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Micellar Properties of Drugs: Determination of Molecular Weight, Size, and Molecular Interactions of Drug Micelles Using the Analytical Ultracentrifuge

JOEL KIRSCHBAUM

Abstract □ This article illustrates the usefulness of the analytical ultracentrifuge in investigating micellar properties of drugs. Also discussed are some examples of micelle forms of drugs and the working hypothesis that the same binding forces that hold together the aggregate found at high concentrations may be responsible for the binding of monomer (at physiological concentrations) to biological structures.

Keyphrases □ Micellar properties of drugs—symposium □ Ultracentrifugation—determination of molecular weight, size, and molecular interactions of drug micelles □ Sedimentation velocity and coefficient—determination of micellar properties of drugs □ Molecular weight of drug micelles—determination from sedimentation data □ Density gradient centrifugation—determination of micellar properties of drugs

The analytical ultracentrifuge is frequently used by physical, biological, and polymer chemists to investigate molecular weights, subunit structure, and purity of molecules and small particles from diverse sources. The investigation of drug micelles is facilitated by the described techniques since ultracentrifugation affords a direct view of some properties of molecules and small particles. For example, a recent controversy (1, 2) concerning whether or not theophylline aggregated in aqueous solutions was resolved when molecular weight studies performed with an analytical ultracentrifuge (3) showed that the self-association of theophylline is concentration dependent; that is, monomers are found at low concentration and multimers (dimers, trimers, and tetramers) are found at higher concentrations. This paper discusses some ultracentrifugal techniques, some physical properties of several drugs that form micelles, and the question of whether aggregation of these agents is a manifestation of a physical property required for activity *in vivo*.

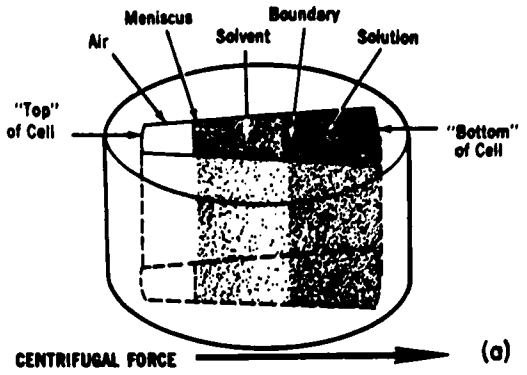
All analytical ultracentrifuges operate on the same principle, *i.e.*, they cause molecules to move toward the bottom (outer edge) of the ultracentrifuge cell under the influence of the artificial gravitational field created by a rotor spinning from 800 to 60,000 rpm.

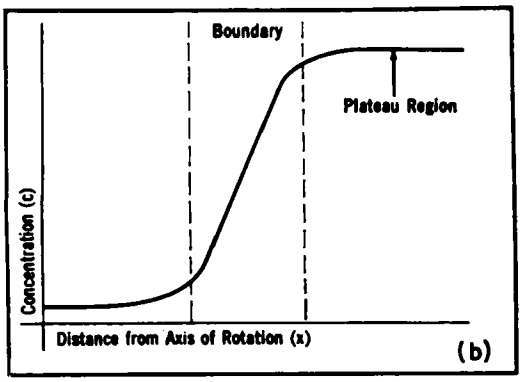
Results obtained with different instruments are similar (4), as expected from calculations based on thermodynamics and mass transfer phenomena.

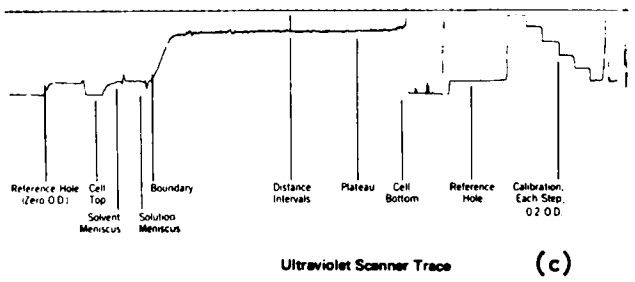
Separations of the sample within the ultracentrifuge cell may be achieved by: *(a)* differences in size and shape, as in the sedimentation velocity methods; *(b)* differences in mass, as in approach-to-sedimentation equilibrium and equilibrium procedures; or *(c)* differences in density, as in the density gradient (isopycnic or isodensity method).

SEDIMENTATION VELOCITY

Figure 1*a* shows a cross section of the filled ultracentrifuge cell. Before the ultracentrifuge run begins, solute is distributed equally throughout the solution. As the cell is accelerated, solute molecules move toward the bottom of the cell. Complete redistribution by diffusion is prevented by maintaining a sufficiently high speed to cause net sedimentation of solute. This speed depends on the size, shape, and partial specific volume (which is related to recip-







