T. J. Greenwalt, Ed., Philadelphia, Pa., Lippincott, p 245.
Shimada, A., and Nathenson, S. G. (1969), *Biochemistry 8*, 4048.
Shimada, A., Yamane, K., and Nathenson, S. G. (1970), *Proc. Nat. Acad. Sci. U. S. 65*, 691.
Shreffler, D. C., David, C. S., Passmore, H. C., and Klein, J. (1971), *Transp. Proc. III*, 176.
Snell, G. D., Cherry, M., and Demant, P. (1971), *Transp. Proc. III*, 183.
Snell, G. D., and Stimpfling, J. H. (1966), *in* Biology of the Laboratory Mouse, Green, E., Ed., New York, N. Y., McGraw-Hill, p 475.
Yamane, K., and Nathenson, S. G. (1970a), *Biochemistry 9*, 1336.
Yamane, K., and Nathenson, S. G. (1970b), *Biochemistry 9*, 4743.

A Proton Magnetic Resonance Study of the Aggregation of Actinomycin D in D_2O†

N. S. Angerman, T. A. Victor,‡ C. L. Bell,§ and S. S. Danyluk*

ABSTRACT: A detailed 220-MHz proton magnetic resonance study has been made of the concentration, pD, salt, and temperature dependence of the actinomycin D spectrum in D_2O. The results confirm that actinomycin D aggregates to form a dimer at the concentration ranges and temperatures covered in this work. Moreover, the measurements show conclusively that the dimer is formed by an interaction between the actinocyl chromophore groups only. Based upon the direction and relative magnitudes of the shift trends for the actinocyl group protons it is further concluded that the actinocyl groups stack vertically in the dimer with one chromophore inverted with respect to the other. An interpretation of the dimer structure and the resultant shift trends is given in terms of the diamagnetic shielding anisotropy of the actinocyl chromophore group. The chemical shift–concentration curves for the actinocyl signals have been analyzed by a least-squares fitting procedure, to obtain dimerization equilibrium constants of 2.70×10^3 M^{-1} and 1.40×10^3 M^{-1} at 4° and 18° (pD = 7.2), respectively. Finally, the nuclear magnetic resonance (nmr) measurements also show that the structure and stability of the dimer are altered by temperature and by solvent properties (pD, ionic strength).

Actinomycin D, an important inhibitor of mRNA synthesis (Reich and Goldberg, 1964), forms relatively stable aggregates in aqueous solution. Initial discrepancies regarding the stoichiometry of the aggregates (Gellert *et al.*, 1965; Müller and Emme, 1965; Berg, 1965) have been resolved recently by careful equilibrium centrifugation measurements which show conclusively that the dimer form predominates at concentrations $>10^{-4}$ M with no detectable formation of higher aggregates, even at concentrations approaching saturation (Crothers *et al.*, 1968). Although the existence of an actinomycin D dimer is, therefore, well established, the orientation of the two actinomycin D molecules in the dimer is still not clear. It has been suggested, based upon optical (Crothers *et al.*, 1968) and hydrodynamic work (Müller and Emme, 1965), that dimer formation results from interaction between the pentapeptide rings; but the possibility of an involvement of the actinocyl chromophore group cannot be ruled out entirely.

Actinomycin D is in many respects an ideal model compound for studying the types of inter- and intramolecular processes likely to occur in much larger biological molecules. Any additional information relating to the conformation of the dimer and the influence of extrinsic factors such as temperature and pH upon the dimerization process would be of considerable interest. With these points in mind we have reinvestigated the aggregation of actinomycin D in aqueous solution using the proton magnetic resonance (pmr) method.

The pmr method has been widely used in studies of solute–solvent and solute–solute interactions of relatively small molecules, *e.g.*, H bonding, π complexing, donor–acceptor interaction (Jackman and Sternhell, 1969). In favorable cases equilibrium parameters can be determined directly from an analysis of chemical shift–concentration curves. Such an approach can be extended in principle to much larger biological molecules, but in practice it is limited by the complexity and extensive overlapping of signals in proton spectra for these molecules.

Several factors, however, favor the use of pmr measurements for studying the aggregation of actinomycin D. Firstly, a detailed assignment of the proton spectrum has been reported at 60 MHz (Victor *et al.*, 1969) and 100 MHz (Arison and Hoogsteen, 1970) in several nonaqueous solvents and a partial assignment has been made in D_2O. Secondly, the likelihood that actinomycin D aggregates in one of two widely different structural orientations permits a general prediction of expected shift changes. For example, if the dimer is formed by intermolecular interaction between the pentapeptide rings then dimerization-induced chemical shift changes would be expected for key groups (*i.e.*, CH_3, methylene) on these rings but no changes would occur for actinocyl group protons. If

† From the Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Illinois 60439. *Received February 4, 1972*. Work supported by the U. S. Atomic Energy Commission.

‡ Department of Molecular Biology, Division of Medicine, Walter Reed Army Institute of Research, Washington, D. C.

§ Supported in part by NIH fellowship (1F03 GM 45084-01); permanent address, University of Illinois at the Medical Center, Chicago, Ill.

