Table III—Effect of Topical Application of Lycopene and Ionones on Wound Healing

Group	No. of Animals	Drugs Applied	Mean Tensile Strength, g. ± *SE*
I	14	NIB Control	451 ± 9
II	8	1% Lycopene in NIB	418 ± 5
III	9	1% β-Ionone in NIB	497 ± 11
IV	8	1% α-Ionone in NIB	453 ± 6

wound-healing promotion activity studies indicate that β-ionone ring and conjugated double-bond hydrocarbon side chain and terminal carboxylic group are responsible for the full healing-promotion activity.

REFERENCES

(1) K. H. Lee and T. G. Tong, *J. Pharm. Sci.*, **58**, 773(1969).
(2) K. H. Lee, *ibid.*, **57**, 1042(1968).

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Tetrameric Structure and Conformation of Heat-Microaggregated Human Serum Albumin

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Abstract □ ^{131}I-labeled heat-microaggregated human serum albumin is a colloid used in a method that measures liver blood flow through a determination of the rate of removal of microaggregates from the circulation. The heat-microaggregated material, either labeled or unlabeled, has a molecular weight (mol. wt.) of 273,000 daltons and a sedimentation coefficient of 8.6 *S*. Since the precursor, human serum albumin, has a molecular weight of 67,000 daltons and a sedimentation coefficient of 4.6 *S*, the microaggregate is a tetramer. The tetramer may be converted to subunits with a molecular weight of 72,000–76,000 daltons and a sedimentation coefficient of 4.7 *S* by the addition of buffered urea, guanidinium chloride, or formamide or by decreasing the pH to 2.2. Immunological studies indicate that this subunit has a different conformation from that of native human serum albumin. Hydrodynamic calculations indicate that the colloid has a particle size between 50 × 50 × 200 Å and 50 × 100 × 100 Å.

Keyphrases □ Albumin, human serum, heat-microaggregated—tetrameric structure, conformation, physical constants □ Human serum albumin, heat-microaggregated—structure, physical constants □ Ultracentrifugation—sedimentation coefficients □ Conformational studies—heat-microaggregated human serum albumin

Heating human serum albumin results, under certain conditions, in the formation of two protein aggregates (1, 2), made clinically useful by subsequent labeling with ^{131}I. One preparation, particulate in nature (2), is known as heat-macroaggregated human serum albumin. The other preparation is known as heat-microaggregated human serum albumin (3). The rate of disappearance of injected colloidal microaggregated particles from the circulation of mammals permits an estimation of the phagocytic capacity of the reticuloendothelial system, because this removal involves principally the Kupffer cells of the liver. Knowledge of phagocytic capacity appears to be useful in determining the extent of such diseases as pneumococcal pneumonia, typhoid fever, and Hodgkin's disease. This paper describes some physical properties of the metabolizable microaggregate, either unlabeled or labeled with ^{131}I.

EXPERIMENTAL

Source of Heat-Microaggregated Human Serum Albumin—The aggregation of human serum albumin (HSA) was performed according to the method of Iio and Wagner (3), as modified from the method of Benacerraf *et al.* (1). HSA at a concentration of 3% protein in 0.9% NaCl adjusted to pH 10 with NaOH was shaken vigorously for 20 min. at 70° and then for 15 min. at 79°. After rapid cooling, the precipitate that formed from the solution was resuspended in 0.1 *M* $NaHCO_3$. After storage, the precipitate dissolved and the solution of heat-microaggregated HSA was diluted to 10 mg./ml.

Sedimentation Coefficients—By the use of a Spinco model E analytical ultracentrifuge, the sedimentation coefficients of ^{131}I-labeled and unlabeled heat-microaggregated HSA at 20° and the viscosity and density of water, $s^0_{20,w}$ (4), were calculated at 29,500, 42,040, and 50,740 r.p.m. with 0.2 *M* NaCl–0.02 *M* sodium phosphate buffer (pH 6.85) as diluent. The viscosity was measured with the aid of either capillary or rotating (5) viscometers. A 10-ml. pycnometer at 20 ± 0.002° (Fisher Isotemp water bath) was used for density measurements. Viscosity and density corrections for aqueous solutions of urea and guanidinium chloride were also obtained from the data of Kawahara and Tanford (6).