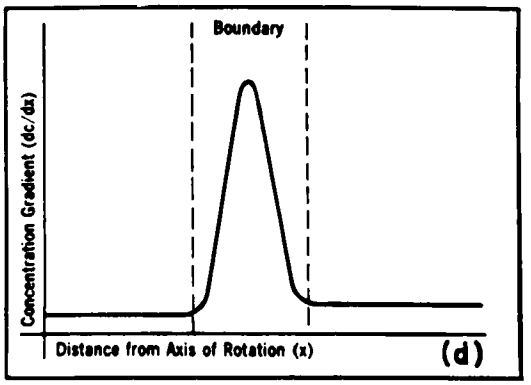


Figure 1—(a) *The centerpiece filled with solution in a sedimentation velocity experiment.* (b) *The variation in solute concentration in the centerpiece after a sufficiently high speed has been reached to sediment solute from the meniscus and these changes in concentration with distance as visualized by the* (c) *UV scanner and* (d) *schlieren optical system. (Portions of this figure are courtesy of Beckman Instruments, Inc.)*

rocal density) of the solute and the density of the solvent. When there is zero solute concentration at the meniscus (Fig. 1*b*), the UV optical system, which is based on absorptivity, visualizes this as shown in Fig. 1c. The schlieren optical system, which produces images based on differences in indexes of refraction caused by

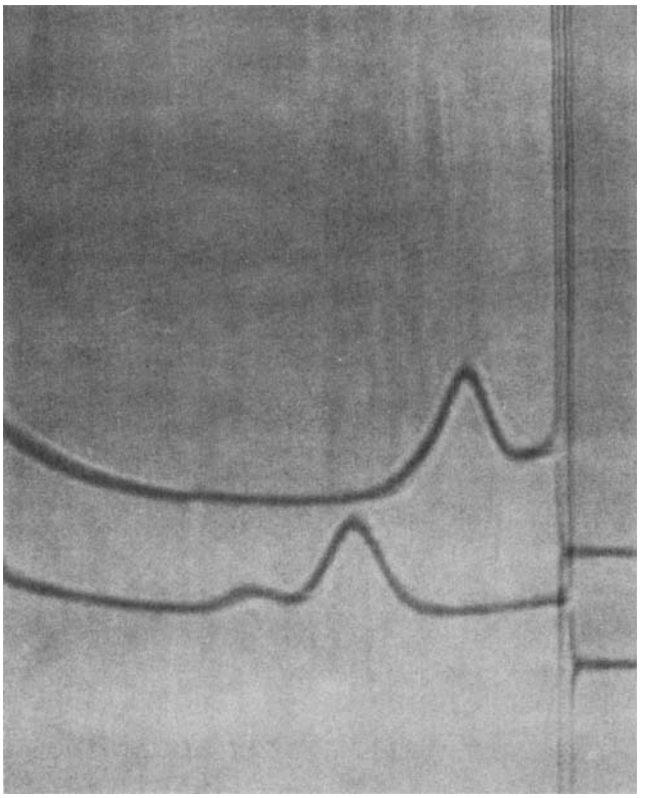


Figure 2—*Upper portion: aged γ-globulin in 5* M *NaCl solution. Lower portion: aged γ-globulin in tromethamine buffer. Sedimentation is from right to left.*

local differences in concentration, visualizes the region of increasing solute concentration as a peak (Fig. 1*d*). As sedimentation continues, the region devoid of solute increases and the plateau region (Fig. 1*b*) decreases. The peak is seen to travel toward the bottom of the cell.

Such an ultracentrifuge run can aid in determining the purity of such sedimenting species, as illustrated in Fig. 2. This figure is from a photograph of aged γ-globulin in dilute tromethamine buffer. The lower tracing shows two peaks; the larger one is the slower moving monomer, and the smaller peak is a faster moving dimer. The upper tracing is an aliquot of the same γ-globulin in 5 *M* NaCl. No dimer is visible, because it has been cleaved to the monomer by this concentrated salt solution. If the logarithm of the distance of the peak from the center of rotation is plotted *versus* time and the material is approximately 95% pure (depending on the molecular weight or size differences between compound and impurity), a straight line usually results. The slope of this line is used to calculate the sedimentation coefficient *(s)*:

$$s = \frac{\log_e x_t - \log_e x_0}{\omega^2(t_t - t_0)} \qquad \text{(Eq. 1)}$$

in which $(t_t - t_0)$ = time interval during which the boundary moves from x_0 to x_t, and ω is the angular speed of rotation, which is dependent on revolutions per minute. Such a determination requires 10–180 min of centrifugation, depending on the size of the macromolecule or particle. In Eq. 1, s is usually given in units of 10^{-13} cm sec^{-1} (S) or Svedbergs. The value of s is usually corrected for the density, ρ, and viscosity, η, of water at 20° (6) and is then written as $s_{20,w}$.

Particles as large as viruses can be investigated by this method. For example, a virus preparation with s values of 400 and 1400 S was interpreted[1] as containing both individual viruses and clumps of viruses, and the interpretation was subsequently verified by electron microscopy.

It is mandatory to calculate sedimentation coefficients at different concentrations of solute when one is examining micelles,

[1] Unpublished studies from this laboratory.

