filtered off and 50 ml. of a 4 *M* aqueous solution of sodium ethylmercaptide was added. The solution was allowed to stir for 1 hr. at room temperature. The ether layer was separated and the aqueous layer was extracted with two 50-ml. portions of ether. The ethereal layers were combined and dried overnight with anhydrous magnesium sulfate. The magnesium sulfate was removed by filtration and the ether removed by distillation. The residual oil was then distilled under reduced pressure and the fraction distilling between 59–60°/.075 mm. was collected. The product weighed 11.95 Gm. (71.98%) and its refractive index was 1.5690 at 20.5°. The reported boiling point is 83–84° at 2.5 mm. and refractive index is 1.5678 at 25° (5).

α-Alkyloximinocarboxylic Acid Thiol Esters—The title compounds were prepared in the manner described for the preparation of ethyl thiolbenzoate. Pertinent data on the compounds are summarized in Table I.

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Keyphrases

α-Alkyloximino acid thiol esters—synthesis
Mercaptan decomposition of acyl alkyl carbonates
Refractive index
Antimalarial activity

Molecular Association of the Antibiotic Prasinomycins

By JOEL KIRSCHBAUM

The molecular weight of the antibiotic prasinomycins in ethanol is 1600–2300. In aqueous solvents the prasinomycins associate, forming two aggregates with molecular weights of approximately 31,000 and 61,000. The aggregates may be disrupted by organic solvents, dilution below 0.012 percent, or the addition of salts.

THE prasinomycins are a family of closely related antibiotics isolated from *Streptomyces prasinus* (1). Although the molecular weight of one major component of the mixture, as calculated from phosphate group analysis (1), was 1550 ± 10%, the inability to dialyze these antibiotics through a cellulose membrane indicated the possibility of molecular association. The molecular weight in ethanol, determined by means of an analytical ultracentrifuge,[1] ranged between 1600 and 2300. In aqueous solvents the apparent molecular weights were 31,000 and 61,000. These solvent-induced differences in molecular weight indicate association or aggregation. Some of the properties of these aggregates will be described in this paper.

EXPERIMENTAL

Sedimentation coefficients of prasinomycin solutions of concentrations from 10 mg./ml. to 1.25 mg./ml. were measured in 4°, 12-mm. cells centrifuged in an An-D rotor at 42,040 r.p.m. and 20.7°. The phaseplate angle of the schlieren optical system was 75°. For solutions with concentrations of 1.25 mg./ml. to 0.25 mg./ml., the sedimentation coefficients (s) were determined in a 12-mm. synthetic boundary centerpiece in an interference cell.

Molecular weights were determined using equilibrium and Archibald approach-to-sedimentation equilibrium methods.

For the Archibald (2) method, 5–10 mg./ml. solutions in 12-mm. interference cells were centrifuged in an An-D rotor at 20.7°. Concentration gradients were formed at speeds of 6995, 8766, 12,590, 20,410, 29,500, and 42,040 r.p.m. to test for any dependence of molecular weight on speed, which, in turn, affects concentration of the sedimenting material. The schlieren optical system was used with a phaseplate angle of either 75° or 80°. The concentration gradients at the meniscus and cell bottom were measured from data recorded on Kodak metallographic plates magnified 10-fold on a Nikon magnifier. Engelberg's technique (3) for evaluating the concentration gradient integral was used. Total concentrations were determined in separate experiments using a synthetic boundary centerpiece. An IBM 1620 computer was used for some of the repetitive calculations.

Equilibrium centrifugation (4) aided in obtaining the molecular weights of prasinomycin solutions of 0.15 mg./ml. and lower in concentration. Using a calibrated syringe, 0.03 ml. of prasinomycin solution was injected into one compartment of an interference cell fitted with sapphire windows and layered with fluorochemical FC 43 (Beckman Instruments, Inc.). Solvent was injected into the reference compartment. The final percentage

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[1] Model E Analytical Ultracentrifuge, Spinco Division, Beckman Instruments, Inc.

of prasinomycin in the solution compartment was determined by two methods: (*a*) photographically measuring the height of liquid in each compartment of the rotating cell, before and after flow of solvent into the solution compartment and measuring the cell centerpiece cross-section, and (*b*) combining the weight of solvent and solution added to a cell with the densities of liquids.

