for native albumin content using UV spectroscopy. Incubation for 90 min at 80°C or with cross-linking agent at room temperature resulted in complete denaturation as indicated by the total absence of native albumin in the supernatant. In the formaldehyde experiments, the mixture was equilibrated for 90 min with stirring to precipitate the albumin particles. The denatured dispersion was cooled to room temperature with continuous stirring for heat-denatured systems and without stirring for systems denatured with the cross-linking agent. The solid particles were then decanted and washed in 100 ml of an ethanol/methanol mixture (95:5) with gentle agitation for 5 min. This procedure was repeated twice and the suspension was then filtered. The solid particles were dried at 40°C overnight under a gentle stream of nitrogen to facilitate complete removal of residual solvents and stored in tightly sealed vials.

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Microscopic Evaluation of the Microspheres

Scanning electron microscopy (SEM) was used to study the surface and particle size of the microspheres. The samples were treated for 90 sec in an atmosphere of argon with gold–palladium (Pelco Model 3 Sputter Coater) and examined with a scanning electron microscope (JEOL JSM 35C).

Evaluation of the Surface Properties of Albumin Microspheres

The surface properties of the albumin microspheres were evaluated using five fluorescein dyes of varying hydrophobicity. Twenty-five milligrams of albumin microspheres (25 ± 5 μm) was accurately weighed and suspended in 12.5 ml of phosphate buffer, pH 7.4 (ionic strength, 0.165), containing dye concentrations ranging from 10^{-3} to 10^{-7} *M*. The suspensions were stored in tightly sealed culture tubes wrapped in aluminum foil to prevent degradation by light. The suspensions were equilibrated at room temperature with continuous shaking for 24 hr.

After equilibration, the microspheres were separated by centrifugation at 10,000 rpm. Dye content was spectrophotometrically determined in aliquots of the supernatant solution. Standard curve for the fluorescein dyes over a wide range of dye concentrations were constructed. Absorbances were measured at 541.7, 525.1, 514.8, 489.1, and 551.9 nm for rose bengal, erythrosin, eosin, fluorescein, and rhodamine B, respectively. Assay of drug content in adsorption experiments was carried out after appropriate dilution to ensure that absorbances were well within the linear range of standard absorbance–concentration plots.

A spectral scan of the supernatant solution after centrifugation of unloaded albumin microspheres incubated in phosphate buffer with continuous shaking for 24 hr indicated the total absence of albumin. Scanning electron microscopy was employed to determine any changes in surface morphology of bovine albumin microspheres before and after equilibration with the fluorescein dyes.

Treatment of Adsorption Data

The Langmuir model assumes that (a) all sites available for adsorption are energetically equivalent, (b) the adsorbed phase is confined to a monolayer, (c) there are no lateral interactions between adsorbate molecules, (d) the adsorbate solution is very dilute, (e) there is no mixed film formation at maximum solute adsorption, and (f) adsorption is reversible. Since the adsorption of dyes onto albumin microspheres appeared to satisfy the above conditions, especially in dilute solutions, the adsorption data were analyzed according to the Langmuir model.

The Langmuir equation is given by

$$x/m = k_1k_2C_{eq}/1 + k_1C_{eq} \quad (1)$$

where x is the amount of dye adsorbed, m is the mass of microspheres, C_{eq} is the concentration of unadsorbed dye at equilibrium, k_1 is the affinity constant of the dye for the microspheres, and k_2 is a constant indicating the capacity of the microspheres for a given dye.