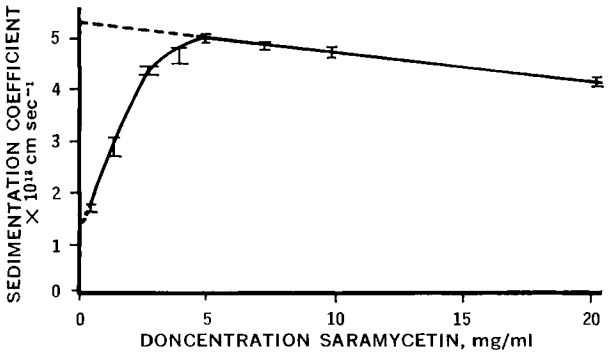


Figure 3—*Plot of the dependence of the sedimentation coefficient of saramycetin at different concentrations of 0.2* M *sodium chloride–0.02* M *sodium phosphate buffer, pH 6.85, with both regions of the curve extrapolated to zero concentration.*

because dilution will eventually disrupt the aggregate. Figure 3 illustrates the different sedimentation coefficients found for the antifungal antibiotic saramycetin at various concentrations of solute. At concentrations greater than 5 mg saramycetin/ml 0.2 *M* sodium chloride–0.02 *M* sodium phosphate buffer, pH 6.85, the micelle has an $s^0_{20,w}$ value of 5.3 S. The sedimentation coefficient increases initially, due to diminishing solute–solute interactions. At lower concentrations of solute, the micelle is disrupted to yield an $s^0_{20,w}$ value of 1.2 S. These data are supported by diffusion coefficient measurements; with disruption, the diffusion coefficient (*D*, Ref. 8) increases from 6×10^{-7} to 34×10^{-7} cm^2 sec^{-1}.

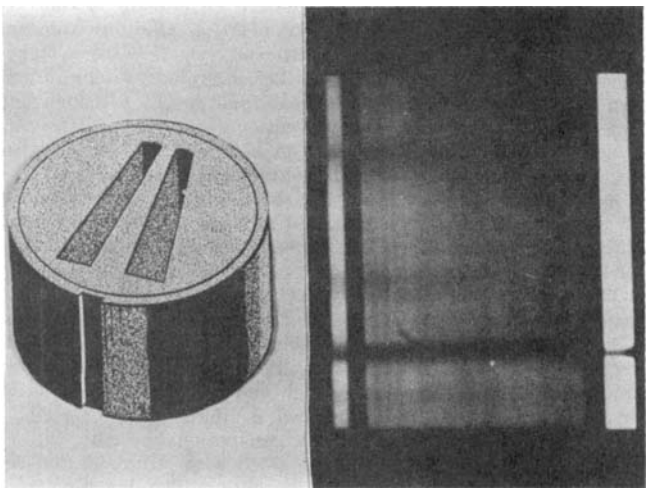
MOLECULAR WEIGHT DETERMINATIONS

The values of M_{app} from various methods used in determining the apparent molecular weight from sedimentation data are similar, as expected from the common thermodynamic basis of the calculations. The commonly used methods, their advantages, and disadvantages are summarized as follows.

Estimation from Sedimentation Coefficient—The approximate M_{app} of spherical micelles (8) can be determined as follows:

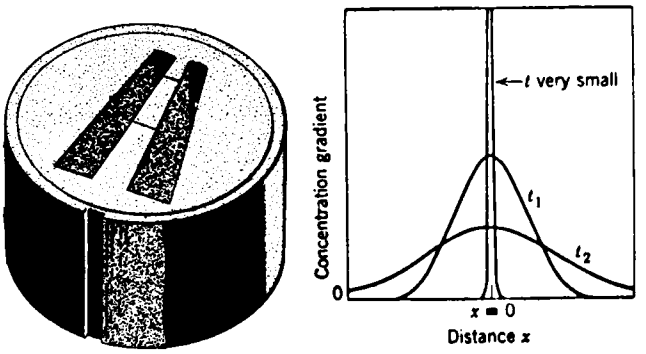
$$\frac{s^0_{20,w}\,(\text{unknown})}{s^0_{20,w}\,(\text{standard})} = \frac{M_{app}\,(\text{unknown})}{M_{app}\,(\text{standard})} \qquad \text{(Eq. 2)}$$

where $s^0_{20,w}$ is the sedimentation coefficient corrected for the viscosity and density of the solvent at 20°. This method is fast but relatively inaccurate.



(a) (b)

Figure 4—(a) *Double-compartment centerpiece used to measure the concentration gradient,* dc/dr, *for determining the apparent molecular weight by the approach-to-sedimentation equilibrium technique.* (b) *Concentration gradients at the meniscus and the cell bottom as visualized by the schlieren optical system.*



(a) (b)

Figure 5—(a) *Double-compartment capillary centerpiece used to determine total concentration.* (b) *Representation of peak produced by schlieren optical system. Note that the peak broadens and decreases with time due to diffusion.*

Sedimentation–Diffusion Method—The value of M_{app} can be determined using Eq. 3, where:

$$M_{app} = \frac{RTs}{D(1 - \bar{v}\rho)} \qquad \text{(Eq. 3)}$$

in which R is the gas constant, T is the absolute temperature, D is the diffusion coefficient, $\bar{v}$ is the partial specific volume of the solute, and ρ is the density of the solvent. The partial specific volume can be determined by pycnometry (9), Archimedes principle using sinkers or floats (9), and microdrop density in organic fluids (10) or from two ultracentrifuge runs, one in water and the other in a heavy isotope of water, followed by simultaneous solution of the two equations (11).