Diffusion constants (5) were evaluated in the analytical ultracentrifuge at 20.7°, using a synthetic boundary centerpiece and the schlieren optical system. Initial solute concentrations were from 10 to 2 mg./ml. The first image was recorded at two-thirds of the final speed of 29,500 r.p.m. This first picture was considered to be the zero-time image for diffusion constant calculations

The partial specific volume was determined at 20° in a 1-ml. pycnometer. Viscosities were determined with the aid of capillary and Zimm (6) viscometers.

RESULTS AND DISCUSSION

The minimum molecular weights of the prasinomycins, as determined by approach-to-equilibrium sedimentation in either 100% ethanol or in 90% ethanol–10% 0.2 *M* NaCl–0.02 *M* sodium phosphate buffer (pH 6.85), are identical within experimental error and are between 1,600 and 2,300 daltons (Table I). The small quantity of inorganic buffer in the ethanol diminishes charge-charge interactions that may lead to 5 to 20% erroneously lower molecular weight. A minor component, molecular weight approximately 7,200, is evident which may be a tetramer of the prasinomycins. In NaCl–sodium phosphate buffer the molecular weight of the prasinomycins was calculated to be 31,000 daltons, within a 3% experimental error. This is an aggregate since almost all of the solute prasinomycin could be accounted for as the 31,000 dalton component. Occasionally, traces of a component of higher molecular weight were evident; apparent mol. wt. 60,000 daltons. In 0.072 *M* borate buffer, pH 8.6, only the higher molecular weight component, mol. wt. = 61,500, was present. No dependence of molecular weight on speed was found.

The 31,000 dalton aggregate of prasinomycin is stable in various aqueous buffers ranging from pH 4.05 to pH 10.4 (Table I). In pH 12 glycine–NaOH buffer the aggregate is disrupted since the molecular weight is approximately 2,000, with a trace of a 3,600 molecular weight component present.

Verification of the 31,000 dalton molecular weight determined in aqueous solvents and the 1,600–2,300 molecular weight determined in ethanolic solutions was achieved by combining the sedimentation and diffusion constant measurements (7). The aggregate sedimentation constant (*s*) of 4.0 *S* ($S = 10^{-13}$ cm. sec.$^{-1}$) at zero concentration and 20°, together with the diffusion constant, $D^0_{20,w} = 9.0 \times 10^{-7}$ cm.^{2} sec.$^{-1}$ of the aggregate, yields a mol. wt. of 30,000 daltons. The diffusion constant of prasinomycin in ethanol is 2.2×10^{-6} cm.^{2} sec.$^{-1}$ (which itself indicates that prasinomycin in ethanol has a lower molecular weight than prasinomycin in aqueous solvents). Combination with the $D^0_{20,w}$ value of $0.82–1.02 \times 10^{-13}$ cm.^{2} sec.$^{-1}$ gives an approximate mol. wt. of 2,100 daltons for unaggregated prasinomycin, in good agreement with the 1,600–2,300 mol. wt. determined by approach-to-equilibrium sedimentation.