Equation (1) can be rearranged as

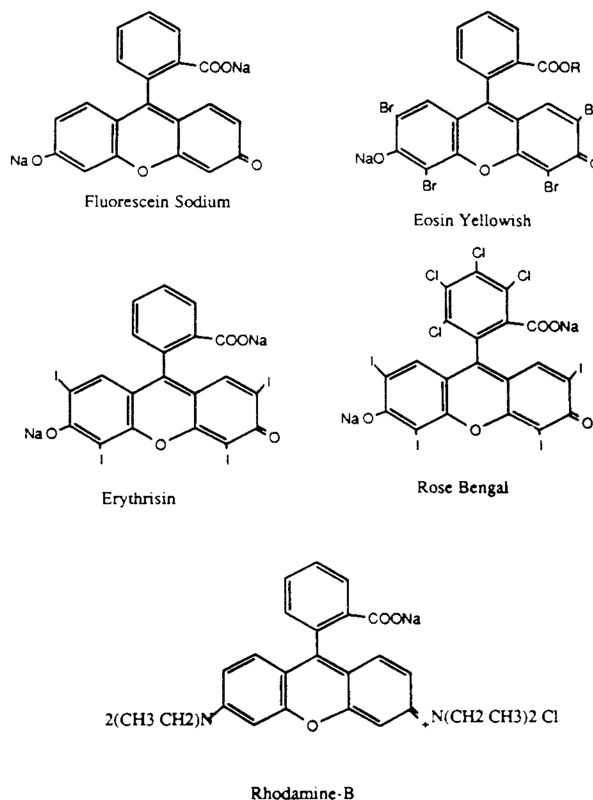

Fig. 1. Chemical structures of the fluorescein probes.

$$C_{eq}/(x/m) = 1/k_1k_2 + 1/k_2C_{eq} \quad (2)$$

A plot of $C_{eq}/(x/m)$ versus C_{eq} should yield a slope of $1/k_2$ and an intercept of $1/(k_1k_2)$. Adsorption data were analyzed using linear regression fits of the data to Eq. (2).

RESULTS

The chemical structures of the family of fluorescein probes used in the study are shown in Fig. 1. Table I lists the physicochemical properties of the fluorescein dyes. It is evident from the table that the relative polarizabilities as well as hydrophobicities (calculated using Hansch subsituent effects) increase linearly with increasing halosubstitution. The dimerization constants of the dyes also increase with increasing halosubstitution. Thus, it appears that both polar-

Table I. Physicochemical Properties of Fluorescein Dyes

Fluorescein dye	Relative polarizability $\Sigma \alpha_i$[a]	Dimerization constant k[b]	Partition coefficient octanol/water, log PC[c]
Rose bengal	29.56	250	11.36
Erythrosin	20.44	140	8.52
Eosin	13.36	110	7.48
Fluorescein	0.00	5	4.05
Rhodamine B	—	2100	5.54

[a] Calculated according to Rohatgi and Mukhopadhyay (12).
[b] From Rohatgi and Mukhopadhyay (12) and Lopez *et al.* (18).
[c] Calculated using Hansch substituent effects (19).

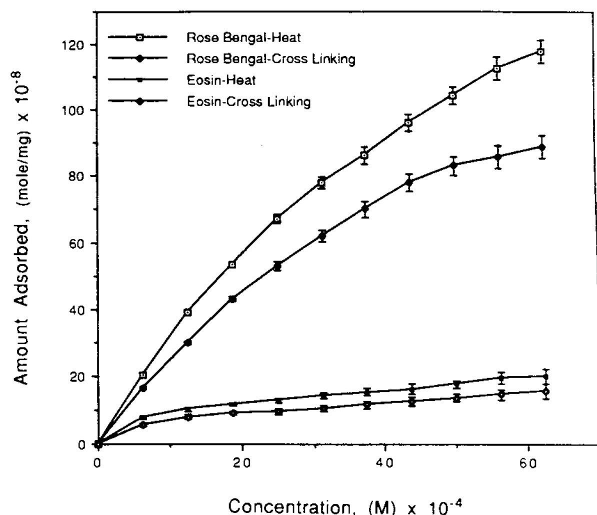

Fig. 2. Comparison of the adsorption of rose bengal and eosin on unloaded bovine albumin microspheres prepared using heat and cross-linking agent denaturation.

