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Studies Concerning the Behavior of Actinomycin in Solution*

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ABSTRACT: Dimerization and conformational change of actinomycin are investigated by equilibrium centrifugation and optical rotatory dispersion. A specific dimer forms in aqueous solutions of actinomycin, with a dimerization constant of roughly 10^3 M^{-1}. The heat and entropy of dimerization are relatively large and negative. The optical rotatory dispersion spectrum of actinomycin depends on the nature of the solvent, presumably reflecting differences in molecular conformation. Changes in the optical rotatory dispersion spectrum seem to be correlated with the surface tension of the solvent. Results are discussed in terms of some qualitative ideas about the nature of hydrophobic interactions.

Actinomycin, an oligopeptidic substance, has a structure intermediate in complexity between small molecules and proteins. In our studies of the complex formation between actinomycin and DNA (Müller and Crothers, 1968), we have found it useful to regard this system as a very simple model for the interaction of protein with nucleic acid. This analogy would require some similarity between the solution properties of actinomycin and more complicated protein molecules. Many proteins undergo conformation changes as a result of changes in external conditions, and also are able to form specific aggregates at appropriate concentrations. In this paper we show that actinomycin exhibits both of these characteristics. A further purpose of the work reported here is to aid the interpretation of experiments on the actinomycin–DNA complex by elucidating the properties of one of the components.

Recent work (Müller and Emme, 1965) has disclosed that actinomycin aggregates in aqueous solutions but not in most organic solvents. These studies, employing the ultracentrifuge for molecular weight determination, concluded that actinomycin C_3 (see Brockmann, 1960,

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for nomenclature) exists as a specific dimer at low (3.8×10^{-5} M) and moderate concentrations, and that higher aggregates occur as the solubility limit is approached. A polarographic study (Berg, 1965) furthermore reported a tetrameric apparent molecular weight, M_{app}, for 3×10^{-4} M actinomycin C_1 (=D) in aqueous solution. On the other hand, Gellert *et al.* (1965), employing equilibrium centrifugation, reported a monomeric M_{app} at low concentration, and an M_{app} intermediate between monomer and dimer at higher concentrations (without noticeable aggregation across the centrifuge cell). In the present study, equilibrium sedimentation was employed systematically over a wide concentration range to resolve these contradictions. We confirm the existence of a specific dimer, with a (temperature-dependent) dimerization constant of roughly 10^3 M.$^{-1}$ At the concentrations that actinomycin is usually used ($<10^{-5}$ M), the dimer is therefore present in only very small amount. At 5° we find little tendency to form higher aggregates, even at 6×10^{-3} M.

The presence of optically active centers in the actinomycin peptide rings permits the advantageous use of optical rotatory dispersion to investigate conformational alterations involving these flexible structures. Two types of such alterations seem possible: those accompanying dimerization, and those related to the intramolecular ordering of the rings of individual monomers in solution. These two conformational variations have been proposed to be governed by hydro- or solvophobic forces (Müller and Emme, 1965), but were not distinguished from each other experimentally. The present study reports a means of differentiating the two alterations by optical rotatory dispersion and supports the proposal that solvophobic interaction is important in determining the conformational form.

Materials and Methods

Materials and Solutions. Crystalline actinomycin C_3 was obtained from *Streptomyces* cultures and separated chromatographically from other actinomycins (Brockmann, 1960). Calf thymus DNA (Sigma Chemical Co., type 1, highly polymerized) was used without further purification.

Aqueous solutions were made up from glass-distilled water and were buffered at pH 6.95 by 0.008 M sodium phosphate containing 0.18 M NaCl. Other solvents, used without further purification, were: *p*-dioxane, spectroquality reagent, Matheson Coleman and Bell; formamide, 99%, Matheson Coleman and Bell; glycerine, analytical reagent, Malinkrodt; ethylene glycol, reagent grade, Fisher; and methanol, reagent grade, Fisher.

Actinomycin concentrations were determined spectrophotometrically, using a molar extinction coefficient at 444 mμ of 24,600 for methanol solutions. Extinction coefficients in other solvents were determined either by evaporating a known volume of a methanol solution followed by dissolution of the residue in another solvent, or by diluting a very concentrated methanol solution into a large excess of the other solvent. This latter procedure is necessary for poor solvents like glycerol for which a quantitative dissolution of the residue after methanol evaporation was not possible, due to adsorption of the antibiotic to the walls of the flask. Measured fluctuations in extinction coefficients from one solvent to another were not more than a few per cent.