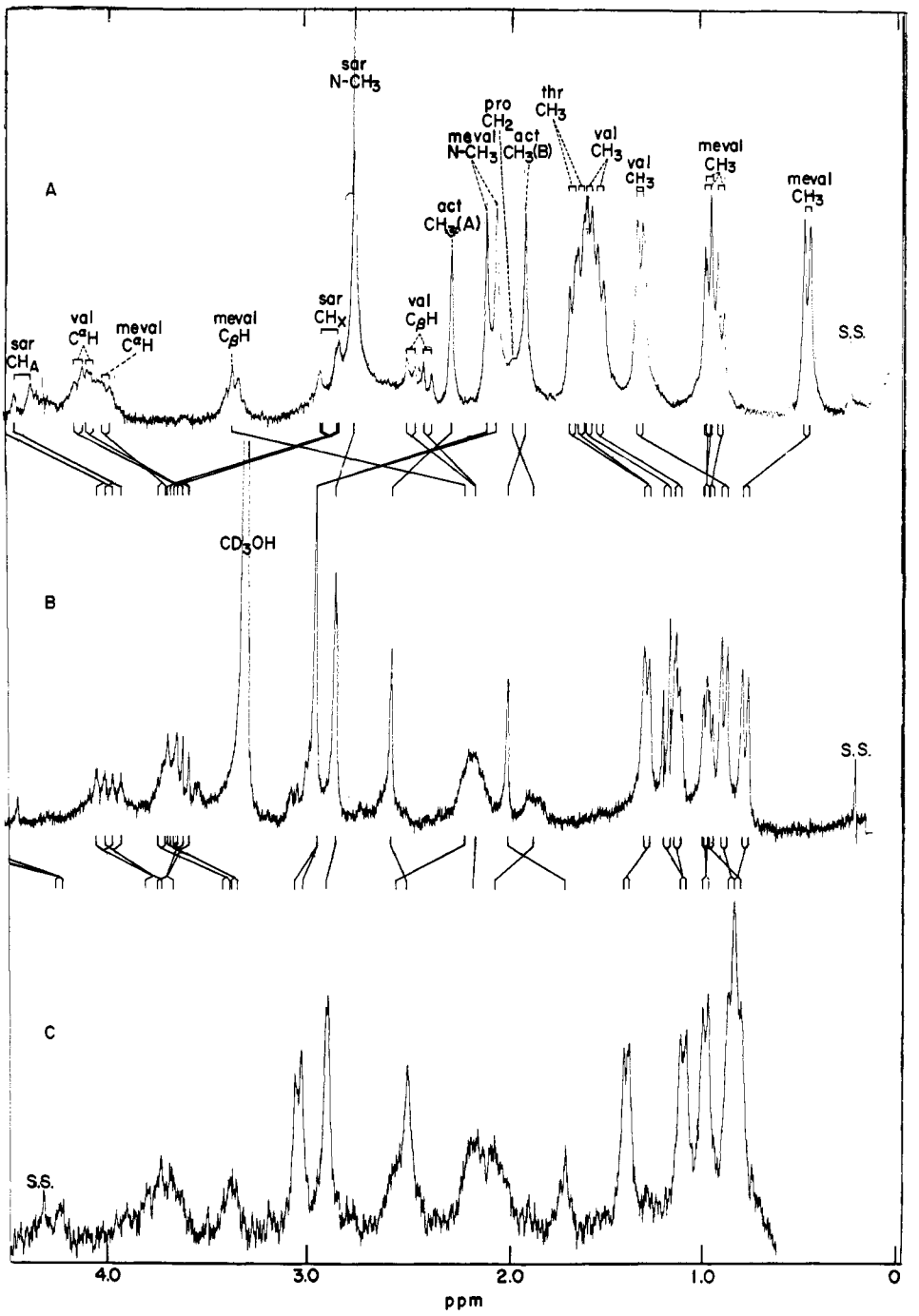

FIGURE 1: High-field region of the 220-MHz proton spectrum of actinomycin D in A, benzene-d_6; B, methanol-d_4; C, D_2O (pD 7.2) at 18°. Note: the structural formula and labeling scheme for actinomycin D is given in Figure 8.

the dimer involves an interaction between the actinoçyl group chromophores then actinocyl group protons would show shift changes while pentapeptide protons would not.

An extensive study was therefore made of the effect of concentration, pH, and temperature upon the proton spectrum of actinomycin D at 220 MHz. The pmr measurements confirm that actinomycin D aggregates to the dimer form only, and dimerization constants derived from the data are in good agreement with values reported using other techniques. Based upon the trends and magnitudes of the dimerization shift changes it is further concluded that the actinomycin D dimer involves a vertical "stacking" interaction of the actinocyl group chromophores with one chromophore inverted with respect to the second. A preliminary account of several aspects of this work has been given elsewhere (Bell *et al.*, 1971).

Experimental Section

Materials. Actinomycin D used in this study was obtained from two sources. One lot was kindly supplied by Merck, Sharpe and Dohme, Rahway, N. J., while a second lot was purchased from Nutritional Biochemicals Corp. The second lot contained up to 5% water. Spectra from both lots were identical and no evidence of impurities (other than water) was apparent. Deuterium oxide (100.0 atom % deuterium) was

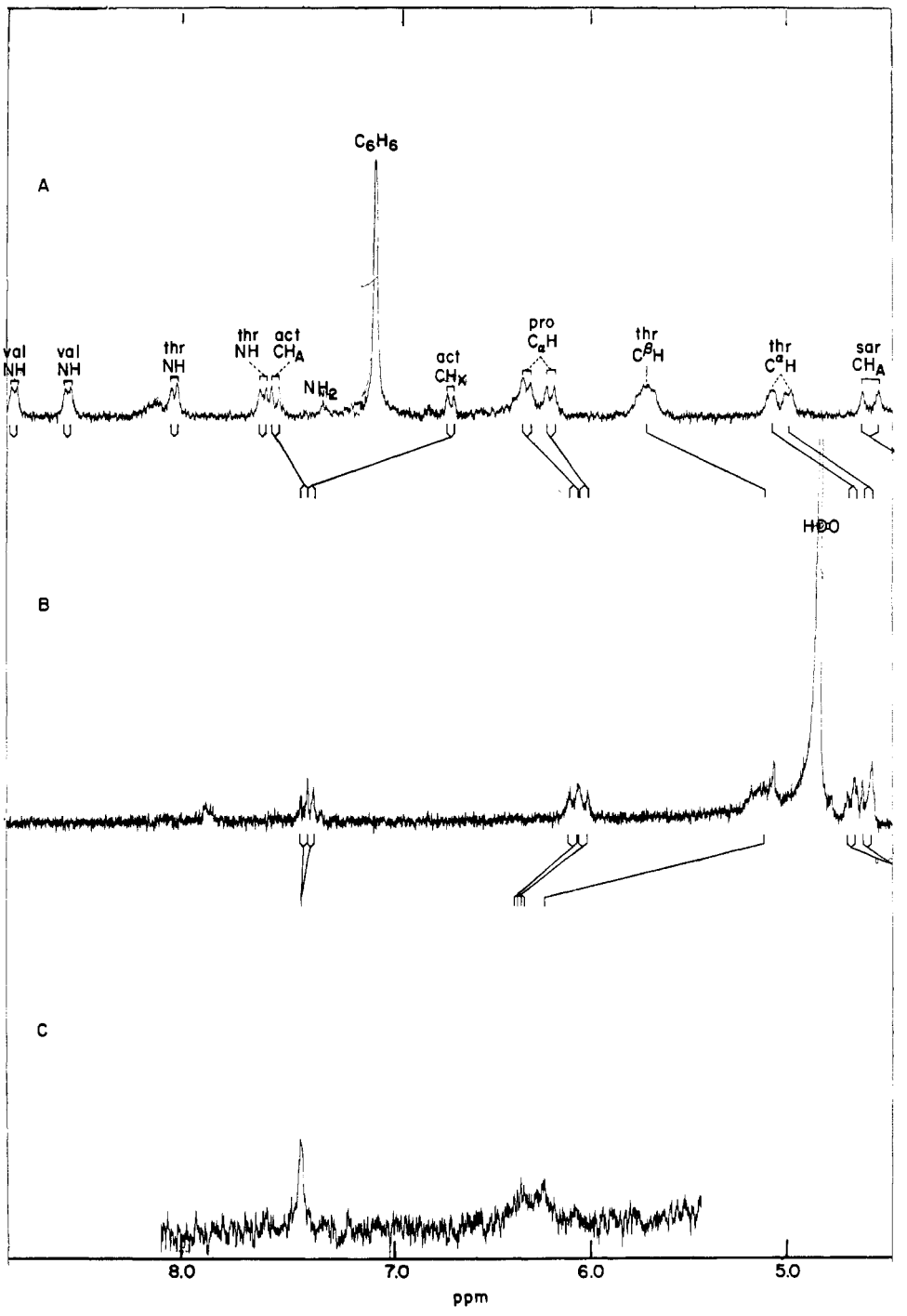

FIGURE 2: Low-field region of the 220-MHz proton spectrum of actinomycin D. Conditions are the same as in Figure 1

purchased from Diaprep Inc. Sodium 3-trimethylsilylpropionate-2,2,3,3-d_4, used as an internal reference, was purchased from Merck, Sharp and Dohme.

Preparation of Solutions. Standard solutions of actinomycin D were prepared by weighing actinomycin D into a Teflon vial and dissolving with a known weight of phosphate-buffered D_2O (phosphate buffer prepared with known amounts of Na_2DPO_4 and NaD_2PO_4 in D_2O). Pmr solutions were then made up by carefully weighing out actinomycin D solutions directly into 5-mm precision-bore spinning tubes and then diluted to 0.5 ml by adding a weighed amount of phosphate-buffered D_2O and sodium 3-trimethylsilylpropionate-2,2,3,3-d_4. pDs[1] were measured for a number of solutions after preparation and no changes were noted upon actinomycin D addition.