This method is good for mixtures and large molecules or micelles. The disadvantage is that both s and D must be determined at several different concentrations. The concentration of solute needed depends on methods of visualization available.

Archibald Approach-to-Sedimentation Equilibrium—In this procedure (12), M_{app} is determined using Eq. 4:

$$M_{app} = \frac{RT}{\omega^2(1 - \bar{v}\rho)} \frac{dc}{dr} \frac{}{Cr} \qquad \text{(Eq. 4)}$$

where ω is the angular speed of rotation, which depends on revolutions per minute, C is the concentration, dc/dr is the concentration gradient, and r is the distance from the center of rotation; C, dc/dr, and r are determined separately at the meniscus and cell bottom. Here, dc/dr is determined in a two-compartment centerpiece (Fig. 4*a*); one side is filled with solvent and the other with solution. Figure 4*b* shows the schlieren visualization of dc/dr—the heights of the schlieren curves at the meniscus and cell bottom.

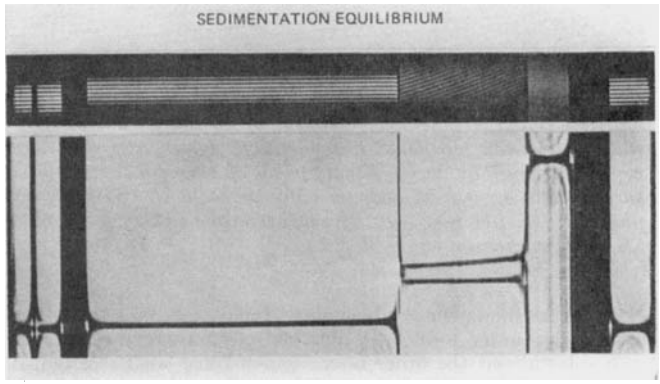


Figure 6—*Visualizations by Rayleigh interference (upper portion) and schlieren optical (lower portion) systems of low-speed equilibrium method for determining molecular weight. (After Schachman, Ref. 15.)*