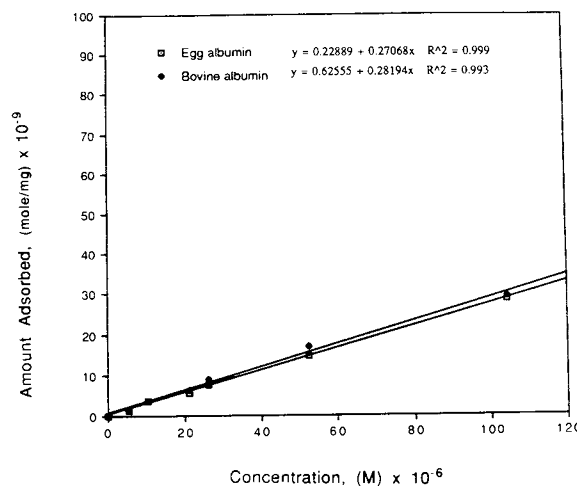

Fig. 4. Adsorption of rhodamine B from dilute solutions by unloaded egg and bovine albumin microspheres prepared using heat denaturation.

izability and hydrophobicity contribute to dimerization effects.

The mean diameter of unloaded bovine and egg albumin microspheres prepared using heat denaturation or denaturation via cross-linking agents was determined using scanning electron microscopy (SEM). Micrographs were obtained under various magnifications. The mean diameter obtained from a population of 100 microspheres was 25 ± 5 μm for all the microspheres presented in this study.

Previous studies (10,6) on albumin microspheres denatured with heat or cross-linking agent showed that storage under 4°C for up to 6 months had no influence on the morphology particle size, and *in vitro* drug release rates.

Figure 2 shows a typical plot of adsorption of rose bengal and eosin on bovine albumin microspheres prepared using (a) heat denaturation and (b) cross-linking agent (formaldehyde). The adsorption capacity of the cross-linked derived microspheres for the dyes appears to be around 20–25% lower than that of microspheres obtained using the heat denaturation method.

Figure 3 shows a plot of amount of rose bengal adsorbed from dilute solutions by egg albumin microspheres as a function of equilibrium concentration of the dye. The adsorption isotherm profiles for eosin and erythrosin were similar to that shown for rose bengal. Rhodamine B adsorption behavior was qualitatively dissimilar (Fig. 4). Fluorescein did not adsorb to egg or bovine albumin microspheres from dilute solutions. Figure 5 shows a typical Langmuirian plot of $C_{eq}/(x/m)$ versus C_{eq} for rose bengal with bovine albumin microspheres. Table II lists the adsorption parameters, k_1 and k_2, for rose bengal, erythrosin, and eosin with egg and bovine albumin microspheres along with the correlation coefficients, r.

Figures 6 and 7 show the amounts of rose bengal, erythrosin, eosin and fluorescein adsorbed or incorporated from concentrated solutions, with egg and bovine albumin microspheres, respectively. Figure 8 shows the adsorption or in-

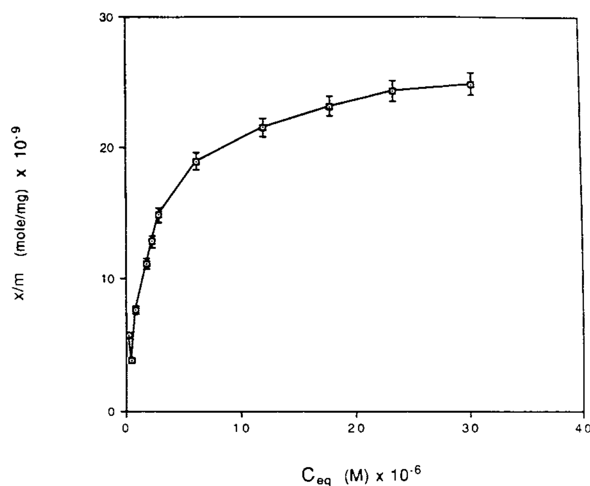

Fig. 3. Adsorption of rose bengal from dilute solutions by unloaded egg albumin microspheres prepared using heat denaturation.