Measurement of Spectra. All of the proton spectra were measured at 220 MHz with a Varian 220-MHz spectrometer. In order to maximize the spectral signal-to-noise ratio special care was taken to obtain the best probe null point, and optimum conditions of R_F power and receiver gain. Typically, spectra were recorded at scan rates of 0.4–1 Hz per sec. Signal

[1] pDs are reported in this work and are related to pH by pD = pH (meter) + 0.4 (Glasoe and Long, 1960).

averaging (Varian SS-100 system) was used to improve the signal-to-noise ratio for solutions with concentrations $<10^{-3}$ M.

All of the spectra were calibrated by the audio side-band method using a Hewlett-Packard Model 4204A (10 Hz–1 MHz) digital oscillator and Hewlett-Packard Model 5245L electronic counter. The chemical shifts were determined by interpolation and are accurate to ±2.0 Hz.

Results

Assignment of the Actinomycin D Spectrum in D_2O. A meaningful pmr study of intermolecular interactions between biological molecules such as actinomycin D requires as its starting point a detailed assignment of the pmr spectrum for the molecule, with signals for key groups identified unambiguously. Although assignments have been reported in nonaqueous solvents (Victor *et al.*, 1969; Arison and Hoogsteen, 1970) and partial assignments have been made in D_2O (Victor, 1969, Arison and Hoogsteen, 1970) a more detailed assignment was desirable for the present aggregation study.

A complete assignment of the spectrum in D_2O cannot be made directly from measurements in D_2O alone since one of the key first steps, an identification of the Thr and Val NH signals, is prevented by exchange of these protons with solvent. Therefore, an indirect approach employing measurements in selected binary solvent systems of varying composition was adopted. An initial spectral assignment was made at 220 MHz using benzene-d_6 as solvent, Figures 1A and 2A, and following procedures (deuterium exchange, spin decoupling, etc.) described in the earlier work at 60 MHz. Complete agreement was noted between the assignments at the two frequencies.

In the second step the signals were monitored over a series of binary CD_3OD–C_6D_6 solutions with increasing CD_3OD concentration, and led to an assigned spectrum in neat CD_3OD, Figures 1B and 2B. Finally, the actinomycin D signals were followed in binary CD_3OD–D_2O solutions (with increasing D_2O concentration) until the spectrum was obtained in neat D_2O (Figures 1C and 2C). An assignment of the spectra and a representation of the shift trends for key signal groupings in going from C_6D_6 to D_2O is given in Figures 1 and 2.

As an additional check of the assignment-decoupling experiments were carried out in D_2O for most of the residues with spin-coupled sets of protons. These measurements confirmed the assignments for Val $(CH_3)_2CHCH$, MeVal[2] $(CH_3)_2CHCH$, Thr CH_3CH–, Sar (H_AH_X), Pro $CH_2CH_2CH_2CH$, actinocyl group CH_3(A), and actinocyl group (H_A,H_B) protons.

Figures 1 and 2 show that variation of the solvent produces striking and characteristic shift changes for a number of the protons in actinomycin D. The changes arise in part from a solvent-induced perturbation of the pentapeptide ring conformation, alterations in hydrogen-bonding interactions (between Sar C=O and Val NH), and solvent anisotropy effects. A detailed discussion of the solvent effects is given elsewhere (Victor, 1969). One interesting point is the somewhat greater line widths for signals in D_2O solutions. A line-broadening effect of this sort is commonly observed in pmr spectra of aggregating molecules.

A compilation of chemical shifts for actinomycin D in D_2O is given in Table I.

Concentration Dependence in D_2O. The self-aggregation of solute molecules in aqueous solution generally shows up as a concentration dependence for signals in the pmr spectrum (Jackman and Sternhell, 1969). A detailed 220-MHz study was therefore made of the concentration dependence of the actinomycin D spectrum in D_2O (10^{-4} M to saturation[3]) at 4° and 18° and at a number of different pH values.

An illustration of the concentration dependence for protons in the pentapeptide rings and the corresponding results for the actinocyl group protons are shown in Figure 3. In each case the concentration ranged from approximately 5×10^{-4} M to saturation. Figure 3 shows that no chemical shift changes of any significance occur for the pentapeptide ring protons when the concentration is varied.[4] This behavior is in decided contrast with that observed for the actinocyl group protons. All three sets of the latter proton signals shift *upfield* as the actinomycin D concentration is increased with the trends increasing in the order actinocyl group (H_A,H_B) $<CH_3$(A) $<CH_3$(B). The most marked rate of chemical shift change occurs at the low end of the concentration range, $<10^{-3}$ M, and the rate levels off distinctly as the concentration approaches saturation.

The upfield shift changes are accompanied by a noticeable

TABLE I: Pmr Shifts of Actinomycin D in a Buffered D_2O Solution at 4°.[a]

Proton Type	Shift (ppm[c] ± 0.01 ppm)
MeVal CH_3(1)[b]	0.82
Val CH_3(1)[b]	0.85
MeVal CH_3(2)[b]	0.99
Val CH_3(2)[b]	1.10
Thr CH_3	1.38
Actinocyl group CH_3(B)	1.69
Pro CH_2	2.05
Val $C_\beta H$	2.15
Actinocyl group CH_3(A)	2.49
MeVal $C_\beta H$	2.55
Sar N-CH_3	2.90
MeVal N-CH_3(1)[b]	3.01
MeVal N-CH_3(2)[b]	3.05
Val $C_\alpha H$	3.35
MeVal $C_\alpha H$	3.40
Sar CH_X	3.70
Sar CH_A	3.77
Thr $C_\alpha H$	4.24
Thr $C_\beta H$	6.24
Pro $C_\alpha H$ (1)[b]	6.35
Pro $C_\alpha H$ (2)[b]	6.37
Actinocyl group H_A, H_B	7.47

[a] Concentration of ACD = 3.90×10^{-3} M; μ = 0.40 M. [b] The numbers refer to nonequivalent groups. [c] Shifts are in ppm relative to internal sodium 3-trimethylsilylpropionate-2,2,3,3,-d_4.

[2] Abbreviation used is: MeVal, methylvalyl.

[3] Actinomycin D formed saturated solutions at 4.6×10^{-3} M and 3.0×10^{-3} M at 4° and 18°, respectively.

[4] Although Figure 3 shows the δ–concentration data for the key CH_3 groups only, similar results, *i.e.*, no concentration dependence, were observed for methylene and methine protons of the pentapeptide groups.