Molecular Weights—The molecular weight was determined by two methods. The Archibald approach-to-sedimentation equilibrium method (7) was used with the modification of Engelberg (8) to evaluate the integral of the concentration gradient. The Yphantis meniscus-depletion (9) analyses were performed in a capillary-type double-sector cell at 20° at a speed of 20,410 r.p.m. The cell bottom was layered with FC-43 fluorochemical oil (Beckman Instruments), and 0.03 ml. of 0.1% dialyzed solution was layered over the oil. The solvent was the last dialysate.

Immunological studies utilized rabbit and horse antihuman serum albumin (Hyland Laboratories, Los Angeles, Calif.).

Polyacrylamide gel electrophoresis was performed with the Canalco model 6 apparatus (Canal Industrial Corp., Rockville, Md.).

Table I—Sedimentation Coefficients of Human Serum Albumin, Heat-Microaggregated Human Serum Albumin, and ^{131}I Heat-Microaggregated Human Serum Albumin in Various Solvents

Solvent	HSA[a]	$s^0_{20,w} \times 10^{13}$ sec. Aggregated HSA[b]	$s^0_{20,w} \times 10^{13}$ sec. ^{131}I-Aggregated HSA[c]
0.2 *M* NaCl–0.02 *M* phosphate buffer, pH 6.85	4.6	8.6	8.5
Buffered 8 *M* urea, pH 6.85	4.0	4.0	4.0
Buffered 8 *M* urea, pH 6.85, followed by dialysis against neutral buffer	4.5	4.7	4.5
Buffered 9 *M* formamide, pH 6.85	4.1	4.0	4.2
Buffered 5 *M* guanidinium chloride, pH 6.85	3.3	3.3	3.2

[a] Human serum albumin. [b] Heat-microaggregated human serum albumin. [c] ^{131}I heat-microaggregated human serum albumin.

RESULTS AND DISCUSSION

Hydrodynamic Properties of the Heat-Microaggregated Human Serum Albumin Molecule—The heat-microaggregated HSA was considered sufficiently pure for hydrodynamic studies, because polyacrylamide gel electrophoresis indicated that approximately 95% of the heat-microaggregated HSA migrated as one component and the sedimentation velocity studies showed only one peak with almost no asymmetry. The sedimentation coefficient of unlabeled heat-microaggregated HSA at 20°, corrected to the viscosity of water and extrapolated to zero concentration, was 8.56 ± 0.04 *S* (*S* = 10^{-13} sec.) between pH 4.7 and 10.2 (Table I). The sedimentation coefficient of the heat-microaggregated ^{131}I-labeled HSA was 8.45 ± 0.08 *S*. The heat-microaggregated preparation was sensitive to small changes in duration of heating; an increase of 5 min. in the heating time at 79° led to the formation of a particle with a molecular weight of 880,000 daltons.

The partial specific volume of heat-microaggregated HSA was experimentally determined (10) to be 0.737 cm.^{3}/g., compared with 0.733–0.736 cm.^{3}/g. for native HSA (11, 12).

According to the Archibald method, approximately 95% of the sedimenting material had a molecular weight of 279,000 ± 10,000 daltons at the meniscus and cell bottom; the remaining 5% possessed an approximate molecular weight of 132,000 ± 9000 daltons, which corresponds to the naturally occurring dimer of HSA found in serum albumin at similar concentrations of 5–8% (12). The molecular weight found by the Yphantis method at 20,410 r.p.m. was 267,000 ± 8000 daltons. Since the molecular weight of HSA is 67,000 (12, 13), four molecules of HSA must have combined to give one molecule of microaggregated HSA. The addition of ^{131}I has no apparent effect on molecular weight or sedimentation coefficient.

The diffusion constant, $D_{20,w}$, was calculated to be 2.84 × 10^{-7} cm.^{2} sec.$^{-1}$ from the Svedberg equation (14), $D_{20,w} = RTs/M(1 - \bar{v}\rho)$, where R is the gas constant, T is the temperature in °K., s is the sedimentation coefficient, M is the molecular weight, $\bar{v}$ is the partial specific volume, and ρ is the density. Using this value for $D_{20,w}$, $f/f_{min.}$ was calculated (15) to be 1.7, where $f = kT/D$ (k is Boltzmann's constant) and $f_{min.} = 6\pi\eta\ (3M\bar{v}/4\pi N)^{1/3}$ (η is the viscosity of solvent and N is Avogadro's number). This indicates that the heat-microaggregated HSA molecule is slightly asymmetric, since HSA is globular with a calculated $f/f_{min.}$ value of 1.3–1.4 (15, 16).