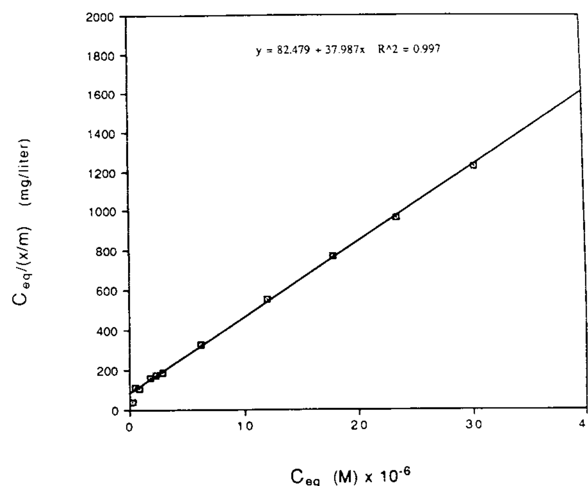

Fig. 5. Linear regression fit of the adsorption of rose bengal from dilute solutions by unloaded heat denatured egg albumin microspheres using Eq. (2).

Table II. Parameters from Linear Regression Fit for Adsorption of Dyes on Albumin Microspheres Using the Langmuir Equation (2)

Albumin	Dye	k_1 (liters/mol * 10^5)	k_2 (mol/mg * 10^{-8})	r
EGG	Rose bengal	4.60	2.63	0.998
	Erythrosin	2.12	2.34	0.997
	Eosin	0.69	1.09	0.998
Bovine	Rose bengal	6.74	3.53	0.998
	Erythrosin	5.79	3.45	0.988
	Eosin	0.47	1.53	0.997

corporation of rhodamine B from concentrated solutions with egg and bovine albumin microspheres. The amounts of the dyes adsorbed or incorporated appear to increase linearly with dye concentration (r^2 = 0.995).

Figures 9–12 show scanning electron micrographs of bovine and egg albumin microspheres prepared under various conditions. A few general trends are evident from the micrographs.

(a) Heat-denatured microspheres are more porous than cross-linked denatured microspheres (Figs. 9 and 10).

(b) Egg albumin microspheres are more porous than bovine albumin microspheres (Figs. 9 and 11).

(c) Bovine albumin microspheres incubated with dye solutions for 24 hr are unaffected by the incubation process (Figs. 9 and 12).

DISCUSSION

Fluorescent probes of varying hydrophobicity were used to investigate the surface characteristics and the microstructure of bovine and egg albumin microspheres. The selected dyes differed in the level of halogen substitution (Fig. 1). The dye hydrophobicities and the polarizabilities in ascending order were fluorescein < eosin < erythrosin < rose bengal (Table I). The self-association of these probes in dilute solutions (less than 10^{-5} *M*) is negligible (11). The reported self-association constants and thermodynamic parameters for rose bengal and the other fluorescent probes derived from spectroscopic studies are shown in Table I. It has been postulated that the halogen substituents, especially the bulky and highly polarizable iodine atoms in rose bengal and erythrosin and the bromine in eosin, play an important role in the unique behavior of rose bengal, erythrosin, and eosin. These halogen substituents augment the hydrophobic character and the polarizability of the hydrocarbon skeleton and sterically increase the stability of the noncoplanar conformation (12). Further, they decrease the specific hydration capacity of the solvent water molecules since the large volume of the bulky substituents in the crowded molecule prevents the solvent from approaching the negatively charged phenoxide group (13).