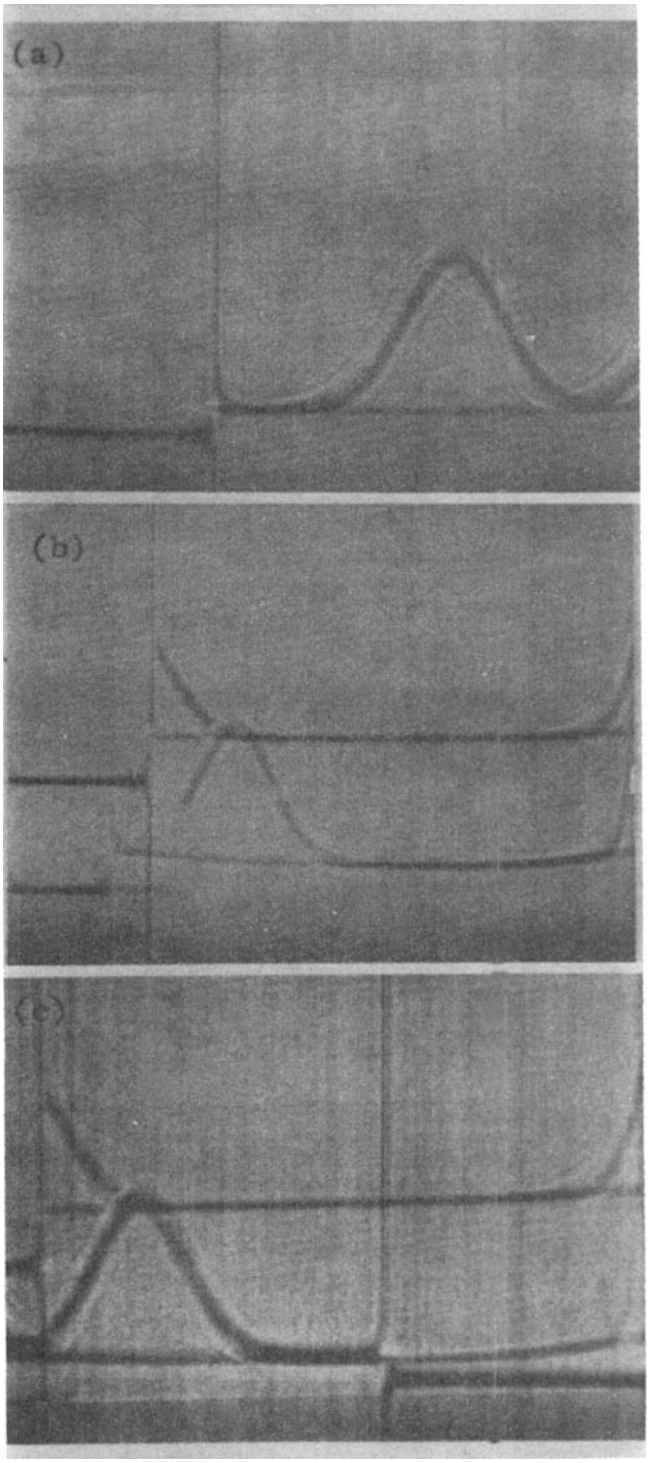


Figure 7—(a) *Total concentration of 10 mg saramycetin/ml ethanol, as seen by the schlieren optical system.* (b) *Schlieren peak produced by the sedimentation of this concentration of saramycetin in 0.2* M *sodium chloride–0.02* M *sodium phosphate buffer, pH 6.85.* (c) *The superimposition of* (a) *on* (b). (*After Kirschbaum* et al., *Ref. 22.*)

Figure 5*a* shows the double-compartment capillary centerpiece used to determine total concentration; one compartment is filled with solvent and the other is one-fourth filled with solution. As the ultracentrifuge cell is accelerated, solvent is forced into the solution compartment by its increasing apparent weight. Figure 5*b* shows the schlieren visualization of the different concentrations in the cell (originating from differences in indexes of refraction). With time, diffusion causes the peak height to diminish

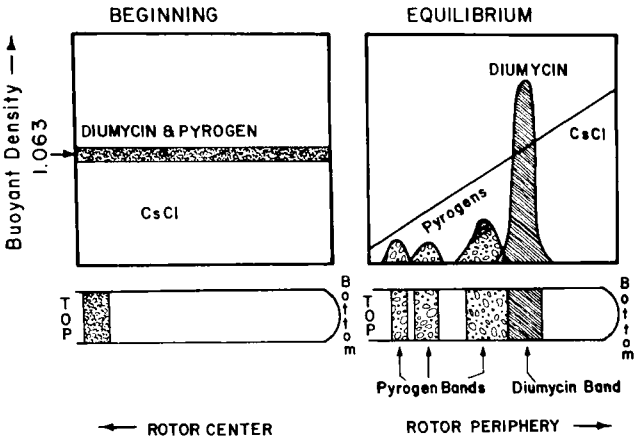


Figure 8—*Diagram of preparative centrifuge tube and distribution of diumycin, contaminating pyrogen, and cesium chloride in the tube prior to the start of centrifugation and after equilibrium had been achieved in the final density gradient.*

and the peak to broaden. Total concentration minus area under the schlieren curve at the meniscus (Fig. 4*b*) equals C at the meniscus. Total concentration plus area under the schlieren curve at the cell bottom equals C at the cell bottom. Note that the schlieren optical system visualizes concentrations at the meniscus and the cell bottom as positive, or upward, deflections. The advantages of this method are that as little as 1 hr of operating time is required, D can be calculated from the total concentration data, and M_{app} values as low as 100 can be calculated. Similar M_{app} values at both the meniscus and the cell bottom usually indicate homogeneity. The major disadvantage is that the values of the concentration gradients (height of schlieren curves at the meniscus and the cell bottom) are often difficult to measure, occasionally introducing large errors.

Sedimentation Equilibrium—The value of M_{app} can also be measured (13, 14) using Eq. 5:

$$M_{app} = \frac{2RT}{(1 - v\rho)\omega^2} \frac{d \ln c}{dr^2} \qquad \text{(Eq. 5)}$$

where $d \ln C/dr^2$ is the slope of the Rayleigh fringe (Fig. 6, upper part) or schlieren (Fig. 6, lower part) displacement *versus* radius squared. This method is accurate, but up to 3 days may be needed to achieve equilibrium, which may lead to new intermolecular interactions (16).

Several modifications exist to shorten the amount of time required for an accurate determination of M_{app}. One procedure involves accelerating the rotor, or overspeeding (17), to a higher speed than has been calculated as being needed for equilibrium and then decelerating to the desired final speed. Although less time is needed than with the conventional method, more distortion of cell windows is found and the method is limited to higher M_{app} values for solutes than is the conventional method.

In the short column method (18) the height of solution is limited to 1–3 mm. Less time is needed to achieve equilibrium than with the conventional method, but only one to three good values for M_{app} can usually be obtained.

The meniscus depletion method (19) involves running the centrifuge faster than is needed for the conventional equilibrium procedure to deplete the meniscus of solute. The advantages are that less time is needed, the total concentration can be calculated at any point in the ultracentrifuge cell, and the method is sensitive to polydispersity (20). This method is limited to higher M_{app} values than can be determined by the conventional method.

Data for M_{app} can often be combined with sedimentation velocity data to yield other useful information. The deviation from sphericity of a micelle can be approximated (21) from M_{app}, $s^0_{20,w}$, solvent viscosity (η), $\bar{v}$, and ρ. First, the diffusion coefficient is calculated (if it has not been determined directly) by a rearrangement of Eq. 3: $D = RTs/M_{app}(1 - \bar{v}\rho)$. Then, f/f_{min} is calculated, where $f = kT/D$ (k is Boltzmann's constant), and $f_{min} = 6\pi\eta\,(3M_{app}\,\bar{v}/4\pi N)^{1/3}$ (N is Avogadro's number). Numbers much above 1 indicate asymmetry.