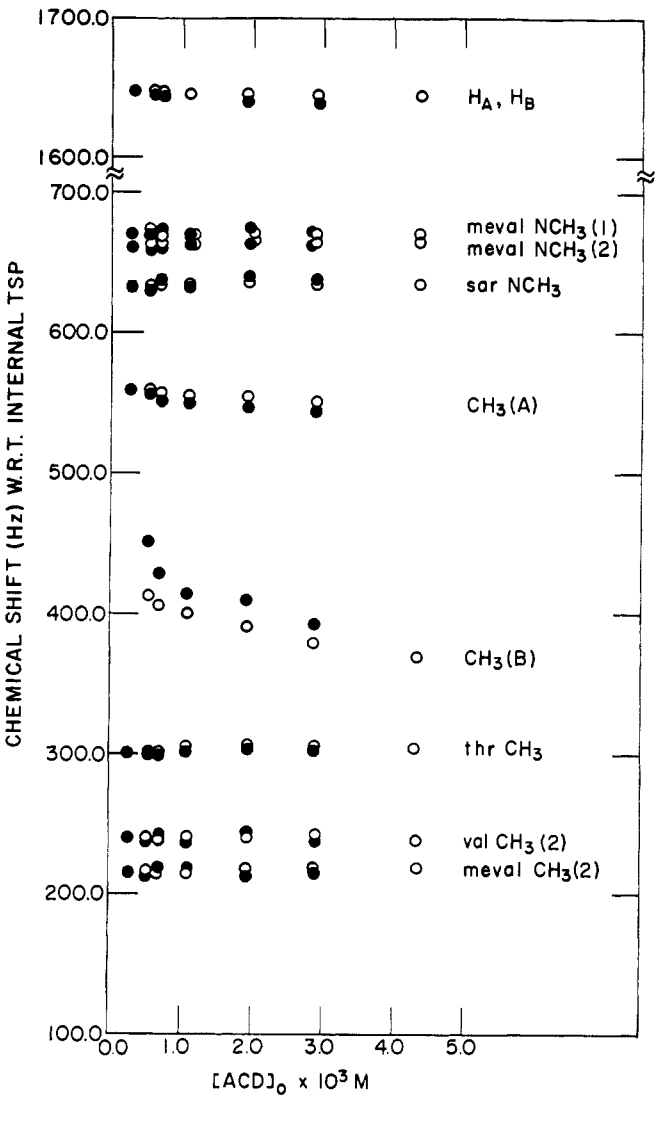

FIGURE 3: Concentration dependence of key groups of actinomycin D in D_2O at 4° (○) and 18° (●); pD 4.7; μ = 0.40 M.

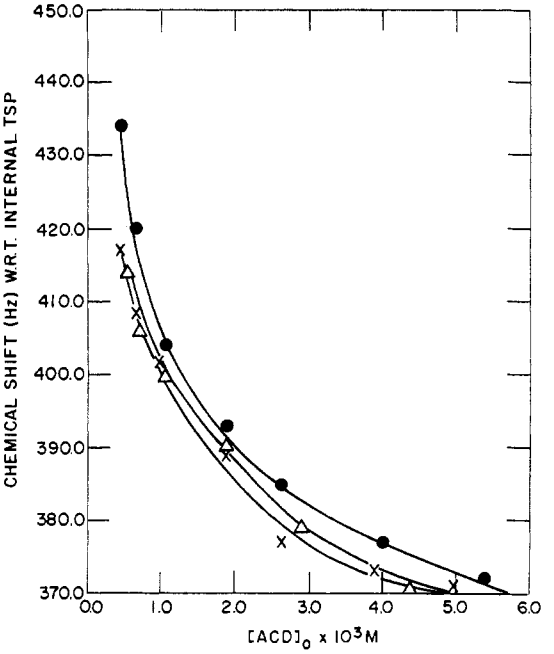

FIGURE 4: Effect of pD upon the concentration dependence of the CH_3(B) protons. ●, pD 7.16; Δ, pD 4.55; ×, pD 8.04; μ = 0.40 M.

line broadening of the actinomycin D signals, indicating that the process responsible for the shift changes also affects the relaxation times of the protons, *e.g.*, by slowing of the motional properties of the actinomycin D molecules, as would occur in an aggregate. Both the actinocyl group chemical shift changes and the line broadening at a given pD and temperature are suggestive of a solute–solute interaction, *i.e.*, aggregation, any other cause being highly unlikely at the concentrations studied. A further indication that the shift changes reflect an aggregation is the rough correlation between the observed chemical shift–concentration changes and M_{app} and $\Delta[a]$ changes (Crothers *et al.*, 1968).

Effect of Temperature. No temperature effect of any significance was found for the pentapeptide ring proton chemical shifts, with the concentration dependences at 4° and 18° being virtually identical, Figure 3. This result differs from the behavior in nonaqueous solvents where sizeable medium- and temperature-induced effects, directly associated with conformational changes of the pentapeptide rings occur.

The actinocyl group protons, in marked contrast, show very definite and individual temperature dependences (Figure 3). Thus, the CH_3(B) signal is displaced very noticeably to *lower* field in going from 4° to 18°, the displacement at 5×10^{-4} M amounting to 10–40 Hz depending upon the pD. For CH_3 (A) and H_A, H_B the trend is in the *opposite direction* with an increase in temperature causing an *upfield* displacement over the entire concentration range. As in the case of the concentration effects the temperature dependence of the actinocyl group shifts can be related to the aggregation equilibrium, and to the orientation of the actinomycin D molecules in the dimer.

pD Effects. A check of a possible pD effect upon the δ–concentration curves was made by measuring the concentration dependence of the actinomycin D spectra at several pDs in the range 4.50–8.04. As with temperature no pD-induced δ changes were noted for pentapeptide protons at either 4° or 18°. For the actinocyl group protons a small but detectable displacement was observed for the δ–concentration curves as illustrated for CH_3(B) at 4° in Figure 4. Although the observed shifts show some scatter, it is evident that the curve at pD 7.16 is displaced slightly to lower field compared with the curves at pD 4.55 and 8.04. Differences were also noted in the solubility of actinomycin D at various pDs with the lowest solubility occurring at pD 4.55.

Addition of electrolyte (phosphate buffer) had a very pronounced effect upon the actinocyl group CH_3(B) and CH_3(A) signals in aqueous solution (Figure 5). In each case the signals are shifted by up to 30 Hz relative to unbuffered solutions.

Calculation of Equilibrium Constants from Chemical Shifts. Chemical shift–concentration data have been used to determine equilibrium constants for a wide variety of molecular interactions including hydrogen-bonding, donor–acceptor complexing, and π-complex formation (Jackman and Sternhell, 1969; Laszlo, 1967). One of the main requirements for this method to be feasible for a particular system is a sufficient chemical shift difference for protons in the free and complexed molecular species, a condition which is fulfilled by the actinocyl protons in actinomycin D.

In the usual procedure an initial assumption is made about the stoichiometry of the interaction, and the resultant theoretical δ–concentration curves, calculated for different values

TABLE II: Parameters Determined from a Nonlinear Least-Squares Analysis of the δ–[Actinomycin D]$_0$ Data at 4° ± 1°.

