Thermodynamics of Chelation by Tetracyclines

By LESLIE Z. BENET* and JERE E. GOYAN

Thermodynamic data were determined for the complexation of five tetracycline analogs with cupric ion. Free energy values were calculated from data previously published by the authors. Enthalpy determinations were made in a Dewar calorimeter. Entropy values were determined from ΔG° and ΔH° at 25°. For the formation of 1:1 complexes, ΔH° was approximately −12 Kcal./mole and ΔS° was approximately −4 e.u. for the three chlor-analogs studied (4-epi-chlor-, demethylchlor-, and chlortetracycline), while a ΔH° of −7.6 Kcal./mole and a ΔS° of +10.2 e.u. was obtained for tetracycline. It is suggested that all of the analogs studied form inner sphere 1:1 complexes with cupric ion but that the chlorine at C-7 compels the three chlor-derivatives to undergo a more severe conformational change than tetracycline in order to reach a favorable configuration for chelation. All of the above analogs showed a small negative enthalpy change and a large positive entropy change for 2:1 complex formation. It is suggested that all four analogs form outer sphere or ion-pair complexes during 2:1 complexation.

IN A PREVIOUS paper (1), the authors calculated the thermodynamic dissociation and stoichiometric stability constants for five tetracycline analogs with cupric ion. In light of the experimental evidence presented, it was felt that all five analogs investigated, tetracycline·HCl, chlortetracycline·HCl, demethylchlortetracycline·HCl, 4-epi-chlortetracycline·HCl, and 4-epi-anhydrotetracycline·HCl, formed 2:1 (ligand–metal) complexes with cupric ions. The present study was undertaken to further elucidate the thermodynamic relations involved in this complexation.

In order to reach accurate conclusions regarding the nature of the forces operating within complexes during their formation in solution, it is necessary to know the energy changes accompanying the reactions in question. Stability constants are related directly only to the change in free energy.[1]

$$\Delta G^\circ = -2.303\ RT \log \beta_i \qquad \text{(Eq. 1)}$$

Where β_i is the over-all stability constant. The values for the change in free energy for the tetracycline chelations studied may be calculated from the data presented in *Reference 1*.

A knowledge of the entropy change during the complexation reaction allows the investigator to estimate the effects of certain steric factors which may be involved in complex bonding. In order to calculate the entropy of complexation, the enthalpy of complexation must also be determined as seen in Eq. 2.

$$\Delta S^\circ = (\Delta H^\circ - \Delta G^\circ)/T \qquad \text{(Eq. 2)}$$

The enthalpy of complexation was determined by direct calorimetry. Although this method requires more experimentation than the determination of enthalpy by the variation of stability constants with temperature, the accuracy gained in the calorimetric method justifies the work expended, especially in this study where the maximum heat evolved for any one measurement was only 0.5 cal. Rossotti (2) presents an enlightening discussion as to the accuracy of the two methods.

EXPERIMENTAL

Materials.—Tetracycline · HCl, chlortetracycline · HCl, demethylchlortetracycline · HCl, 4-epi-chlortetracycline · HCl, and 4-epi-anhydrotetracycline · HCl were donated by Lederle Laboratories. All solutions were prepared as described in *Reference 1*.

Calculation of Dissociation and Stability Constants.—Dissociation constants and concentration of tetracycline present were determined by the method of nonlogarithmic titration curves (1, 3).

Received April 25, 1966, from the School of Pharmacy, University of California, San Francisco.

Accepted for publication July 13, 1966.

Presented to the Basic Pharmaceutics Section, A.PH.A. Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.

This investigation was supported in part by training grant 5 TO1 GM 0728 05 from the Division of General Medical Sciences, National Institutes of Health, U.S. Public Health Service, Bethesda, Md.

* Fellow of the American Foundation for Pharmaceutical Education, 1963–1965. Present address: College of Pharmacy, Washington State University, Pullman. 99163.

[1] The choice of the standard state is of great importance in determining ΔG°, ΔH°, and ΔS°. Many authors choose as the standard state a hypothetical 1 molal solution with the properties of an infinitely dilute solution. This choice of standard state eliminates the effect of ionic strength and the ambiguity which arises when comparing values with other work. However, this advantage is usually outweighed by the inaccuracy inherent in the extrapolation of data to values at zero ionic strength. In recent years, therefore, use has been made of a less hypothetical molar solution of a given ionic strength (μ = constant) at 25°. This choice of standard state was used in this work. However, it must be pointed out that all determinations, calculations, and comparisons of thermodynamic functions must be made with reference to the same standard state.