Dissociation of the Aggregate—The apparently mild conditions required for aggregation suggested that dissociation of the aggregate to monomers of HSA was possible. As will be shown later, it was found that although the monomeric molecular weight of the dissociated microaggregate was similar to that of the monomer, the conformation changed. As seen in Table I, the sedimentation coefficients of microaggregated HSA and monomeric HSA dissolved in buffered 8 *M* urea, 9 *M* formamide, or 5 *M* guanidinium chloride were identical within the limits of experimental error (17). These values indicated that heat-microaggregated HSA (mol. wt. 273,000) had dissociated to monomeric HSA. The molecular weight in 8 *M* urea, 9 *M* formamide, and 5 *M* guanidinium chloride was between 72,000 and 76,000 daltons. Ten milligrams microaggregated HSA/milliliter 0.2 *M* glycine–HCl buffer (pH 2.2) showed two components. The $s^0_{20,w}$ values of 4.5 and 9 *S* indicated some breakdown of the aggregate. Aggregated HSA, HSA, or mixtures of both HSA and aggregated HSA all produced schlieren images with one broad peak, indicating not only breakdown of the aggregate but also some denaturation of the monomeric HSA (18). After removal of urea by dialysis, the schlieren peaks still appeared broad, indicating that denaturation of HSA is only partially reversible.

Conformation of Heat-Microaggregated Human Serum Albumin—Although both monomeric HSA and monomeric HSA first dissolved in buffered 8 *M* urea and then dialyzed against H_2O yield strong precipitin bands, both aggregated HSA and urea-dissociated, aggregated HSA (dialyzed subsequently against H_2O and buffer to remove urea) fail to form precipitin bands with rabbit or horse antihuman serum albumin. This failure indicates that the HSA monomer formed from dissociated tetrameric microaggregated HSA has a different conformation from that of human serum albumin.

Since: (*a*) the HSA molecules are globular (15) with a diameter of approximately 50 Å [radius = $(3M\bar{v}/4\pi N)^{1/3}$]; (*b*) the conformation of each of the HSA molecules after heating is still generally spherical, as seen by the sedimentation coefficient of 4.5 *S* found after the addition of urea and subsequent dialysis (Table 1); and (*c*) the aggregate is asymmetric, then the aggregate may consist of either: (*a*) a chain 4 subunits long (200 × 50 × 50 Å); (*b*) a tetragon, two subunits wide and 2 subunits long (100 × 100 × 50 Å), or an intermediate conformation. One forbidden conformation is the symmetrical tetrahedral pyramid.

Binding Forces of the Aggregate—The disaggregation of the tetramer caused by buffered urea, guanidinium chloride, formamide, or low pH indicates that the denaturing agent may be interacting with peptide groups through hydrogen and hydrophobic bonds (19). The aggregate may have been formed by the reverse process, with heat causing the cleavage ("melting") of some interpeptide bonds and the subsequent cooling of the mixture resulting in the partial intertwining of the protein chains from four molecules.

REFERENCES

(1) B. Benacerraf, G. Biozzi, B. N. Halpern, C. Stiffle, and D. Mouton, *Brit. J. Exp. Pathol.*, **38,** 35(1957).

(2) G. V. Taplin, D. E. Johnson, E. K. Dore, and H. S. Kaplan, *J. Nucl. Med.*, **5,** 259(1964).

(3) M. Iio and H. A. Wagner, Jr., *J. Clin. Invest.*, **42,** 417 (1963).

(4) H. K. Schachman, "Ultracentrifugation in Biochemistry," Academic, New York, N. Y., 1959, p. 81.

(5) B. H. Zimm and D. N. Crothers, *Proc. Natl. Acad. Sci., U. S.*, **48,** 905(1962).

(6) K. Kawahara and C. Tanford, *J. Biol. Chem.*, **241,** 3228 (1966).

(7) W. J. Archibald, *J. Phys. Colloid Chem.*, **51,** 1204 (1947).

(8) J. Engelberg, *Anal. Biochem.*, **6,** 530(1963).

(9) D. A. Yphantis, *Biochemistry*, **3,** 297(1964).

(10) H. K. Schachman, *Methods Enzymol.*, **4,** 70(1957).