Molecular Weight Determinations. Apparent weight-average molecular weights were determined by equilibrium sedimentation in a Spinco Model E analytical ultracentrifuge. Absorption optics were used at concentrations below 1000 μg/ml, and schlieren optics at higher concentrations. In preliminary experiments at low to moderate concentrations, occasional very erratic results were found. We believe these were caused by contaminants in the centrifuge cell, since a routine procedure of filling the assembled cell with an actinomycin solution and soaking for several hours, followed by rinsing and replacement with fresh solution before centrifugation, gave consistent results.

In experiments with absorption optics, the optical path length (centerpiece height) was varied from 1.5 to 30 mm and two different wavelengths (260 and 365 mμ) were used in order to provide a level of absorbance appropriate for photographic detection. AM[1] concentrations varied from 4 to 1000 μg/ml. The liquid column height was 4–5 mm, and most runs were made at 29,500 rpm for 24 hr. After each run the cell was cleaned and filled with buffer and accelerated to the speed of the run and a blank photograph was taken. Films were scanned on a recording densitometer, and the tracings from the solution and blank runs were superimposed in the air region of the cell. The optical density of the AM solution relative to solvent was then assumed proportional to the measured difference between the (logarithmic) density tracings in the liquid portions of solution and solvent runs. Since the deviations from Beer's law which we could detect were small, it was assumed that optical density is linearly related to actinomycin concentration. Apparent molecular weights were determined in the usual manner from the slope of a plot of the logarithm of concentration, *c*, *vs.* the square of the distance, *r*, from the rotational axis. The partial specific volume was set equal to 0.786 (Müller and Emme, 1965).

Experiments using schlieren optics were performed in a similar manner, except that the blank cell was centrifuged long enough to establish an equilibrium density gradient in the buffer. The proportionality between index of refraction gradient and concentration gradient was calculated from the average of two synthetic boundary runs at different concentrations. Concentration at various points in the cell was then determined by a graphical integration procedure. At the highest concentrations reached (about 1%) the assumption of ideal solutions is doubtless a poor one, but activity coefficients are unfortunately not available for actinomycin. We therefore refer throughout to molecular weights determined with this assumption as apparent molecular weights, M_{app}.

Optical rotatory dispersion was measured on a Cary

[1] Abbreviation used that is not given in *Biochemistry* 5, 1445 (1966), is: AM, actinomycin.

60 spectropolarimeter equipped with a nitrogen-flushed cell chamber and programed slit width. Jacketed, quartz cells of path lengths 1.0, 0.1, and 0.01 cm were connected to a temperature-controlled water bath. For buffered aqueous solutions the temperature was kept at 5 ± 1°. For solutions in organic solvents the Cary 60 cooling system was used, maintaining the temperature at 26 ± 2°.

A solvent base line was recorded before and sometimes after each sample rotation. Difficulties were encountered in maintaining a constant base line between blank and sample runs when using 1.0- and 0.1-mm cells, because their construction necessitated their complete removal from the mounting and considerable handling when changing contents. To cancel out partially the inevitable base-line shifts, *amplitudes* of apparent Cotton effects are plotted in units of specific rotation (eq 1), where α is the observed rotation in degrees, c

$$\Delta[\alpha] = \frac{\alpha_{max} - \alpha_{min}}{cl} \quad (1)$$

the actinomycin concentration in g/cm^3, and l the path length in decimeters. Occasional samples were run in duplicate to check instrument reproducibility. Where results differed slightly the averages were plotted. When measurements were made in a series of solvents the data were corrected for variation of solvent refractive index, n, by multiplying the amplitude of the Cotton effect by the Lorentz correction factor, F, where the value of n re-

$$F = 3/(n^2 + 2) \quad (2)$$

fers to the wavelength around which the Cotton effect is centered (eq 2).

Spectra taken in glycerol are subject to somewhat greater uncertainty than those in other solvents because of the tendency of actinomycin to adsorb to glass surfaces from glycerol solution.