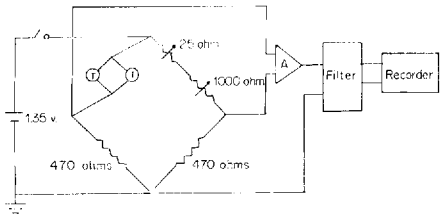

Fig. 1.—Circuit diagram for thermistor bridge. Key: T, 1000-ohm thermistor; A, amplifier.

Stability constants were determined as described in *Reference 1*. All determinations were carried out at an ionic strength of 0.01.

Calorimetry.—The design of the calorimeter was influenced by the desire to measure the heat of complexation for the same reactions from which the stability constants were determined. The work of Atkinson and Bauman (4, 5) was of special interest since they had used very dilute solutions. Therefore, modifications were made on a basic Wheatstone bridge in order to improve on the accuracy which they reported. A circuit diagram of the "thermometer circuit" is shown in Fig. 1. The bridge was driven by a Mallory 1.35-v. mercury battery (RM-12-R) which could be switched in and out of the circuit. Two arms of the bridge were fixed with matched 470-ohm, 0.5-w. carbon resistors. The temperature-sensing arm contained two 1000-ohm thermistors (Fenwall GB 31 P2) connected in parallel. The fourth arm contained two 10-turn wire wound potentiometers in series, the first a 1000-ohm potentiometer and the second a 25-ohm potentiometer. These potentiometers were used to zero the voltage output of the bridge at the beginning of a titration run. The voltage output of the bridge then goes to a model 1755 low-noise d.c. instrumentation amplifier[2] which is operated as a differential amplifier. The voltage output of the amplifier then passes through a model 7500 d.c. active filter[2] which attenuated all signal frequencies above 0.1 c.p.s. The voltage output was then fed into a Varian model G-10 graphic recorder.[3]

The calorimeter was constructed with a Dewar flask, based on the design proposed by Schlyter (6) as modified by Bauman (5), and was equipped with a thermostated buret for enthalpy titrations. The buret device was constructed from a microburet identical with the one utilized in the potentiometric procedures (1, 3). The entire device holds approximately 1.7 ml. of titrant, of which 1 ml. may be delivered to the solution. A micro-heater was utilized to calibrate the Dewar flask and its contents (7). The procedure was as follows.

The solutions to be titrated were placed in the Dewar flask, the pipet was filled with titrant, the calorimeter was closed and placed in a constant-temperature bath maintained at 25.00 ± 0.005°. The solution was either cooled or heated as was required to bring the temperature of the solution to 25°. At this time the bridge was balanced and a constant drift rate was established on the recorder. Heat was added by applying a voltage across the known resistance of the heater for a period of time which was electronically recorded (7). A steady drift rate is again obtained and the number of spaces between the two drift lines is measured. The heat sensitivity per space (C_s) can then be calculated from Eq. 3.

$$C_s = \frac{E^2 t}{(4.1840 \text{ joules/cal.})\, R\, (\Delta \text{ spaces})} \qquad \text{(Eq. 3)}$$

where C_s = calories per space,
E = voltage across heater,
R = heater resistance, 120.39 ohms,
t = heating time in sec.,
Δ spaces = number of spaces drift curve is displaced.

In the beginning of the calorimetric work, electrical calibrations were made before and after each addition of titrant. The average of the two calibrations was then taken as the C_s for that particular addition of titrant. However, when following this procedure, the time spent in joule calibrations in any one run was well over an hour, which was undesirable since epimerization takes place during the titration. Therefore, the average values of C_s for 49 calibrations with 158-ml. volume and 25 calibrations with 153-ml. volume were calculated.[4]

$C_s = (1.551 \pm 0.039) \times 10^{-2}$ cal./space for 158 ml.

$C_s = (1.504 \pm 0.037) \times 10^{-2}$ cal./space for 153 ml.

During the course of the work sample determinations of C_s were run to ascertain that the calculated values had not changed.

Most titrations were carried out with a total volume change of less than 0.6 ml.; therefore, it was felt that any change in C_s due to volume change would be imperceptible considering the size of the standard deviation. All joule calibrations were made on solutions with $\mu = 0.01$, so that it was assumed that C_s was the same no matter which tetracycline was being titrated.