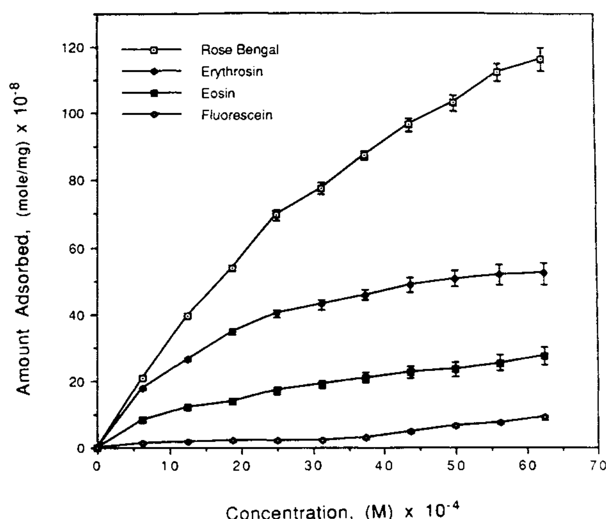

Fig. 7. Adsorption and incorporation of rose bengal, erythrosin, eosin, and fluorescein from concentrated solutions by unloaded egg albumin microspheres prepared using heat denaturation.

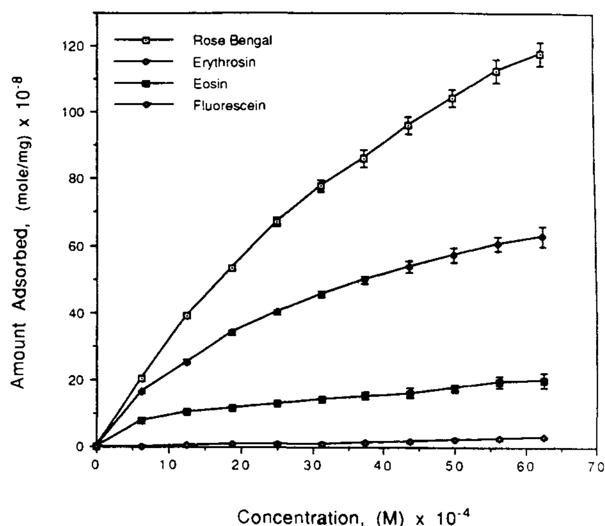

Fig. 6. Adsorption and incorporation of rose bengal, erythrosin, eosin, and fluorescein from concentrated solutions by unloaded bovine albumin microspheres prepared using heat denaturation.

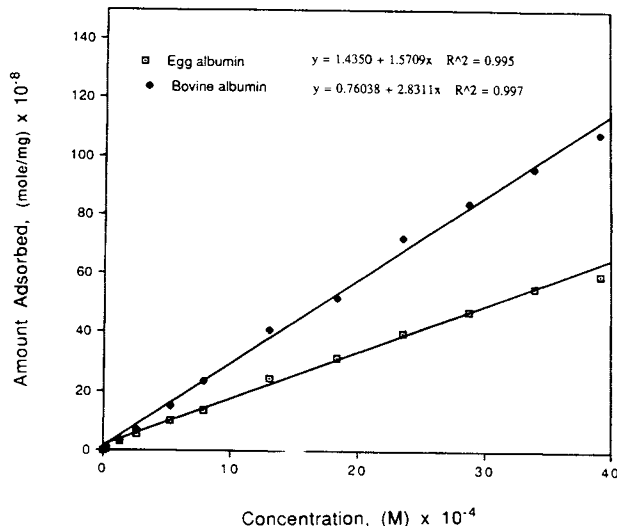

Fig. 8. Adsorption and incorporation of rhodamine B from concentrated solutions by unloaded egg and bovine albumin microspheres prepared using heat denaturation.