Proton Type	$K_d \times 10^{-3}$ M^{-1} ± 10%	δ°_A (Hz ± 5 Hz)	$\delta^\circ_{A_2}$ (Hz ± 5 Hz)	pD[a] ± 0.05
CH_3(B)	2.77	493	315	8.00
	2.70	493	321	7.16
	3.00	495	314	4.50
	2.79	451	350	7.16 (unbuffered)
CH_3(A)	2.53	575	535	8.00
	2.80	576	537	7.16
	2.90	570	540	4.50
	2.50	568	537	7.16 (unbuffered)
Actinocyl	2.61	1664	1632	8.00
group	2.66	1662	1634	7.16
(H_A,H_B)	2.74	1657	1634	4.50

[a] Ionic strength = 0.40 M.

of K_d and limiting shifts, are then compared with observed curves until a best fit is obtained. For the actinomycin D system the centrifugation measurements show the dominant process to be a simple dimerization for which the equilibrium can be written as

$$2A \underset{}{\overset{K_d}{\rightleftharpoons}} A_2 \qquad (1)$$

with the equilibrium constant given by

$$K_d = \frac{[A_2]}{[A]^2}\ M^{-1} \qquad (2)$$

where $[A_2]$ and $[A]$ are the molar equilibrium concentrations for the dimer and monomer, respectively. If $[A]_0$ is the initial actinomycin D concentration then eq 2 can be rewritten in the form

$$[A_2] = K_d([A]_0 - 2[A_2])^2 \qquad (3)$$

and solution of the quadratic gives an expression for the molar concentration of the dimer, eq 4[5]

$$[A_2] = \frac{1}{8K_d}\{4K_d[A]_0 + 1 \pm (8K_d[A]_0 + 1)^{1/2}\} \qquad (4)$$

Since the actinomycin D spectrum showed only one set of signals for the actinocyl protons the actinomycin D molecules must be exchanging rapidly (on the pmr time scale) between monomer and dimer forms and the observed chemical shifts are therefore given by

$$\delta_{obsd} = X_A\delta^\circ_A + X_{A_2}\delta^\circ_{A_2} \qquad (5)$$

[5] Only the minus sign is used in eq 4 since plus would not give convergence in the least-squares analysis.

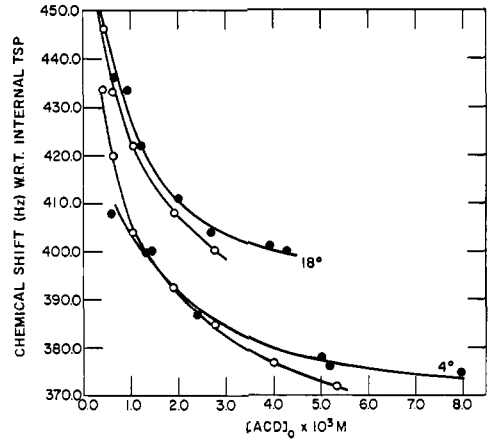

FIGURE 5: Effect of phosphate buffer μ = 0.40 M, upon the CH_3(B) concentration dependence at 4° and 18°, pD 7.16; solid circles represent points for unbuffered solutions: solid curves give the best-fit theoretical curves for a dimerization equilibrium; μ = 0.40 M.

where δ°_A and $\delta^\circ_{A_2}$ are the limiting shifts and X_A, X_{A_2} the equilibrium mole fractions of the monomer and dimer forms. Equation 5 can be rewritten in the form

$$\delta_{obsd} = \frac{[A]_0 - 2[A_2]}{[A]_0 - [A_2]}\delta^\circ_A + \frac{[A_2]}{[A]_0 - [A_2]}\delta^\circ_{A_2} \qquad (6)$$

and in combination with eq 4 can be solved to obtain K_d, δ°_A and $\delta^\circ_{A_2}$ values which fit the observed δ–concentration curve. Calculations were carried out on the IBM 360-75 computer and a nonlinear least-squares program was used to obtain the parameters which gave the best agreement between calculated and observed curves.

A summary of the final calculated K_d, δ°_A and $\delta^\circ_{A_2}$ values at 4° and 18° and at various pDs is given in Tables II and III, while Figures 6A–C illustrate the fit obtained between the observed and calculated δ–concentration curves for the actinocyl group protons.

TABLE III: Parameters Determined from a Nonlinear Least-Squares Analysis of the δ–[Actinomycin D]$_0$ Data at 18° ± 1°.

Proton Type	$K_d \times 10^{-3}$ M^{-1} ± 10%	δ°_A (Hz ± 5 Hz)	$\delta^\circ_{A_2}$ (Hz ± 5 Hz)	pD[a] ± 0.05
CH_3(B)	1.40	490	320	8.04
	1.40	490	320	7.23
	1.60	497	317	4.55
	1.00	473	337	7.16 (unbuffered)
CH_3(A)	1.32	572	512	8.04
	1.31	574	513	7.23
	1.54	568	525	4.55
Actinocyl	1.21	1656	1620	8.04
group	1.39	1663	1621	7.23
(H_A,H_B)	1.59	1653	1625	4.55

[a] Ionic strength = 0.40 M.

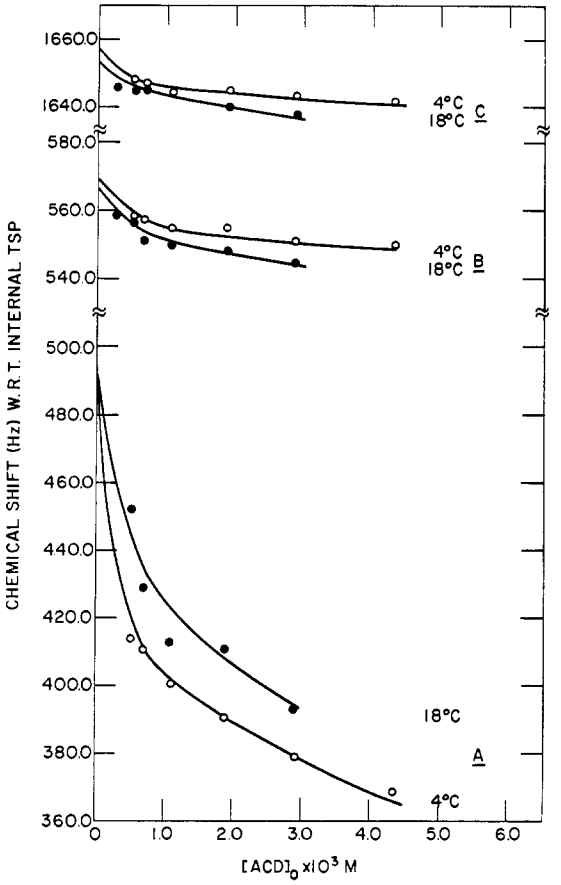

FIGURE 6: Comparison of calculated concentration dependence (solid curves) and observed points; pD 4.55; μ = 0.40 M A, CH_3(B); B, CH_3(A); C, (H_A, H_B).

Discussion

Dimer Equilibrium Constants and Limiting Shifts. The good agreement between observed and calculated δ-concentration curves at all the temperatures and pD values confirms the presence of a dimerization process for actinomycin D in aqueous solutions in the concentration ranges covered. A further check that the shift changes do indeed reflect a dimerization process is made possible by the fact that the actinocyl group gives rise to three separate δ-concentration curves (due to the magnetic nonequivalence of the CH_3(A), CH_3(B), and H_A, H_B protons). Three independent calculations can therefore be made of the equilibrium parameters at a given temperature. The final K_d value should be the same for all three sets of data if the shift changes are directly due to the dimerization. Comparison of the three K_d values at 4°, Table II, shows quite close agreement, yielding a dimerization constant of 2.70 $\times$ 10^3 M^{-1} at pD 7.16. Similar agreement is obtained for the sets of K_d values at 18° giving a K_d of 1.40 $\times$ 10^3 M^{-1} at pD 7.23. These pmr K_d values are surprisingly close to the values derived from centrifugation measurements (3.6 $\times$ 10^3 M^{-1} at 5°, and 9.0 $\times$ 10^2 M at 20° (Crothers *et al.*, 1968); the minor discrepancies probably arising from differences in temperature and pD.