To test the reliability of the calorimeter, the heat of neutralization of HCl by KOH was examined. The average of 27 determinations at 0.01 N HCl gave a value of 13.95 ± 0.46 Kcal./mole, which when corrected to infinite dilution (8) was 13.76 ± 0.46 Kcal./mole. Because the range was slightly higher than those values reported by calorimetry for very dilute solutions (9), all the parameters used in the determination were carefully rechecked. Since the heat of neutralization of hydroxide ions is used in the enthalpy determinations to be presented, it was decided to use the value obtained in this calorimeter, since any systematic error would then be carried through all of the calculations.

For each addition of titrant, the heat evolved, Q, in cal./L., is given by Eq. 4.

$$Q = \frac{C_s\,(\Delta \text{ spaces}) - (\text{heat of dilution of KOH titrant})}{\text{total vol., L.}} \qquad \text{(Eq. 4)}$$

The heat of dilution for 1 ml. of 0.4405 M KOH added to 158 ml. of ionic strength 0.01 was cal-

[2] California Electronic Mfg. Co., Inc., Alamo, Calif.
[3] Varian Associates, Palo Alto, Calif.

[4] Calibrations were necessary at both of these volumes, since all calorimetric determinations were carried out under identical conditions as the potentiometric determinations for the stability constants, which were carried out at both of these volumes.

culated from the values tabulated by Rossini (8) and found to be 7.3×10^{-2} cal./ml. of titrant.

The heat evolved in the titration of a solution of tetracycline·HCl and cupric ions is the sum of the heats evolved in the five reactions listed below.

$H_3T^+ + OH^- \rightleftharpoons H_2T + H_2O \quad \Delta H^\circ_1$ Reaction *1*

$H_2T + OH^- \rightleftharpoons HT^- + H_2O \quad \Delta H^\circ_2$ Reaction *2*

$HT^- + Cu^{++} \rightleftharpoons Cu(HT)^+ \quad \Delta H^\circ_3$ Reaction *3*

$HT^- + Cu(HT)^+ \rightleftharpoons Cu(HT)_2 \quad \Delta H^\circ_4$ Reaction *4*

$H^+ + OH^- \rightleftharpoons H_2O \quad \Delta H^\circ_5$ Reaction *5*

If $\Delta[\ ]$ is defined as the concentration initially minus the concentration after titrant (base) is added, and noting that multiplying the enthalpy for each reaction times the change in one of the reactants gives the heat evolved by this reaction, we can write Eq. 5.

$$Q = \Delta[H_3T]\,(\Delta H^\circ_1) - \{\Delta[HT^-] + \Delta[Cu(HT)^+] + \Delta[Cu(HT)_2]\}\,(\Delta H^\circ_2) - \{\Delta[Cu(HT)^+] + \Delta[Cu(HT)_2]\}\,(\Delta H^\circ_3) - \Delta[Cu(HT)_2]\,(\Delta H^\circ_4) + \Delta[H^+]\,(\Delta H^\circ_5) \quad \text{(Eq. 5)}$$

Before the values of interest in this work ΔH°_3 and ΔH°_4 can be calculated, the heats of reaction ΔH°_1 and ΔH°_2 must be determined. These heats can be found by measuring the heat of reaction for the titration of a pure tetracycline·HCl with base. This was done for each of the five tetracycline analogs studied here. The calorimeter was filled with a solution of the particular tetracycline hydrochloride. Simultaneously an exact duplicate of the solution in the calorimeter was titrated potentiometrically. To obtain a larger number of values for ΔH°_1 and ΔH°_2 the concentrations of the tetracycline analogs used in the titrations without metal were about 3 times as great as those used for the work reported in *Reference 1*. This procedure gave good values for all the tetracyclines except 4-epianhydro-, which is considerably less soluble than the other four derivatives.

The heat of reaction for Reactions *1* and *2* may be determined from Eq. 6.

$$Q = \Delta[H_3T^+]\,(\Delta H^\circ_1) - \Delta[HT^-]\,(\Delta H^\circ_2) + \Delta[H^+]\,(\Delta H^\circ_5) \quad \text{(Eq. 6)}$$

From the parallel potentiometric titration, $[H^+]$, $[H_3T^+]$, and $[HT^-]$ may be determined as described in *Reference 1*. Since the first and second dissociation constants for the tetracyclines are widely separated, $[HT^-]$ will be negligible at high hydrogen-ion concentrations and ΔH°_1 may be determined. At lower $[H^+]$, ΔH°_2 may be determined.