Since the molecular weight of the prasinomycins in ethanolic solutions is 1,600–2,300 and the apparent molecular weight of the prasinomycins in aqueous solvents is 31,000, an obvious question is: what is the apparent molecular weight of the prasinomycins in mixtures of ethanol and aqueous solvents. In Fig. 1 are the curves depicting the changes in molecular weight of the prasinomycins as (*a*) the free acid of prasinomycin dissolved in ethanol is diluted with NaCl–sodium phosphate buffer, and (*b*) sodium salt, of prasinomycin, initially dissolved in NaCl–sodium phosphate buffer is diluted with ethanol. Molecular weights were determined after a 3–18 hr. equilibration. Per-

TABLE I—MOLECULAR WEIGHTS OF THE THREE MAJOR PRASINOMYCINS A, B, AND C DISSOLVED IN VARIOUS SOLVENTS

Form of Prasinomycin	Prasinomycin	Solvent	Mol. Wt. Major Component	Mol. Wt. Minor Component
Free acid	A	Ethanol–phosphate buffer	2300	7300
Methyl ester	A	Ethanol	2100	6400–7600
Free acid	A	Ethanol	2000	7400
NH_4^+ Salt	A	Ethanol–phosphate buffer	2150	6800
Free acid	B	Ethanol	1600	7400
Free acid	B	Ethanol–phosphate buffer	1850	7500
Na^+ Salt	B	Ethanol–phosphate buffer	1700	7400
Free acid	C	Ethanol–phosphate buffer	2200	7100
Free acid	C	Ethanol	1850	7200
Na^+ Salt	C	Ethanol	1650	...
Methyl ester	C	Ethanol	2100	7200
K^+ Salt	A,B	Ethanol–phosphate buffer	1700	6000
Free acid	A,B,C	Phosphate buffer	31000 ± 3%	61000 ± 5%
Na^+ Salt	A,B,C	Phosphate buffer	31000 ± 3%	61000 ± 5%
Na^+ Salt	B	pH 4.05, 1 *M* acetate buffer	32000	
Na^+ Salt	B	pH 10.4, 0.05 *M* glycine–NaOH buffer	32000	
Na^+ Salt	B	pH 12, 0.17 *M* glycine–NaOH buffer	2000	3600
Na^+ Salt	B	Isotonic buffer, containing K^+, Na^+, Ca^{2+}, Mg^{2+}, Cl^-, $SO_4^=$ and $PO_4^=$	31000	

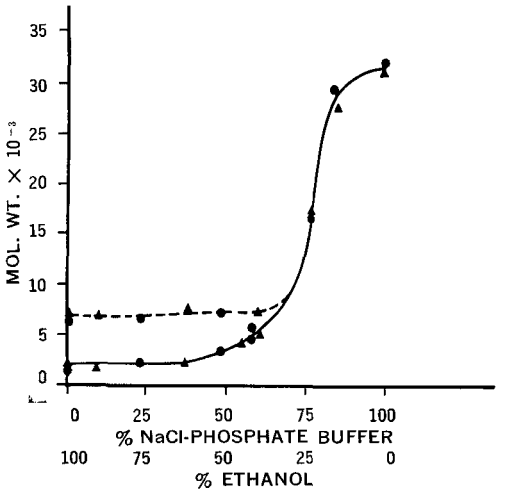

Fig. 1—Changes in mol. wt. of prasinomycin caused by changing the solvent: ●, free acid of prasinomycin initially dissolved in ethanol diluted with NaCl-sodium phosphate buffer; ▲, sodium salt of prasinomycin dissolved in NaCl-sodium phosphate buffer diluted with ethanol. Note existence of tetramer of mol. wt. 7,200–7,600 daltons (broken line) at ethanol concentrations from 30 to 100%.

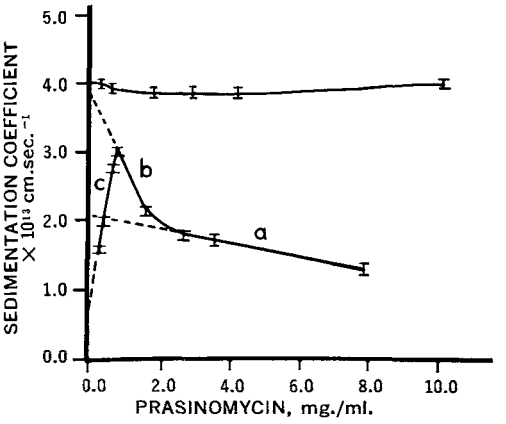

Fig. 2—Dependence of the sedimentation coefficient of prasinomycin on its concentration in 0.2 M NaCl–0.02 M sodium phosphate buffer, pH 6.9, upper curve and in 5.0 M NaCl–0.02 M sodium phosphate buffer, pH = 6.9, lower curve.