From Tables II and III it is apparent that temperature and ionic strength have a marked influence upon the dimerization while pD has a marginal effect at most. A temperature change can affect the actinomycin D aggregation in at least two ways: the monomer:dimer ratio will change, and the orientation of actinomycin D molecules in the aggregate may alter. Earlier centrifugation measurements (Crothers *et al.*, 1968) showed that aggregation is less favored at higher temperature with the overall process having a relatively large negative enthalpy change. The present pmr results are in agreement with this work with the pmr K_d value increasing by almost twofold in going from 18° to 4°. The pmr measurements also indicate that the structures of the dimers differ slightly at the two temperatures. This conclusion follows from a consideration of the limiting monomer and dimer shifts. Although direct measurement of δ°_A and $\delta^\circ_{A_2}$ is impossible the limiting values can be derived from the analysis of the δ-concentration curves. Within the limits of the computational procedure ($\pm$5 Hz), no changes occur with temperature for any of the limiting monomer shifts of CH_3(A), CH_3(B), and H_A, H_B in buffered solution. This result is expected since the solvation of actinomycin D (monomer) is unlikely to change much in the temperature range covered. In contrast, the limiting dimer shifts show a somewhat different behavior with $\delta^\circ_{A_2}$ of CH_3(B) showing no temperature effect but $\delta^\circ_{A_2}$ of CH_3(A) shifting to lower field (20 Hz) with decreasing temperature. The latter result and the related trends in δ-concentration curves with temperature, Figure 6, point to a small but detectable alteration in the dimer structure as the temperature increases. A qualitative explanation of such structure-induced shift changes can be given in terms of the shielding anisotropy of the actinocyl chromophore (following section).

Although an enthalpy change derived from the pmr data is not strictly valid because the dimer structures differ at the two temperatures, the temperature dependence of the K_d values nevertheless suggests that there is a rather large negative enthalpy for the dimerization. Negative enthalpy changes are particularly characteristic of aggregation processes involving stacking interactions between π electron systems such as purine derivatives and acridine-type dyes (Leng and Felsenfeld, 1966; Brahms *et al.*, 1966; Blears and Danyluk, 1967). The precise origin of these changes is not completely clear, though the unique solvent properties of water, and, to a lesser extent, the dipole character of the solute, appear to be involved.

Further indication of the importance of solvent properties in the aggregation process is provided by the unusual behavior of the equilibrium parameters in going from unbuffered to buffered solutions of similar pD (~7.2), Tables II and III. Addition of electrolyte (phosphate buffer, μ = 0.40 M) to the solution affects not only the monomer:dimer equilibrium but also the limiting shifts for CH_3(B) in both the monomer and dimer. No changes occur for δ°_A, $\delta^\circ_{A_2}$ of CH_3(A).

Salts are known to affect the structures of proteins (Nemethy and Scheraga, 1962) and nucleic acids (Prestegard and Chan, 1969) and the stabilities of biomolecular aggregates (Brahms *et al.*, 1966) in aqueous solution. The salt influence may be direct, *i.e.*, formation of ionic complexes with the biomolecule, or indirect, as would be the case where the salt modifies the solvent structure (Worley and Klotz, 1966). The latter effect would appear to be the dominant factor altering the actinomycin D dimer stability; however, the data are not of sufficient scope to permit a more definite conclusion. Further studies of this aspect of the problem are currently underway.

In addition to an alteration in dimer stability, the pmr results in buffered and unbuffered solutions also suggest the possibility of a weak interaction between the solvated anion and the quinoid ring of the actinocyl group in the monomer and dimer. The evidence for such an interaction lies in the

surprisingly large salt-induced changes in $\delta°_A$ and $\delta°_{A_2}$ of $CH_3(B)$[6] and the temperature dependence of $\delta°_A$(buffered) − $\delta°_A$ (unbuffered) of CH_3(B).

Ionic salts often produce sizeable shift changes for protons on uncharged solutes, particularly in cases where ion–dipole interactions are possible (Hammonds *et al.*, 1969). Since the CH_3(B) region of the quinoid ring is likely to be charge deficient a weak ion–dipole type interaction can occur between the anion and the quinoid ring and would result in a deshielding of the CH_3(B) protons as observed for the monomer. The reasons for a shift in $\delta°_{A_2}$ of CH_3(B) on going to buffered solution are more puzzling. However, this could arise from differences in orientation of the actinomycin D molecules in the dimer in buffered and unbuffered solutions. It is interesting to note that the change in dimer structure produced by a temperature variation differs from that resulting from ion effects; the former affects the CH_3(A) protons predominantly while the latter affects CH_3(B), *cf.* following section.

Actinomycin D does not have any readily ionizable group (*i.e.*, on the chromophore) in the pD range 4.5–8.0 (Cavalieri and Nemchin, 1964) and the lack of any significant changes in the equilibrium parameters is therefore not surprising. The slight shift of the δ–concentration curves and a decrease in actinomycin D solubility at pD = 4.5 are probably due to small differences in solvation properties of the solvent medium.

Structure of the Actinomycin D Dimer. Although several general features of the actinomycin D monomer structure have been derived from pmr measurements in nonaqueous media (Victor *et al.*, 1969; Victor, 1969; Danyluk and Victor, 1970) a more detailed conformational model for actinomycin D in aqueous solutions is lacking.[7] Nevertheless, it is possible to arrive at a realistic model for the dimer structure consistent with the present δ–concentration data. In order to do this it is first useful to consider hypothetical dimers which might form and compare the shift changes which would result for such dimers.

A variety of interactions could give rise to an aggregation of the actinomycin D molecules and out of these Müller and Emme (1965) have summarized the more plausible aggregate structures. Two types appear to be the most likely: the first (i) involves an interaction between the pentapeptide rings only, while the second (ii) involves an interaction between the actinocyl groups with no participation of the pentapeptide rings. The former is typical of interactions between nonpolar residues of oligopeptides and proteins. An interaction of this type is favored in aqueous solution and would lead to a compact clustering of the pentapeptide rings either by intercalation or by a face-to-face alignment. It has been suggested, mainly from the insignificant concentration dependence of the optical spectrum for the actinocyl group chromophore, that the dimer structure is type i (Crothers *et al.*, 1968).

A type ii dimer could result either from a hydrogen-bonding interaction or a stacking interaction between the actinocyl group rings. In the former case the actinocyl group rings would orient in a coplanar arrangement by formation of intermolecular hydrogen bonds between the NH_2 and C=O groups. However, water molecules would exert a destabilizing effect on the hydrogen bonds and a complex of this type is considered to be unlikely in aqueous solution. Alternatively, the actinocyl group chromophores may stack vertically in a manner analogous to that reported for a wide variety of molecules containing delocalized π-ring systems, including purine derivatives and acridines (Chan *et al.*, 1964; Broom *et al.*, 1967; Blears and Danyluk, 1967). Such stacking interactions are generally strongly favored in aqueous solution. Optical studies on simple actinomycin analogs show changes characteristic of stacking interactions for a number of these compounds, though actinomycin D itself does not (Müller and Crothers, 1968).

A choice between type i and ii dimer conformation can be made from observed δ–concentration curves as follows. For a type i dimer a variation in actinomycin D concentration would produce shift changes for pentapeptide ring protons but no change in actinocyl group shifts. A type ii dimer, in contrast, would give rise to actinocyl group shift changes only. Moreover, based on observations on nucleotides and acridines a stacked dimer specifically would lead to *upfield* shifts for the actinocyl group signals with *increasing* actinomycin D concentration. The present δ–concentration data, thus, very definitely support the conclusion that actinomycin D forms a stacked dimer in aqueous solution. No other interaction can account for the upfield shift trends with concentration in the range covered.