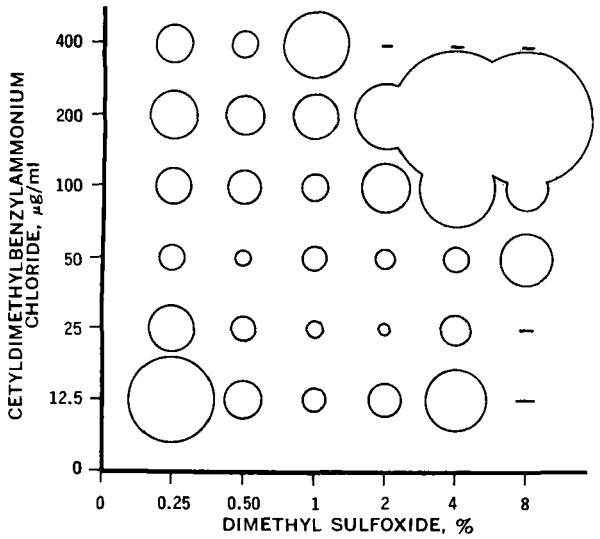


Figure 9—*Relative sedimentation coefficients of 100 μg amphotericin B/ml of various concentrations of cetyldimethylbenzylammonium chloride and dimethyl sulfoxide.*

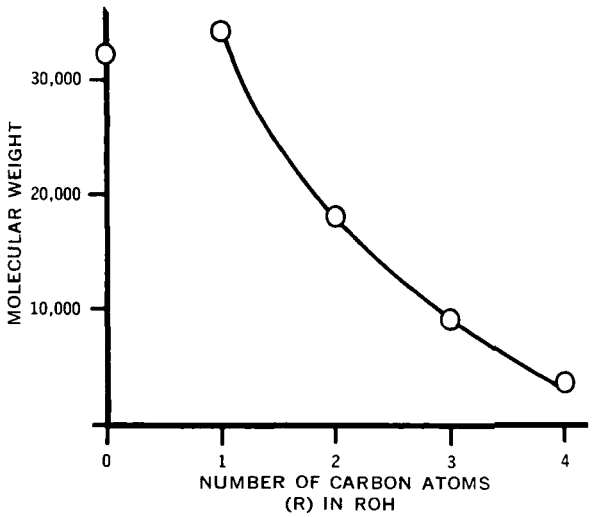


Figure 10—*The M_{app} values of diumycin in 95% 0.2 M sodium chloride–0.02 M sodium phosphate buffer, pH 6.85, containing 5% ROH, where R = CH_3—, CH_3CH_2—, $CH_3CH_2CH_2$—, or $CH_3CH_2CH_2CH_2$—.*

Sedimentation velocity and total concentration data can also be used to determine how much of the solute is in the form of the aggregate. Figure 7*a* is a reproduction of the photographic plate (22) recording the total concentration of 10 mg saramycetin/ml ethanol (M_{app} = 2100 daltons or molecular weight units). Figure 7*b* is a reproduction of the photograph of the schlieren peak produced during the sedimentation-velocity study of the same concentration of saramycetin in 0.2 *M* sodium chloride–0.02 *M* sodium phosphate buffer, pH 6.85; the solute is in the form of an aggregate (M_{app} = 55,000 daltons). Figure 7*c* is the superimposition of part *a* on *b*. The exact superimposition demonstrates that essentially all saramycetin in the aqueous buffer is in the form of the aggregate.