Results

Equilibrium Centrifugation. The calculated apparent weight-average molecular weights of actinomycin C_3 in aqueous solutions of different concentrations are listed in Table I. Considering that the molecular weight of actinomycin C_3 is 1283, the results indicate that as the concentration approaches zero, monomers predominate, but with increasing concentration, M_{app} rises to an apparent plateau near 2566, the dimer molecular weight. This plateau is good evidence that aggregation beyond the dimer is insignificant up to dye concentrations of 9.0 mg/ml (7×10^{-3} M). The optical rotatory dispersion results reported below also support this conclusion. There is some evidence for formation of higher aggregates at the highest concentrations studied, since a slightly curved line was obtained in the log c *vs.* r^2 plot for the most concentrated solution; nevertheless, the slope at the back of the cell, where the concentration was about 14 mg/ml, yielded an M_{app} of only 3000. Possibly this curvature is a manifestation of solution nonideality or experimental error.

TABLE I: Apparent Weight-Average Molecular Weights from Sedimentation Equilibrium for Solutions of Actinomycin C_3 in pH 6.95 Sodium Phosphate Buffer (0.008 M) and NaCl (0.18 M).

Temp (°C)	Initial Concn (μg/ml)	M_{app} (g)	Method
5	20.1	1444	Absorption optics
5	109	1621	Absorption optics
5	200	1750	Absorption optics
5	476	1975	Absorption optics
5	992	2248	Absorption optics
5	2280	2510	Schlieren optics
5	5880	2900	Schlieren optics
5	7760	2650	Schlieren optics
20	4.2	1307	Absorption optics
20	9.0	1307	Absorption optics
20	39.6	1328	Absorption optics
20	102	1400	Absorption optics
20	223	1485	Absorption optics
20	299	1672	Absorption optics
20	476	1702	Absorption optics

Thermodynamic Constants of Actinomycin Dimerization. We assume a simple monomer–dimer equilibrium at the concentrations studied, for which the equilibrium constant is

$$K_{dimer} = [AM_2]/[AM]^2 \quad (3)$$

where [AM] and [AM_2] are the molar concentrations of monomer and dimer, respectively. In addition, it is assumed that the apparent molecular weights derived from equilibrium sedimentation may be expressed as

$$M_{app} = 1283y + 2566(1 - y) \quad (4)$$

where

$$y = [AM]/([AM] + 2[AM_2]) \quad (5)$$

from which the concentrations of monomer and dimer may be readily calculated.

Figure 1 shows log–log plots of the concentrations of [AM] and [AM_2] up to a total actinomycin concentration of 1 mg/ml. A line of the expected slope 2.0 is drawn to fit the points, with error bars corresponding to about 7% error in molecular weight determinations. Since the lines of slope 2 fit the data within experimental error, a simple dimerization equilibrium is consistent with our observations. The equilibrium constant can be obtained from the extrapolated value of log [AM_2] at log [AM] = 0, yielding 3.6×10^3 M^{-1} at 5° and 9.0×10^2 M^{-1} at 20°.

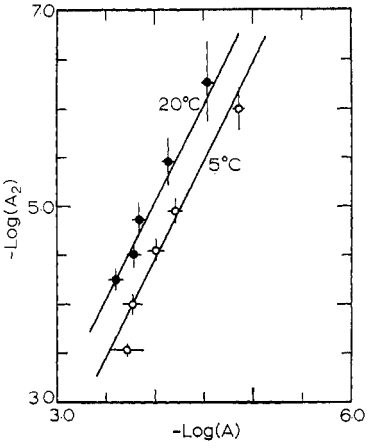

FIGURE 1: Log–log plot of the dependence of the concentration A_2 of the actinomycin dimer on the concentration of monomer, A. Concentrations were calculated from equilibrium sedimentation data, assuming a simple dimerization equilibrium and ideal behavior for each component. Error bars correspond to 7% uncertainty in measured molecular weights. For each temperature the data are fit to a straight line of slope 2.00.