sistently found below 65% NaCl–sodium phosphate buffer–35% ethanol is the 7,200 dalton compound identified tentatively as tetramer. The two molecular weight curves, including tetramer branching, are superimposable, as expected, since this procedure of adding the free acid form to buffered solvent is analogous to adding acid to aqueous sodium phosphate.

As seen in Fig. 2, upper curve, variation of concentration from 10 mg./ml. to 0.25 mg./ml. for prasinomycin dissolved in 0.2 *M* NaCl–0.02 *M* sodium phosphate buffer does not appear to result in the dissociation of the aggregate, since $s_{20,w}$ = 4.0 *S* at both the initial and final concentrations. However, the curvature of the line is indicative of some dissociation or rearrangement. When the NaCl concentration of the buffer was increased to 5 *M*, a complex figure was obtained (Fig. 2 lower curve). If the three portions of the curve, labeled a, b, and c are extrapolated to zero concentration (broken lines) and the *s* values measured, a is equal to 2.05 *S*, b is equal to 3.9 *S*, and c is equal to 1.1 *S*; all values corrected for viscosity and density. The sedimentation coefficient of b, *s* = 3.9 *S*, may be considered to be equivalent to a molecular weight of 31,000 daltons. Substituting these values into the formula, molecular weight = $f_s^{2.2}$, *f* is equal to 1,550. The calculated molecular weight of *a* is approximately 7,500 daltons and that of *c* is approximately 1,700 daltons. These molecular weight values approximate closely monomer and tetramer molecular weights.

Since sedimentation coefficient values of prasinomycin solutions below 0.25 mg./ml. cannot be measured by the schlieren optical system, the interference optical system was used, and molecular weights were determined by sedimentation equilibrium methods. At a concentration of 0.012%, two components, with approximate molecular weights of 3,600 and 2,000 were calculated. At 0.005% only a 1,800–2,000 molecular weight component was discernible.

The various molecular weights of prasinomycin found in this study are 1,800–2,300, 3,600, 7,200–6,600, 15,000, 31,000 and, under certain conditions, 60,000–62,000 daltons. It appears that aggregation proceeds by repeated dimerization of the monomer to the 31,000 dalton aggregate.

The aggregate may be stabilized by electrostatic (8) interactions since increasing the ionic strength can dissociate the complex. Conversely, by using water alone as solvent (0 ionic strength but for added prasinomycin), the observed sedimentation coefficient is 5.4 *S*. This *s* value is equivalent to the 61,000 dalton aggregate and is the highest molecular weight aggregate observed.

Cleavage (9) of one prasinomycin species gives a 1,400 dalton and a 350 dalton fragment. The 350 dalton moiety was found to be a hydrocarbon (9). The 1,400 dalton fragment did not aggregate, indicating that the association of the prasinomycins may also require hydrophobic bonding (10).

Hydrogen bonding may not have a role in prasinomycin aggregation because 8 *M* urea did not affect the sedimentation coefficient of the aggregate (*s* = 3.9 *S*), after best correction (11) for density and viscosity.

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Keyphrases

Prasinomycins—molecular association

Molecular weight—approach-to-equilibrium method

Sedimentation coefficients—schlieren, interference optical systems

Diffusion constants—centrifuge, schlieren optical system

Gas-Liquid Chromatographic Determination of Lincomycin

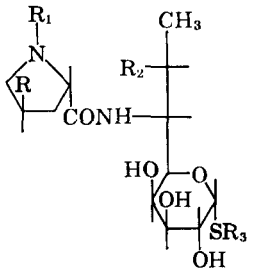
By R. L. HOUTMAN, D. G. KAISER, and A. J. TARASZKA

The gas-liquid chromatography of lincomycin and some of its analogs, determined as trimethylsilyl ethers, is reported. The application of this procedure to quantification of lincomycin in bulk material and pharmaceutical preparations is described.