The absence of any significant concentration, pD, or temperature effects upon the pentapeptide proton shifts is surprising and tends to suggest little if any influence of the stacking interaction upon the pentapeptide ring conformation; *i.e.*, a stacking of the chromophore is not accompanied by intermolecular interring hydrogen bonding or hydrophobic interactions between the pentapeptide rings. This contrasts with the behavior of actinomycin D and guanine nucleotides (Danyluk and Victor, 1970). Here formation of a stacked π complex between the actinocyl group and guanine rings is paralleled by an interaction (hydrogen bonding) between the ribose ring and the pentapeptide rings. Unfavorable stereochemical orientations may prevent such an interaction in the actinomycin D dimer.

The apparent inconsistency between the pmr and optical results may be partly explained by differences in the extent of π-electron overlap in the stacked actinomycin D dimer and in simpler actinocyl analogs. Steric hindrance by the bulky pentapeptide groups would limit such π overlap, thereby moderating the hypochromicity due to stacking of the actinomycin D. Characteristic pmr stacking shifts would still be noticeable in both systems[8] because the shielding regions extend far beyond the periphery of the actinocyl ring system.

Orientation of the Actinocyl Groups in the Dimer. A more detailed model for the orientation of the actinocyl rings in the dimer can be derived from a consideration of the shielding anisotropy of act. Although an accurate quantitative calculation of the shielding values for the entire act ring system is presently a very lengthy calculation, it is nevertheless possible to deduce a satisfactory qualitative shielding model based on a few simple considerations.

The actinocyl group chromophore is comprised of a phenoxazinone ring skeleton with two main ring subsystems of interest; a *benzenoid* ring with π electron delocalization characteristic of simple aromatic rings and a *quinoid* ring with a more localized π distribution typical of quinones. Since the three ring subsystems are unlikely to be coplanar (based on pmr results on the precursor, phenoxazine (N. Angerman and

[6] Preliminary measurements further show that $\delta°_A CH_3$(B) varies with the type of anion, *i.e.*, Cl^-, oxalate, etc.

[7] A crystal structure determination of an actinomycin D–deoxyguanosine complex has been reported very recently (Sobell *et al.*, 1971).

[8] Preliminary pmr measurements of several actinocyl derivatives show upfield shifts of CH_3(A) and CH_3(B) with increasing concentration.

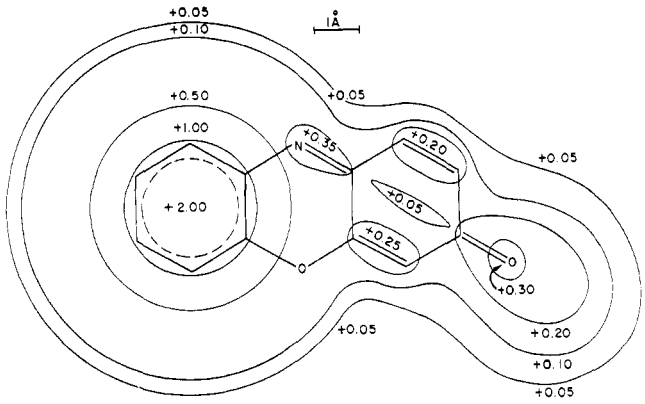

FIGURE 7: Shielding anisotropy diagram for the actinocyl chromophore group for a distance 3 Å above the plane of the actinomycin D chromophore.

S. S. Danyluk, 1972, unpublished results)), little, if any, conjugation is expected between the π systems of the benzenoid and quinoid rings. If it is assumed that the isoshielding values for the benzenoid ring are similar to those in simple benzenes and the shielding in the quinoid ring is more typical of localized C=C and C=O bonds then, using reported isoshielding values for these groups (Jackman and Sternhell, 1969) and summing up the contributions over the entire chromophore, an overall shielding map shown in Figure 7 can be constructed. On this map isoshielding lines are drawn for a horizontal slice located 3 Å above the plane of the chromophore. Although numerical values for the shieldings in Figure 7 have a limited quantitative significance the general features and qualitative shielding trends are likely to be as shown.

The shielding model brings out several features having a bearing on the dimer orientation. Firstly, the shielding over the benzenoid ring is much higher than over the quinoid ring, a situation very similar to that calculated theoretically for isoalloxazines (Giessner-Prettre and Pullman, 1970). Secondly, the shielding is highest over the midpoint of the benzenoid ring and drops off uniformly with increasing distance from the midpoint of the ring. In contrast, the shielding over the quinoid ring is lower in the central region of the ring (a consequence of the localized nature of the C=C and C=O bonds) then increases toward the periphery and finally drops off rapidly with increasing distance from the ring.

The large shielding differences between the benzenoid and quinoid rings make it possible to distinguish between the two most likely stacked actinocyl group orientations;[9] the *symmetric* form in which the two actinocyls are stacked in a face-to-face orientation (benzene–benzene and quinone–quinone), and the *inverted* form where the actinocyls are flipped with respect to each other (benzene–quinone, benzene–quinone). A consideration of Figure 7 shows that the symmetric dimer would produce the largest upfield shift for the CH_3(A) and H_A, H_B protons and only a small change for CH_3(B). A reversal of this behavior would be shown by the inverted dimer with the largest upfield shift occurring for CH_3(B). From the observed δ-concentration curves and the $\delta^\circ_{A_2} - \delta^\circ_A$ values obtained in Tables II and III it is clear that the largest changes occur for CH_3(B). It can, therefore, be concluded that the dimer exists in an inverted stacked form, a schematic representation of which is given in Figure 8.

[9] Both of these orientations have the largest degree of π-electron overlap, one of the essential conditions for stacking.

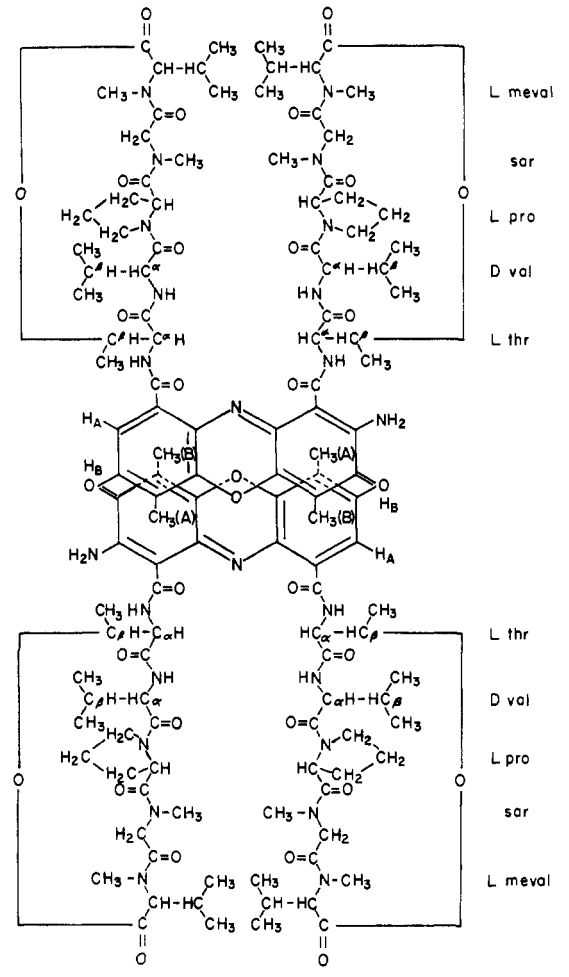

FIGURE 8: Representation of the actinomycin D dimer structure.