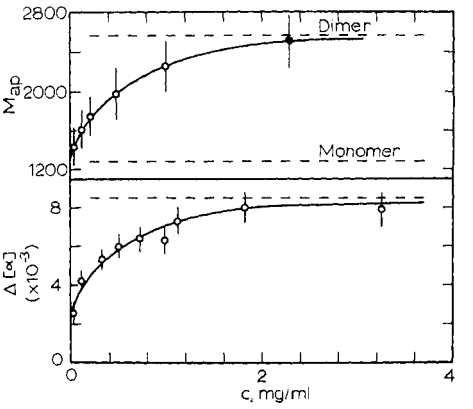

FIGURE 3: Comparison of the concentration dependence of apparent molecular weight, M_{app}, and the specific amplitude, $\Delta[\alpha]$, of the Cotton effect around 269 mμ for a series of aqueous actinomycin solutions (see eq 1 for a definition of $\Delta[\alpha]$). In the upper plot, molecular weight values corresponding to monomer and dimer are indicated, and in the lower plot the plateau value of $\Delta[\alpha]$ at high concentrations is indicated by a dotted line.

Since the dimerization constant was determined at two temperatures, it is possible to estimate the average heat and entropy of dimerization in this temperature region. These calculations yield $\Delta H^\circ_{\text{dimzn}} = -15$ kcal/mole, and $\Delta S^\circ_{\text{dimzn}} = -38$ eu/mole.

Optical Rotatory Dispersion of Actinomycin. The optical rotatory dispersion of actinomycin C_3 is too complex for unequivocal analysis. Apparent negative Cotton effects are observed centered about 449, 376, 269, and 213 mμ (see Figure 2). These curves undoubtedly represent sums of overlapping Cotton effects from individual absorption transitions in the molecule. The weak effect in the visible region (449 mμ) is extrinsic, *i.e.*, induced in the transition of an optically inactive chromophore by an asymmetric molecular environment (Stryer and Blout, 1961; Ulmer and Valee, 1965), in this case by the pentapeptide rings. The center of this apparent Cotton effect is farther from the chromophore absorptions at 425 and 441 mμ than would be expected, but the discrepancy is probably explained by adding and cancelling of overlapping Cotton effects in this region. Also perhaps unexpected is the apparent Cotton effect centered about 376 mμ, near which there are no strong absorption bands. Finally, the Cotton effects in the ultraviolet region represent resultant dispersion related to the optically active amino acid and peptide-bond transitions as well as to the induced asymmetry in ultraviolet transitions of the phenoxazone system.

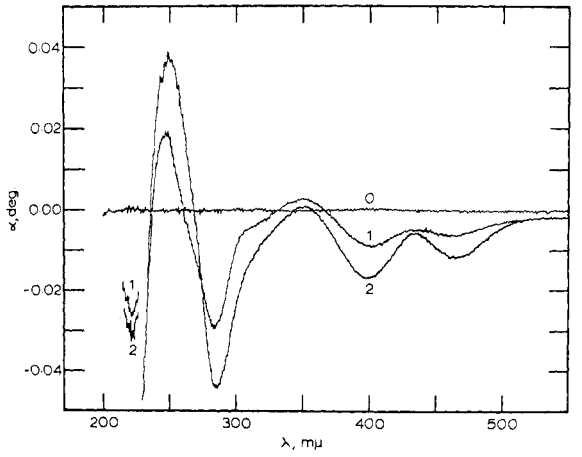

FIGURE 2: Optical rotatory dispersion spectra of actinomycin solutions at a constant (±5%) product of concentration and path length. Curve 0: solvent base line; curve 1: actinomycin C_3 (109 μg/ml) in a 1-cm cell; curve 2: actinomycin C_3 (1120 μg/ml) in a 1-mm cell. The solvent is aqueous sodium phosphate buffer (0.008 M, pH 6.95) containing 0.18 M NaCl. All spectra were measured at 5°. The observed rotation, α, is shown as a function of wavelength, λ. The vertical scale on the rotational troughs in the lower left corner is reduced by a factor of 4.

Figure 2 illustrates the over-all changes in the optical rotatory dispersion with actinomycin concentration. Investigation of a series of solutions showed that the specific rotation (measured as amplitudes) of the Cotton effects centered about 269 and 449 mμ increase with rising concentration until a plateau is reached, while the Cotton effect at 376 mμ is comparatively much less intensified. Parenthetically, the Cotton effect at 449 mμ appears to expand along the wavelength coordinate at the expense of the overlapping effect at 376 mμ, although the midpoint does not change. Figure 3 plots the amplitudes of the Cotton effect centered at 269 mμ as well as the M_{app} values found from equilibrium sedimentation *vs.* concentration. The similarity of these curves indicates that dimerization is responsible for the increase in optical rotation.