The Q values were calculated from the experimental data by use of Eq. 4. A sample determination for ΔH°_1 and ΔH°_2 is given in Table I for tetracycline·HCl. Due to overlap of the second and third dissociation constants, there are fewer determinations for ΔH°_2 and consequently these answers are not as accurate as those for ΔH°_1.

Once ΔH°_1 and ΔH°_2 are known, it is necessary to run two simultaneous titrations of tetracycline with metal—calorimetrically and potentiometrically.

TABLE I.—DETERMINATION OF ΔH°_1 AND ΔH°_2 FOR 153 ml. OF SOLUTION OF TETRACYCLINE·HCl, INITIAL CONCENTRATION (T°) = 14.32×10^{-4}, USING STOICHIOMETRIC DISSOCIATION CONSTANTS $K_1^c = 4.61 \times 10^{-4}$ AND $K_2^c = 2.48 \times 10^{-8}$

Total KOH Added, ml.	$[H^+]$	$[H_3T^+]$	$[HT^-]$	Q cal./L.	ΔH°_1 Kcal./mole	ΔH°_2 Kcal./mole
0.03	5.71×10^{-4}	7.91×10^{-4}	Negligible	...	...	...
0.11	4.84×10^{-4}	7.49×10^{-4}	Negligible	−3.22	−12.2	...
0.19	3.16×10^{-4}	5.84×10^{-4}	Negligible	−2.87	−12.5	...
0.27	2.136×10^{-4}	4.54×10^{-4}	Negligible	−2.89	−11.3	...
0.35	1.27×10^{-4}	3.10×10^{-4}	Negligible	−2.90	−11.7	...
0.47	2.38×10^{-5}	7.03×10^{-5}	1.41×10^{-5}	−4.21	−11.7	...
0.55	2.58×10^{-7}	7.30×10^{-7}	1.25×10^{-4}	...	...	...
0.63	7.07×10^{-8}	1.62×10^{-7}	3.72×10^{-4}	−1.42	...	−5.7
0.75	2.50×10^{-8}	Negligible	7.14×10^{-4}	−1.97	...	−5.7
					−11.9 Av.	−5.7 Av.

TABLE II.—DETERMINATION OF ΔH°_3 AND ΔH°_4 FOR 153 ml. OF SOLUTION OF TETRACYCLINE·HCl, INITIAL CONCENTRATION (T°) = 4.81×10^{-4} M, INITIAL CONCENTRATION OF ADDED HCl (A°) = 3.97×10^{-4}, INITIAL CONCENTRATION OF CUPRIC CHLORIDE (M°) = 2.45×10^{-4} M

Total KOH Added, ml.	$[H^+]$	$[H_3T^+]$	$[HT^-]$	$[Cu(HT)^+]$	$[Cu(HT)_2]$	Q cal./L.	ΔH°_3, Kcal./mole	ΔH°_4, Kcal./mole
0.0300	6.60×10^{-4}	2.27×10^{-4}	5.69×10^{-9}	9.53×10^{-5}	0	...	...	...
0.1047	4.88×10^{-4}	1.94×10^{-4}	9.32×10^{-9}	1.05×10^{-4}	0	−2.90	−5.5	...
0.1670	3.57×10^{-4}	1.59×10^{-4}	1.43×10^{-8}	1.17×10^{-4}	0	−2.40	−6.1	...
0.2334	2.33×10^{-4}	1.15×10^{-4}	2.42×10^{-8}	1.39×10^{-4}	0	−2.63	−10.8	...
0.2971	1.29×10^{-4}	6.75×10^{-5}	4.64×10^{-8}	1.72×10^{-4}	0	−2.38	−5.7	...
0.3340	7.47×10^{-5}	3.96×10^{-5}	8.14×10^{-8}	1.96×10^{-4}	0	−1.47	−9.8	...
0.3802	2.13×10^{-6}	1.05×10^{-5}	2.64×10^{-7}	2.44×10^{-4}	0	...	...	...
0.4095	3.81×10^{-6}	1.47×10^{-6}	1.16×10^{-6}	...	0.56×10^{-4}	−0.78	...	−1.9
0.4349	7.24×10^{-7}	1.73×10^{-7}	3.74×10^{-6}	...	1.24×10^{-4}	−0.57	...	−1.8
							−7.5 Av.	−1.8 Av.