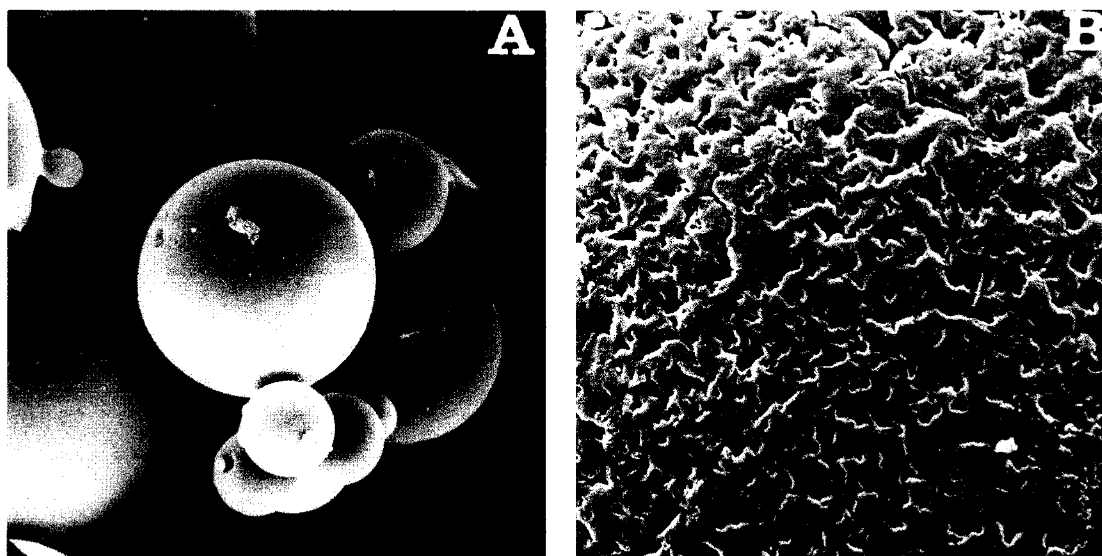

Fig. 9. Scanning electron micrographs of unloaded bovine albumin microspheres prepared using heat denaturation. (A) ×540; (B) ×5400.

Glazer (14), pointed out that strong binding of dyes to proteins (characterized by a dissociation constant of 10^{-5} M or less) occurs in hydrophobic regions, which often overlap with binding sites in substrates, coenzymes, and prosthetic groups.

The amounts of dye adsorbed onto microspheres prepared using the heat denaturation method are higher than those obtained with microspheres of similar size prepared using cross-linking agents (Fig. 2). These differences may be the result of a lower porosity of the microspheres surfaces when the cross-linking method is used (Fig. 10). It is also apparent that the percentage differences between heat-denatured and cross-linked microspheres for a given albumin for all the dyes studied are essentially constant. In view of such a constancy, it is reasonable to evaluate adsorption behavior of the dyes with microspheres prepared by either the cross-linking method or the heat denaturation process. The discussion of adsorption characteristics described hereafter is for albumin microspheres prepared using the heat denaturation. The amounts of fluorescein dyes adsorbed onto unloaded bovine and egg albumin microspheres, at very low dye concentrations (10^{-6} M), were in the order rose bengal > erythrosin > rhodamine B > eosin. Sodium fluorescein did not adsorb to the surface even though it is a more planar molecule than the other dyes. Rose bengal, erythrosin, and eosin exhibited Langmuirian behavior (Figs. 3 and 5). The plateau in the adsorption profiles for these dyes indicates complete monolayer formation on the microsphere surface.

The affinity constant, k_1, of the dyes for the albumin microsphere surface was found to depend on their hydrophobicities (Table II). Highly hydrophobic and polarizable dyes, such as rose bengal, were completely adsorbed onto the surface in dilute solutions, as compared to the lower amounts of adsorption with the less hydrophobic dyes, erythrosin and eosin. The affinity constants for the three dyes appear to be graded with the less hydrophobic egg albumin microspheres, whereas with the highly hydrophobic bovine albumin systems a plateau effect is apparent. Such effects were noted for spectral shifts of the dyes with the albumin systems (15). The trends in k_2 are similar to the trends observed with k_1 for the two systems. It is also clear