THE APPLICATION of gas-liquid chromatography to polyhydroxy compounds has been greatly enhanced by the advent of methods for the quantitative conversion of these compounds to their trimethylsilyl ethers (1, 2). The silylation procedure consists of dissolving the material in pyridine and adding hexamethyldisilazane and trimethylchlorosilane.

Lincomycin (I) (3), a medium spectrum antibiotic containing an octopyranose moiety, was silanized by this procedure and chromatographed as the intact tetra trimethylsilyl ether. U-21699 (II), an antibiotic produced at about the 3% level in the microbiological synthesis of lincomycin (4) was chromatographed under the same conditions and gave a retention time of 0.85 relative to lincomycin. This separation enabled the quantification of small amounts of U-21699 in lincomycin as well as the determination of lincomycin in bulk material and pharmaceutical formulations.

Since the preparation of the trimethylsilyl ether derivatives of these compounds is rapid, quantitative, and can be applied on a micro scale, GLC analysis of lincomycin and some of its analogs has become a practical laboratory procedure.



I. Lincomycin
R = —$CH_2CH_2CH_3$
R_1 = —CH_3
R_2 = —OH
R_3 = —CH_3

II. (U-21699)
R = —CH_2CH_3
R_1 = —CH_3
R_2 = —OH
R_3 = —CH_3

III. (U-21251F)
R = —$CH_2CH_2CH_3$
R_1 = —CH_3
R_2 = —Cl
R_3 = —CH_3

IV. (U-11921A)
R = —$CH_2CH_2CH_3$
R_1 = —CH_3
R_2 = —OH
R_3 = —CH_2CH_3

V. (U-11973E)
R = —$CH_2CH_2CH_3$
R_1 = —H
R_2 = —OH
R_3 = —CH_3

VI. (U-24166A)
R = —$CH_2CH_2CH_3$
R_1 = —CH_2CH_3
R_2 = —OH
R_3 = —CH_3

EXPERIMENTAL

Reagents and Materials—The lincomycin and analogs used in this study were prepared by the Research Division of The Upjohn Company. Hexamethyldisilizane and trimethylchlorosilane were obtained from the Dow Corning Co., Midland, Mich. A solution of tetraphenylcyclopentadienone[1] in pyridine (5 mg./ml.) was employed as the internal standard. The column packing utilized for gas-liquid chromatography was 3% (w/w) SE-30 on 100–120 mesh Gas Chrom Q.[2] All solvents were reagent grade and used as supplied.

Instrumentation—An F & M model 402 high efficiency gas chromatograph,[3] equipped with a flame ionization detector and U-shaped glass columns (2 m. long × 3 mm. i.d.) was utilized throughout the study. Helium was used as the carrier gas at a flow rate of 60 ml./min. Air and hydrogen flow rates were adjusted to give maximum response. The column oven was operated isothermally at 240°, the flash heater at 270°, and the detector at 290° Peak areas were measured with an Infotronics 11HSB/42 electronic integrator.[4]

Procedures—In general, the silanization technique of Makita and Wells (5) was used. For quantification of lincomycin, 50 mg. of the bulk drug was accurately weighed and dissolved in 10.0 ml. of the internal standard solution. One milliliter of hexamethyldisilizane and 0.5 ml. of trimethylchlorosilane were added. The solutions were allowed

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[1] Aldrich Chemical Co., Milwaukee, Wis.

[2] Applied Science Laboratories, Inc., State College, Pa.

[3] F & M Scientific Corporation, Avondale, Pa.

[4] Infotronics Corporation, Houston, Tex.