(11) J. L. Oncley, G. Scatchard, and A. Brown, *J. Phys. Colloid Chem.*, **51,** 184(1947).

(12) G. I. Loeb and H. A. Scheraga, *J. Phys. Chem.*, **60,** 1633 (1956).

(13) T. P. King, D. A. Yphantis, and T. C. Craig, *J. Amer. Chem. Soc.*, **82,** 3355(1960).

(14) T. Svedberg and K. O. Peterson, "The Ultracentrifuge," Oxford University Press, London, England, 1940, p. 5.

(15) C. Tanford, "Physical Chemistry of Macromolecules," Wiley, New York, N. Y., 1963, p. 356.

(16) C. Tanford and J. G. Buzzell, *J. Phys. Chem.*, **60**, 225 (1956).

(17) H. K. Schachman, "Ultracentrifugation in Biochemistry," Academic, New York, N. Y., 1959, p. 82.

(18) H. Neurath, G. R. Cooper, and J. O. Erickson, *J. Biol. Chem.*, **142**, 249(1942).

(19) W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill, New York, N. Y., 1969, p. 323.

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Phospholipid Spherules as a Model to Assess Photosensitizing Properties of Drugs

ALVIN FELMEISTER and SHRILEKHA V. TOLAT

Abstract □ The effect of a series of UV irradiated and nonirradiated phenothiazine drugs on the chromate leakage from lipid spherules has been determined. All of the drugs studied increased chromate leakage to varying degrees prior to irradiation. However, only chlorpromazine and prochlorperazine exhibited a marked increase in chromate leakage after irradiation. These effects are discussed in terms of the *in vivo* photosensitizing properties of the drugs.

Keyphrases □ Photosensitizing properties—phenothiazines □ Lipid spherules, chromate leakage—photosensitizing properties, phenothiazines □ Phenothiazines—photosensitizing properties □ Spectrophotometry—analysis

Nonphysiologic photosensitized reactions have been studied extensively since Raab first observed the photosensitized activity of acridine toward paramecium almost 70 years ago (1). During the intervening time, much has been learned about such reactions, although their exact mechanism of action has not been completely elucidated. However, it has been reasonably well established that changes in the permeability of the membrane of cells or cell organelles are often induced by light-irradiated photosensitizing agents (2).

It was the purpose of this investigation, therefore, to develop a physical model that might be used to assess photosensitizing agents by measuring their influence on membrane permeability. The phospholipid spherule model developed by Bangham *et al.* (3) was selected for this purpose. These workers have shown that phospholipids, when permitted to swell in an aqueous salt solution, form salt-containing compartments bounded by bimolecular membranes. These structures (spherules) exhibit permeability characteristics that are quite similar to those of biological membranes. Furthermore, these spherules have been shown by numerous researchers to be useful tools for studying membrane–drug interactions, particularly when changes in permeability are involved (4). Therefore, changes in permeability of these spherules induced by light-irradiated drugs should be a measure of the photosensitizing property of these drugs.

Five phenothiazine derivatives were selected for this initial study. Two of these compounds, chlorpromazine and prochlorperazine, have been shown unequivocally to be photosensitizers (5). The other three compounds, promazine, triflupromazine, and fluphenazine, rarely if ever produce photosensitization (5–7).

MATERIALS AND METHODS

The phenothiazine derivatives were used without further purification. These were chlorpromazine hydrochloride and prochlorperazine hydrochloride (Smith Kline & French Laboratories); promazine hydrochloride (Wyeth Laboratories); and triflupromazine hydrochloride and fluphenazine dihydrochloride (The Squibb Institute for Medical Research).

The lipid spherules were prepared by the method of Bangham *et al.* (3) with slight modification. Briefly, egg lecithin and dicetylphosphate (90 and 10 μmoles, respectively) were dissolved in chloroform and placed in a 50-ml. round-bottom flask. The solvent was removed under reduced pressure using a flash evaporator. Six milliliters of a 0.145 *M* potassium chromate solution was then added to the flask, and the lipid material was permitted to swell for 4 hr. at room temperature. At the end of 4 hr., any chromate ion not trapped within the spherules was removed by dialyzing the dispersion against a 0.145 *M* KCl solution for 18–20 hr. One milliliter of the dialyzed suspension of chromate-containing spherules was transferred to each of five cells. By use of a micrometer syringe, 0.05 ml. of a 1×10^{-2} *M* solution in 0.145 *M* KCl of the