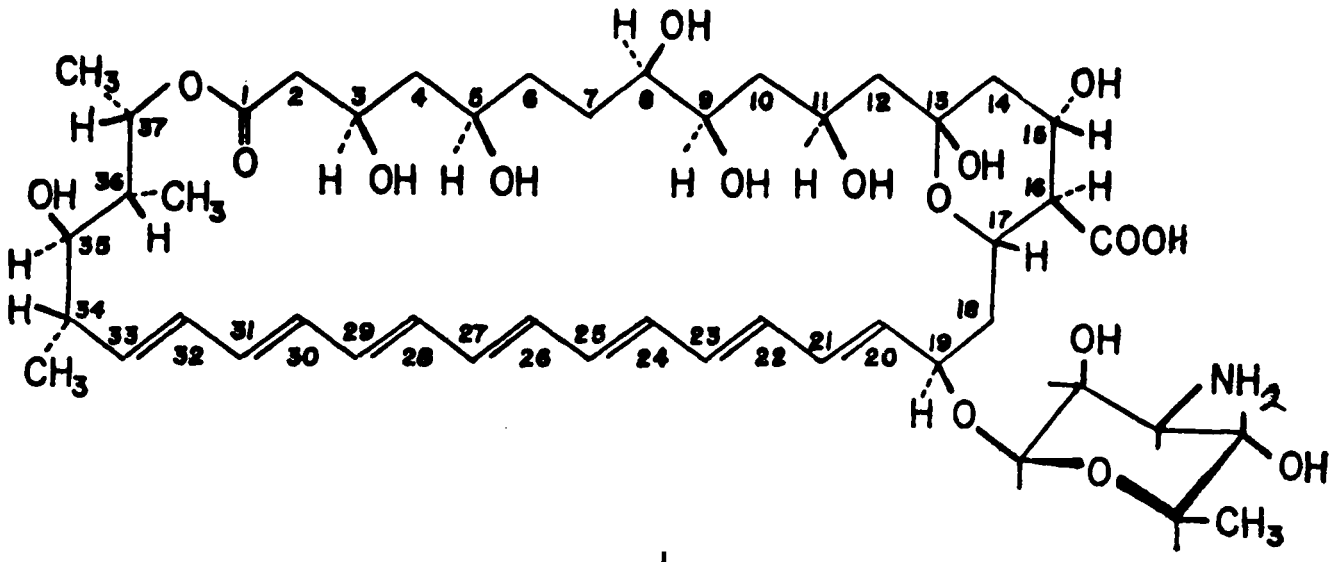
DENSITY GRADIENT CENTRIFUGATION

This method for separating and characterizing macromolecules is also known as isodensity or isopycnic centrifugation. It is based on the tendency of molecules to sink or float in a density gradient until they find the region corresponding to their own density. If a solution of cesium chloride is centrifuged at moderate to high speed (20,000 rpm or more) for 16–48 hr, the salt redistributes to form a density gradient. If a macromolecule is in the cesium chloride solution, it migrates to the region corresponding to its density and forms a band. The width of the solute band is inversely proportional to the molecular weight of the macromolecule; macromolecules or micelles with M_{app} values in the millions form bands 1 mm or less in width. Multiple bands would indicate heterogeneity, and the variety of their widths could be caused by either a wide spread in molecular weights or concentration (23).

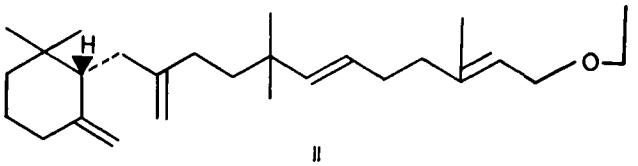
This isopycnic method was performed in a preparative ultracentrifuge to confirm the contamination of a sample of diumycin (26) with pyrogen. Diumycin micelles are essentially intact in cesium chloride. Figure 8 shows the distribution of diumycin, pyrogen, and cesium chloride, both prior to the start of centrifugation and after the attainment of equilibrium. The low molecular weight micelle (M_{app} ~ 30,000 daltons) formed a band about 1 cm wide. Fractions were collected, and the cesium chloride was removed by dialysis. Because most diumycin was in the form of the micelle, it did not diffuse through the cellophane membrane. The pyrogen was found to be heterogeneous, as shown by several fractions or bands that, upon injection, increased the temperature of rabbits.

FURTHER APPLICATIONS OF ULTRACENTRIFUGAL TECHNIQUES TO MICELLAR SYSTEMS

The site of action of polyene antibiotics is the cell membrane (24), at which compounds such as amphotericin B induce alterations in permeability. Amphotericin B (I) contains hydrocarbon

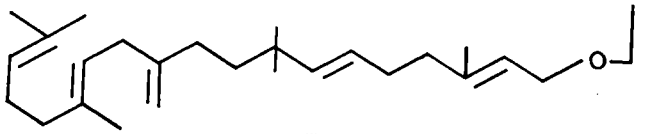


I



II

lipid side chain of diumycin (29)



III

lipid side chain of prasinomycin (30)

regions, hydroxyl groups, and amino and carboxyl groups capable of forming a zwitterion (25). Because of these structural features, it is not unexpected that this molecule forms micelles and that these micelles are sensitive to additions of the detergent cetyldimethylbenzylammonium chloride or of dimethyl sulfoxide, which breaks intermolecular hydrogen bonds. Figure 9 illustrates these changes as reflected in values for the sedimentation coefficient of the micelle in different solutions of cetyldimethylbenzylammonium chloride and dimethyl sulfoxide; small changes in the concentration of either agent greatly affect the sedimentation coefficient.

Diumycin (26) and prasinomycin (27) are closely related mixtures of antibiotics that aggregate in solution. Diumycin and prasinomycin contain 2-amino-1,3-cyclopentanedione, phosphoglyceric acid, phosphate, sugars such as glucose and glucosamine, and a 340-dalton hydrocarbon side chain (II and III). The M_{app} values of the micelles are approximately 30,000 daltons.

Since the molecular weight of each monomer is approximately 1800–1900 daltons, the micelle must be composed of 16 subunits. Figure 10 illustrates the M_{app} values of diumycin as alcohols of increasing side-chain length are used in the solvent system. As the length of the hydrocarbon side chain increases, with the concentration of each alcohol kept constant at 5%, M_{app} values decrease, indicating hydrophobic rather than hydrogen bonding (31). Chemical modifications of the water-soluble portions of the antibiotic, such as esterification or acetylation, or changes in pH or ionic strength of the solvent do not disrupt the micelle, indicating that ionic bonding is not involved. Without the hydrophobic side chains, there is neither aggregation nor microbiological activity. Figure 11 illustrates the most likely structure of the micelle. The core is a tangle of 16 interacting lipid side chains bound by hydrophobic forces. Surrounding this hydrocarbon core is a mantle of water-soluble (or hydrophilic) moieties of the antibiotic. Calculations show that the diumycin micelle can be spherical, since the minimum volume of the hydrophobic core, as calculated (32) from the length of the side chain as the radius (volume = $\frac{4}{3}\pi R^3$), is less than the calculated volume of the 16 lipid side chains.