An attempt was made to detect kinetically the dissociation of actinomycin dimers utilizing the described changes in specific rotation. A 6500-μg/ml actinomycin solution at 5° was rapidly diluted 100-fold into a 1-cm cell, and the rotation at several appropriate wavelengths was recorded *vs.* time by the Cary 60. The

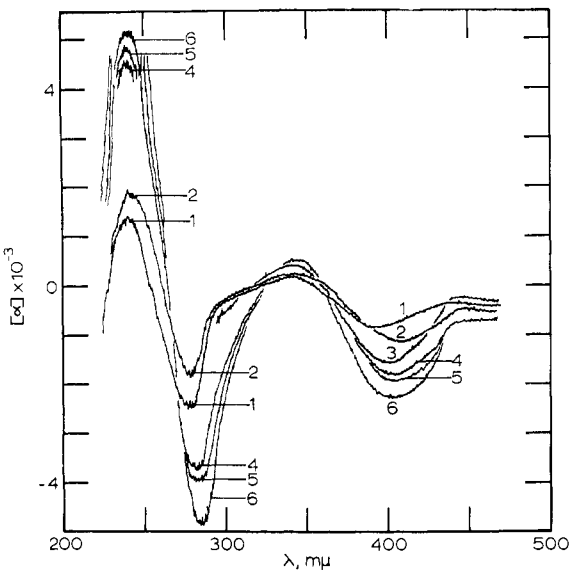

FIGURE 4: Optical rotatory dispersion spectrum of actinomycin in a series of solvents, showing specific rotation $[\alpha]$ as a function of wavelength, λ. The vertical scale for the rotational peaks in the upper left corner is reduced by a factor of 2. Solvents: 1, aqueous buffer (as for the spectra in Figure 2); 2, glycerol; 3, formamide; 4, ethylene glycol; 5, dioxane; and 6, methanol. All spectra were measured at 26°.

uniformly negative results indicate that the reaction is over within 30 sec after dilution.

Varying the solvent at constant actinomycin concentration affects the optical rotatory dispersion in a different manner; these changes seem best correlated with solvent surface tension. Figure 4 shows the optical rotatory dispersion spectrum of actinomycin in several solvents. Aside from an occasional inversion in the water and glycerol spectra, the rotatory dispersion curves change in a steady manner as solvent surface tension changes. This correlation is shown quantitatively in Figure 5, where the specific amplitude of the Cotton effect about 376–383 mμ, corrected for solvent refractive index, is plotted as a function of solvent surface tension. The Cotton effect about 269–272 mμ varies with solvent surface tension in a similar manner, although the correlation is not quite so smooth.

One consequence of these observations is that the solvent-dependent conformational change can be distinguished from dimerization. The former, which we assume to be due to intramolecular ordering of the peptide rings, produces characteristic changes in the Cotton effect around 380 mμ, whereas dimerization produces a Cotton effect around 449 mμ.

Attempts to observe kinetically the solvent-dependent change in the Cary 60 by rapid dilution of aqueous solutions with dioxane were fruitless. The changes apparently occur within seconds after the sudden reduction in surface tension.

Optical Rotatory Dispersion of the Actinomycin–DNA Complex. The optical rotatory dispersion of an aqueous buffered solution at 27° of 100 μg/ml of actinomycin and 500 μg/ml of calf thymus DNA was measured. Because the actinomycin : base pair ratio was about 1 : 12,

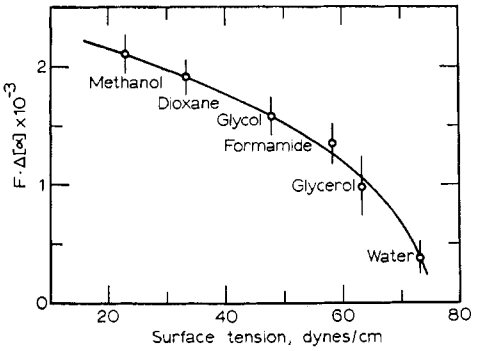

FIGURE 5: Dependence of the specific amplitude, $\Delta[\alpha]$, of the Cotton effect about 376–383 mμ on solvent surface tension, corrected for solvent refractive index by multiplication by the Lorentz factor, F.

essentially all of the dye was bound. From the curve obtained, the rotation of a 500-μg/ml solution of DNA under the same conditions was subtracted. The resultant curve shows a new negative Cotton effect with specific amplitude, $\Delta[\alpha]$, of 1250°, a peak at 431 mμ, and a trough at 474 mμ. Its midpoint at 453 mμ corresponds to the absorption maximum at 452 mμ of the actinomycin–DNA complex. It is analogous to the extrinsic Cotton effects observed by Stryer and Blout (1961) for complexes of optically inactive dyes with α-helical poly-L-glutamic acid.