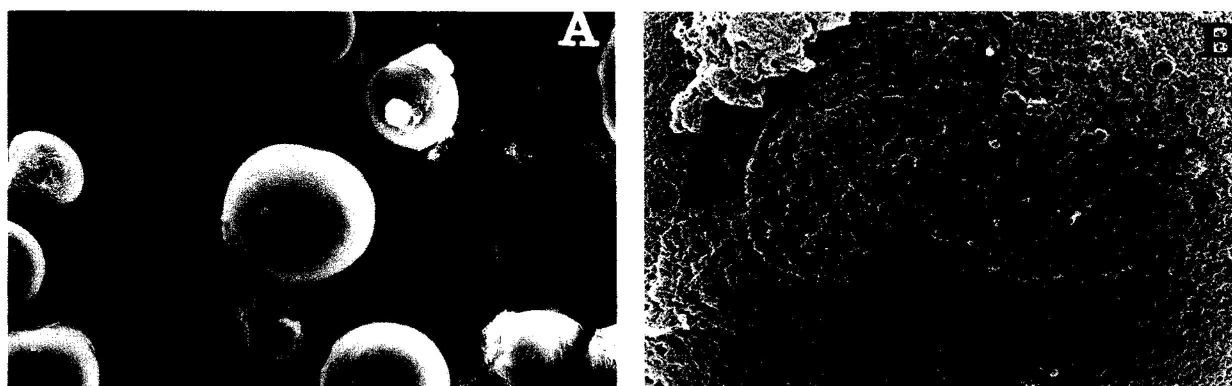

Fig. 10. Scanning electron micrographs of unloaded bovine albumin microspheres prepared using cross-linking denaturation. (A) ×540; (B) ×5400.

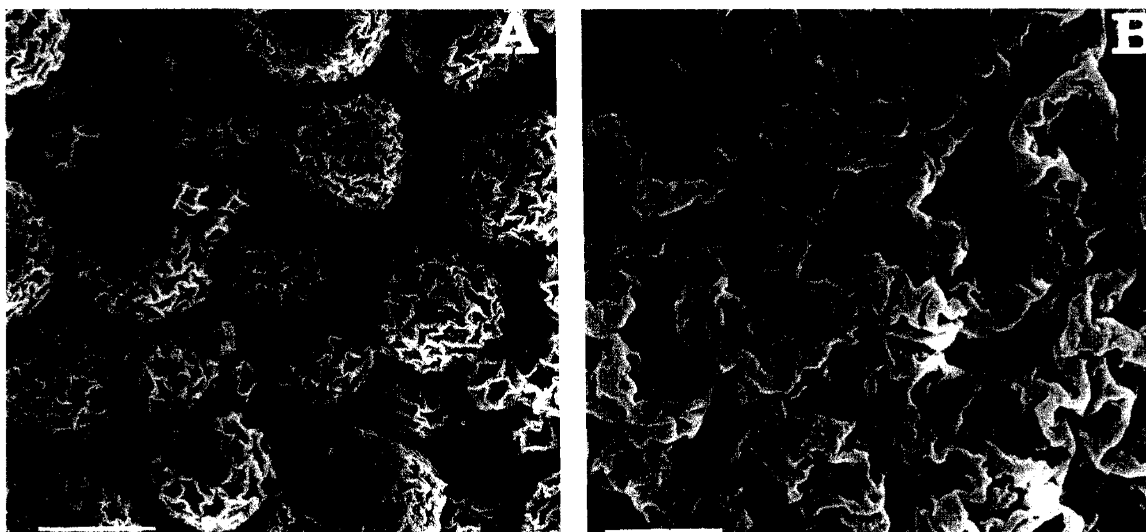

Fig. 11. Scanning electron micrographs of unloaded egg albumin microspheres prepared using heat denaturation. (A) ×540 (bar = 30 μm); (B) ×5400 (bar = 3 μm).

that both k_1 and k_2 for a given dye are higher with bovine albumin microspheres than with egg albumin microspheres even though the porosity of egg albumin microspheres is substantially higher (Fig. 11). This strongly suggests that the major interaction between these dyes and albumin microspheres is hydrophobic in nature. Fluorescein, the least hydrophobic dye in the group, did not adsorb to the microspheres.