The forces binding the micelle appear to be a manifestation of the same physical property of the lipid that is required for drug activity. This working hypothesis is based on evidence[2] that these antibiotics act prior to the site of penicillin action (33) to inhibit cell wall biosynthesis by interfering with the enzyme, peptidoglycan synthetase, that synthesizes phospholipid intermediates of cell wall glycopeptides (34). (The cell wall glycopeptide is synthesized from pentapeptide-phospholipid and acetylglucosamine-phospholipid intermediates.) These antibiotics may resemble the substrate and bind to the enzyme through phospholipid and the hydrophilic moiety, since both portions of the antibiotic are required for microbiological activity.

The lipid side chain may also be responsible for the long half-life *in vivo* of these antibiotics, because both diumycin and prasinomycin bind avidly to human serum albumin and red blood cell membranes, as demonstrated by molecular weight, NMR, and

[2] F. L. Weisenborn (Squibb Institute for Medical Research, Princeton, NJ 08540) and J. L. Strominger, personal communication.

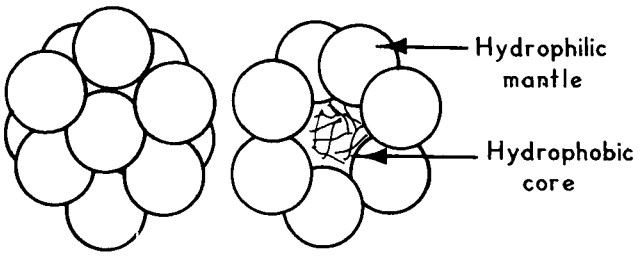


Figure 11—*Representation of the 16-subunit micelle of diumycin, consisting of hydrophobic lipid side chains surrounded by hydrophilic sugars.*

equilibrium-binding studies in this laboratory. The same agents that disrupt the aggregate, such as urea, also displace the antibiotics from human serum albumin.

The enzyme L-asparaginase hydrolyzes the amino acid asparagine to aspartic acid and ammonia and is used in antileukemia therapy. This enzyme (35) is similar to many others (36) in consisting of subunits. Subunit structure is affected by the addition of such compounds as urea, sodium chloride, and sodium lauryl sulfate. The $s^0_{20,w}$ value diminishes from 8.7 S in dilute buffer to 4.0 S in sodium chloride or sodium lauryl sulfate solutions; the M_{app} diminishes from 133,000 to 60,000 daltons. These decreases in M_{app} and $s^0_{20,w}$ and in activity *in vitro*, caused by the addition of sodium lauryl sulfate, can be reversed by adding sodium sulfate, sodium citrate, or potassium dihydrogen phosphate (37) to this solution. Optical rotatory dispersion and UV difference studies show that the spatial relationship of at least some atoms in the molecule is first changed by the addition of detergent and then restored by the addition of the inorganic salt.

MICELLE BINDING FORCES AND INTERACTION OF MONOMER WITH BIOLOGICAL STRUCTURES

At such unphysiologically high concentrations as were used in these studies, many compounds, including drugs, form micelles. The bonding available to maintain the aggregate at these concentrations may be the same forces that bind monomer to biological structures at physiological concentrations. Since all micellar systems of drugs studied in this laboratory dissociate with decreasing concentration and, at physiological concentration, are calculated to be in the form of the monomer (based on uniform distribution of compound in body fluids), the drug substance could bind to the receptor *via* the bonds available to maintain the aggregate at the higher, nonphysiological, concentrations.

Amphotericin B, which forms micelles at the unphysiologically high concentrations used in the model systems *via* bonds affected by the addition of a detergent and/or a hydrogen bond-breaking agent, may, at physiological concentrations, interact with cell membranes *via* these bonds.

Prasinomycin (22) and diumycin (38) form micelles at concentrations higher than those found necessary for antibiotic activity *in vivo* through the lipid side chains that, at physiological concentration, are probably needed only singly to interact with the enzyme to inhibit cell wall biosynthesis.

The enzyme L-asparaginase represents a special group of molecules for which subunit-aggregate equilibrium *in vitro* was previously shown to affect the activity of many enzymes (39, 40), usually through molecular association (41). These macromolecules may have evolved in response to the survival requirements of organisms under various environmental conditions. They provide the final, although special, example of the biological significance of the binding forces that maintain the aggregate.

In summary, the ultracentrifuge has been shown to be useful in investigating some physical properties of drug micelles, including molecular weight, sedimentation coefficients, and mode of binding. The hypothesis is proposed that the intermolecular forces that maintain micellar structure may be a manifestation of the same forces that bind monomers to active sites.

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