Association of an Analogous Compound. The simple actinomycin analog actinomine (Müller and Crothers, 1968), which has triethylamine groups instead of the cyclic peptide side chains, shows strong deviations from Beer's law at relatively low concentrations. The blue shift and hypochromicity at high concentrations (Figure 6) are consistent with aggregation by stacking of the chromophores, as is observed for many dye stuffs. On the other hand, 2,7-diaminophenoxazone-3 (Müller and Crothers, 1968) shows no such spectral changes in the same concentration range. Actinomycin itself shows only a small spectral change on dimeriza-

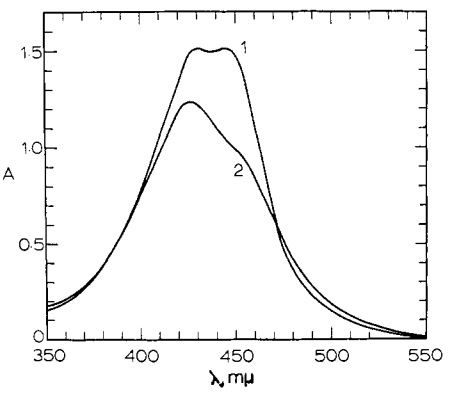

FIGURE 6: Changes in the visible absorption spectrum of actinomine, a simple analog of actinomycin, on aggregation at high concentrations. The spectra are taken at a constant product of concentration and optical path length, and show absorbance, A, as a function of wavelength, λ. Curve 1: 1.13×10^{-5} M in a 40-mm cell; curve 2: 4.53×10^{-4} M in a 1-mm cell. Spectra were taken in the aqueous buffer used for actinomycin solutions at 25°.

TABLE II: Standard Heat and Entropy of Some Reactions of Actinomycin in Aqueous Solutions.[a]

Process	$\Delta H°$ (kcal/mole)	$\Delta S°$ (eu/mole)	Reference
Precipitation from saturated solution at 11.7° (from solubility measurements)	37	136	Gellert *et al.* (1965)
Complex formation with DNA	0	31	Gellert *et al.* (1965)
Complex formation with dGMP	−11	−22	Gellert *et al.* (1965)
Complex formation with deoxyguanosine	−9.1	−15	Gellert *et al.* (1965)
Complex formation with deoxyguanosine	−8.3	−13	Müller and Spatz (1965)
Dimerization	−15	−38	This study

[a] All quantities refer to reaction in the direction of association of actinomycin with itself or some other substance and therefore in the direction of less exposure to actinomycin to water.

tion, with a hypochromicity which is probably less than 10%.

Discussion

Actinomycin has a sufficiently complicated structure that it has many possible conformations in solution. Furthermore, the structure in the crystal has not been determined by X-ray diffraction. It would therefore not be presently possible to do more than suggest hypothetical conformations for actinomycin and its dimer. In this discussion we limit ourselves to the macroscopic changes accompanying dimerization and alterations of actinomycin structure in various solvents.

The dimerization of actinomycin observed in aqueous solutions does not occur in the organic solvents which have been examined (Müller and Emme, 1965). Actinomycin is uncharged at neutral pH, so it seems unlikely that the high dielectric constant of water is responsible for its ability to encourage the aggregation reaction. In addition, any hydrogen bonds in the dimer would almost certainly be less stabilizing in water than in a solvent like dioxane. A strong interaction between chromophores in the dimer must be ruled unlikely in view of the very small shift of the absorption spectrum on dimerization, especially when compared with the large spectral change accompanying aggregation of the analogous compound actinomine. Therefore, it would seem necessary to ascribe the interactions which give the dimer particular stability in water to the hydrophobic, or, more generally, solvophobic class. This interpretation is strengthened by the presence of several nonpolar amino acids in the cyclic peptide side chains.