Rhodamine B, a cationic dye, exhibits non-Langmuirian adsorption behavior [$r^2 = 0.15$ for fit of data to Eq. (2)]. The excellent correlation coefficients for linear fit ($r^2 = 0.99$) suggest that rhodamine B incorporation increases linearly with dye concentration (Fig. 4), indicating that dye uptake may involve absorption into the microsphere pores. It is reasonable to expect that high dye concentrations within the pores would lead to self-association, thereby creating the driving force for dye penetration from the bulk into the pores (dimerization constant = 2100; Table I). At high concentrations (10^{-4} *M*), rose bengal exhibited monolayer adsorption behavior followed by a linear increase in the total amount adsorbed (Figs. 6 and 7). The increase in total amount of dye adsorbed with increasing concentration in the medium could be explained either by dye oligomer or multilayer formation on the microsphere surface (16,17) or by dye penetration into the microsphere pores. The order of the amounts of dyes adsorbed from concentrated solutions remained the same as that observed with dilute solutions.

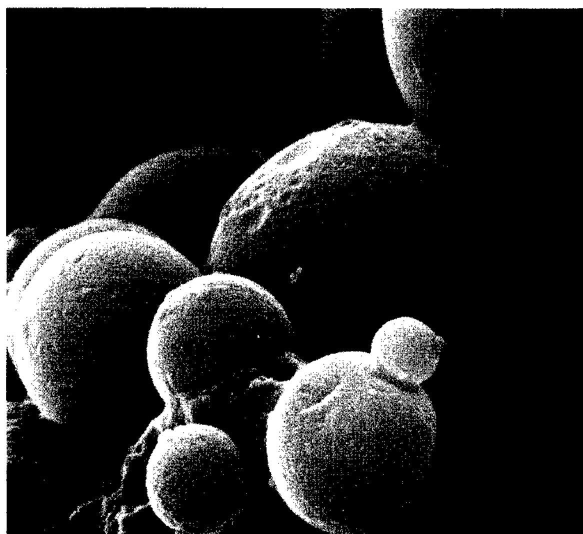

Fig. 12. Scanning electron micrographs of unloaded bovine albumin microspheres prepared using heat denaturation and incubated with dye solutions for 24 hr.

Rhodamine B adsorption behavior in the high concentration range (Fig. 8) exhibited a linear uptake ($r^2 = 0.997$) in which increasing dye concentration promotes further uptake. This is similar to the behavior observed with dilute solutions. It is also clear that self-association effects would be more significant in determining rhodamine B incorporation into albumin microspheres from concentrated solutions. To understand further the forces affecting binding of rhodamine B to bovine albumin microspheres and to explain the large amounts adsorbed or associated with the microspheres despite the low hydrophobicity of the dye, direct spectroscopic studies have been carried out previously (15).

In conclusion, our study demonstrates that the amount of drug uptake by microspheres may depend on both drug polarizability and hydrophobicity, presumably reflecting the heterogeneous character of the microsphere surface. It is not possible, however, to ascertain the relative importance of the two, in determining drug uptake. A detailed investigation of spectral changes of the dyes upon interaction with microspheres and the role of hydrophobicity and polarizability has been described elsewhere (15).

These results can be used to model drug–microsphere interactions. Such a method for predicting drug uptake is of great potential value for screening the suitability of microspheres as carriers for particular drugs, especially for drugs which are highly sensitive to the microsphere preparation conditions, such as heating or cross-linking agents, which might lead to drug decomposition.

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