An inverted dimer also accounts for the unusual trends in the δ-concentration curves with increasing temperature, *i.e.*, the increased shielding of CH_3(A) and the decreased shielding of CH_3(B). If the dimer structures were identical at 4° and 18° then an increase in temperature should displace *both* δ-concentration curves to lower field reflecting the decreased K_d at 18°. The opposite behavior of CH_3(A) cannot result from a change in the monomer:dimer ratio and, therefore, must be attributed to differences in dimer structure at 4° and 18°, a conclusion also supported by the temperature variation of $\delta^\circ_{A_2}$ for CH_3(A). Specifically, the increased thermal motion at 18° would cause a slight reduction in π-electron overlap and a consequent loosening (lateral and vertical displacement) of the actinocyl groups. A lateral displacement would lead to an increased shielding of CH_3(A) in agreement with the observed trend.

Acknowledgments

Grateful acknowledgement is made to Merck Sharp and Dohme, Rahway, N. J., for providing samples of actinomycin D used in this work. We also wish to thank Mr. Ward Rice for assistance in some of the measurements and calculations.

References

Arison, B. H., and Hoogsteen, K. (1970), *Biochemistry 9*, 3976.

Bell, C. L., Victor, T. A., and Danyluk, S. S. (1971), Biophysical Society Abstracts, 15th Annual Meeting, Feb 15–18, New Orleans, La., p 88a.
Berg, H. (1965), *J. Electroanal. Chem. 10*, 371.
Blears, D. J., and Danyluk, S. S. (1967), *J. Amer. Chem. Soc. 89*, 21.
Brahms, J., Michelson, A. M., and Van Holde, K. E. (1966), *J. Mol. Biol. 15*, 467.
Broom, A. D., Schweizer, M. P., and Ts'o, P. O. P. (1967), *J. Amer. Chem. Soc. 89*, 3612.
Cavalieri, L., and Nemchin, R. (1964), *Biochim. Biophys. Acta 87*, 641.
Chan, S. I., Schweizer, M. P., Ts'o, P. O. P., and Helmkamp, G. K. (1964), *J. Amer. Chem. Soc. 86*, 4182.
Crothers, D. M., Sabol, S. L., Ratner, D. I., and Müller, W. (1968), *Biochemistry 5*, 1817.
Danyluk, S. S., and Victor, T. A. (1970), Quantum Aspects of Heterocyclic Compounds in Chemistry and Biochemistry, Pullman, B., and Bergmann, E. D., Ed., Academic Press, p 394.
Gellert, M., Smith, C. E., Neville, D., and Felsenfeld, G. (1965), *J. Mol. Biol. 11*, 445.
Giessner-Prettre, C., and Pullman, P. (1970), *J. Theor. Biol. 27*, 87.
Glasoe, P. K., and Long, F. A. (1960), *J. Phys. Chem. 64*, 188.
Hammonds, C. N., Westmoreland, T. D., and Day, M. C. (1969), *J. Phys. Chem. 73*, 4374.
Jackman, L. M., and Sternhell, S. (1969), Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry, New York, N. Y., Pergamon Press.
Laszlo, P. (1967), Progress in Nuclear Magnetic Resonance Spectroscopy, Emsley, J. W., Feeney, J., and Sutcliffe, L. H., Ed., Vol. 3, New York, N. Y., Pergamon Press, p 231.
Leng, M., and Felsenfeld, G. (1966), *J. Mol. Biol. 15*, 455.
Müller, W., and Crothers, D. M. (1968), *J. Mol. Biol. 35*, 251.
Müller, W., and Emme, I. (1965), *Z. Naturforsch. B 20*, 834.
Nemethy, G., and Scheraga, H. A. (1962), *J. Phys. Chem. 66*, 1773.
Prestegard, J. H., and Chan, S. I. (1969), *J. Amer. Chem. Soc. 91*, 2843.
Reich, E., and Goldberg, I. H. (1964), *Progr. Nucl. Acid Res. 3*, 183.
Sobell, H. M., Jain, S. C., Sakore, T. D., and Nordman, C. E. (1971), *Nature (London) 231*, 200.
Victor, T. A. (1969), Structure and Interactions of Actinomycin D, Ph.D. Thesis, University of Illinois at the Medical Center, Chicago, Ill.
Victor, T. A., Hruska, F. E., Bell, C. L., and Danyluk, S. S. (1969), *Tetrahedron Lett. 53*, 4721.
Worley, J. D., and Klotz, I. M. (1966), *J. Chem. Phys. 45*, 2868.

Biosynthesis of Methylheptadecanes in *Anabaena variabilis*. *In Vitro* Incorporation of *S*-[*methyl*-^{14}C]Adenosylmethionine†

Steven W. G. Fehler and Robley J. Light*

ABSTRACT: Sonication of lysozyme-treated *Anabaena variabilis* cells yielded a cell-free extract which catalyzed the incorporation of the methyl group from *S*-[*methyl*-^{14}C]adenosylmethionine into the branched methylheptadecanes. The apparent K_m for *S*-adenosylmethionine was 1.1×10^{-4} M. The pH optimum was 7.0, and a partial dependence on NADPH could be demonstrated in a short-term dialysis experiment. Activity was inhibited markedly by 10^{-3} M Cu^{2+}, 10^{-3} M Zn^{2+}, 10^{-3} M EDTA, 10^{-3} M dithiothreitol, and by 0.1% solutions of the detergents Triton X-100, sodium deoxycholate, sodium dodecyl sulfate, and celylpyridinium chloride.

In vivo incorporation studies have established that [*methyl*-^{14}C]methionine serves as a radioactive precursor to the methyl-branched alkanes of blue-green algae (Han *et al.*, 1969; Fehler and Light, 1970). Mass spectrometry of the branched alkanes from *Nostoc muscorum* established their structure as a 50:50 mixture of 7- and 8-methylheptadecane by comparison with authentic synthetic material (Han *et al.*, 1968). More recently, gas–liquid chromatography (glc) on a highly efficient 750 ft × 0.02 in. column established the presence of about 10% of 6-methylheptadecane in this mixture (Han and Calvin, 1970). The mass spectrum of the methylheptadecane mixture from *Anabaena variabilis* appears identical with that from *N. muscorum* (Fehler and Light, 1970). *In vivo* incorporation studies with [*methyl*-$^2H_3$]methionine in *A. variabilis* established that the methyl group is incorporated specifically into the branched methyl group of the methylheptadecanes, and that all three deuterium atoms are retained in the transfer.

This report describes a crude cell-free system from *A. variabilis* which catalyzes the incorporation of the methyl group from *S*-[*methyl*-^{14}C]adenosylmethionine into the methylheptadecanes.

Experimental Section

Anabaena variabilis cultures were grown as described previously (Fehler and Light, 1970), except that 4.5 l. of medium was contained in the glass tubes, and cells were harvested at

† From the Department of Chemistry, Florida State University, Tallahassee, Florida 32306. *Received January 20, 1972.* Supported by Grants GB 8325 and GB 26325 from the National Science Foundation and by a Career Development award (to R. J. L.), K3-AM-11,762, from the Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service.