Table II summarizes thermodynamic data on association reactions involving actinomycin. The dimerization reaction as well as complex formation with deoxyguanosine or dGMP all show a large negative heat of reaction. There are other systems for which an association reaction, thought to be mediated at least partly by hydrophobic interactions, shows a similar negative heat of reaction. Examples are the aggregation of tyrocidin (Ruttenberg *et al.*, 1966), the dimerization of insulin (Doty and Myers, 1952), and the stacking of bases in single-stranded nucleic acids (Leng and Felsenfeld, 1966; Brahms *et al.*, 1966; Poland *et al.*, 1966). The source of the large negative heat of reaction may be somewhat puzzling, since in the usual view of hydrophobic bonding the major contribution is an increase in the solvent entropy when two molecules associate in water solution, with only minor changes in the heat (Frank and Evans, 1945; Kauzmann, 1959; Némethy and Scheraga, 1962). The calculations of Sinanoglu and Abdulnur (1965) on the role of solvent effects in stabilizing DNA structure offer a potential explanation for the large negative enthalpy of these association reactions. They pointed out that water is not the only ordered solvent, yet it seems to mediate aggregation reactions more powerfully than any other liquid. They calculated the difference in the free energy of stabilization of certain biopolymers in a range of solvents and found that the term responsible for the major difference among the solvents is the free energy required to form a cavity in the solvent to accommodate the solute. The free energy of cavity formation in a liquid is related to the surface tension, which has a uniquely high value for water. An association reaction in solution leads to a decrease in the cavity surface area since two cavities are merged to one of roughly the same total volume but smaller surface-to-volume ratio. The reaction should be accompanied by a release of heat due to the energetic contribution to the surface free energy. Thus this approach predicts a large negative heat for association reactions driven by solvophobic effects. An experiment designed to test this interpretation of the enthalpy change is reported in another paper (Crothers and Ratner, 1968); the conclusion is that this simple argument cannot be applied generally, since it leads to predictions which are qualitatively incorrect for a specific system. Hence the heat released on actinomycin dimerization must come at least partly from other sources. A likely possibility is the energy contribution from dispersion forces.

On the other hand, the utility of solvent surface tension as a rough measure of solvophobic properties is illustrated by its correlation with optical rotatory dispersion data for actinomycin solutions. It is im-

possible to say on the basis of optical rotatory dispersion measurements just what the underlying structural change is. However, since the optical rotatory dispersion changes occur gradually over a wide range of surface tension, we suppose that the structural modification is a minor one. Our present hypothesis is that in solvents of low surface tension the two peptide rings are held less tightly together than in solvents of high surface tension, where the requirement of minimum cavity surface area would be more demanding.

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Thermodynamic Studies of a Model System for Hydrophobic Bonding*

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ABSTRACT: The free energy, heat, and entropy of complex formation between actinomycin and deoxyguanosine are measured in a series of solvent mixtures with various percentages of methanol and water. The complex is destabilized in the presence of increasing methanol concentration, due to an increasingly negative entropy of complex formation. The results favor the standard view of hydrophobic effects as arising from an ordering of solvent molecules around the solute over the description in terms of varying solvent surface tension (Sinanoğlu, O., and Abdulnur, S. (1965), *Federation Proc. 24*, 5).

It has long been recognized that water has special qualities as a solvent which cause it to encourage certain kinds of interactions between the particles of a dissolved substance. Of particular importance for biochemistry is the tendency of water to promote association between uncharged, and often nonpolar, molecules or parts of molecules in solution. These interactions are usually classed as "hydrophobic" (see Kauzmann (1959) for a general discussion) because of the preference for self-interaction rather than exposure to the solvent. Our understanding of the thermodynamic basis for hydrophobic interactions comes primarily from experiments on the solubility of small nonpolar substances in water (Frank and Evans, 1945), which indicate that exposure of these molecules to the solvent leads to an entropy loss resulting from ordering of water molecules in the neighborhood of the solute. This effect is accompanied by a small evolution of heat (analogous to ice formation) in the case of hydrocarbons, and by practically no heat change when aromatic substances are studied. Such interactions are clearly of importance in biochemical systems, as ex-

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