TABLE III.—THERMODYNAMICS OF DEPROTONATION FOR TETRACYCLINE·HCl ANALOGS

$\Delta G^\circ_6 = -1.364 \log K_1$ $K_1 = [H_2T][H^+]/[H_3T^+]$
$\Delta H^\circ_6 = \Delta H^\circ_1 - \Delta H^\circ_5$

Analog	ΔG°_6 (Kcal./mole)	ΔH°_6 (Kcal./mole)	ΔS°_6 (e.u.)
Chlortetracycline·HCl	+4.46	+2.35	−7.1
Demethylchlortetracycline·HCl	+4.50	+2.35	−7.2
Tetracycline·HCl	+4.55	+2.05	−8.4
4-Epi-chlortetracycline·HCl	+4.90	+2.25	−8.9
4-Epi-anhydrotetracycline·HCl	+4.75	+1.95	−9.4

$\Delta G^\circ_7 = -1.364 \log K_2$ $K_2 = [HT^-][H^+]/[H_2T]$
$\Delta H^\circ_7 = \Delta H^\circ_2 - \Delta H^\circ_5$

Analog	ΔG°_7 (Kcal./mole)	ΔH°_7 (Kcal./mole)	ΔS°_7 (e.u.)
Chlortetracycline·HCl	+10.03	+6.75	−11.0
Demethylchlortetracycline·HCl	+9.79	+7.75	−7.5
Tetracycline·HCl	+10.51	+8.25	−7.6
4-Epi-chlortetracycline·HCl	+10.34	+7.65	−8.7

TABLE IV.—THERMODYNAMIC DATA FOR COMPLEXES OF TETRACYCLINE ANALOGS WITH CUPRIC IONS

Analog	ΔG°_3 (Kcal./mole)	ΔH°_3 (Kcal./mole)	ΔS°_3 (e.u.)
Chlortetracycline·HCl	−9.99	−11.7	−4.0
Demethylchlortetracycline·HCl	−10.71	−12.3	−5.4
Tetracycline·HCl	−10.64	−7.6	+10.2
4-Epi-chlortetracycline·HCl	−10.41	−11.5	−3.7

Analog	ΔG°_4 (Kcal./mole)	ΔH°_4 (Kcal./mole)	ΔS°_4 (e.u.)
Chlortetracycline·HCl	−6.95	−1.80	+17.3
Demethylchlortetracycline·HCl	−7.45	−2.60	+16.3
Tetracycline·HCl	−7.28	−1.80	+18.4
4-Epi-chlortetracycline·HCl	−6.92	−2.00	+16.5

Since the chelation steps of tetracycline with cupric ion are separated sufficiently, it was assumed that when $\bar{n} < 1$ only $Cu(HT)^+$ is formed and therefore, the $Cu(HT)_2$ terms may be dropped from Eq. 5. Using the calculations described in *Reference 1*, the concentrations of H^+, H_3T^+, HT^-, and $Cu(HT)^+$ may be calculated after each addition of base.

At $\bar{n} > 1$, $\Delta[Cu(HT)_2] = -\Delta[Cu(HT)^+]$ so that the only chelate term remaining in Eq. 5 will be $-\Delta[Cu(HT)_2]$ (ΔH°_4), and thus the heat of chelation for the second ligand may be calculated by determining $[H^+]$, $[H_3T^+]$, $[HT^-]$, and $[Cu(HT)_2]$ after each addition of titrant. A sample calculation of ΔH°_3 and ΔH°_4 is presented in Table II for tetracycline·HCl.

It should be noted that the ΔH°_3 and ΔH°_4 values show a wide range of individual points, yet when the averages of two different titrations of a particular analog were compared, the average values were very close. This was also noted in the joule calibrations. Since the calorimeter had no way to lose heat except by diffusion through the Dewar, it seemed that the calorimeter sometimes compensated by giving alternate low and high readings. Yet the average was a good measure of the actual heat evolved. Stability constants calculated from the data as presented in Table II showed good agreement with those values reported earlier (1).

RESULTS

As previously explained, the calorimetric work had to be carried out in two parts. Initially the heats of deprotonation for the first and second dissociating hydrogens were determined. The thermodynamic parameters of the first dissociating hydrogen are designated with a subscript *6*; the thermodynamic parameters of the second dissociating hydrogen are designated with a subscript *7*. The values for these parameters are presented in Table III. The values for K_1 and K_2 are the stoichiometric dissociation constants determined in the parallel potentiometric titrations (*vide supra*) and correspond to the thermodynamic dissociation constants reported previously (1). The values for ΔH°_1 and ΔH°_2 are determined experimentally as demonstrated in Table I. The value for ΔH°_5 is taken as −13.95 Kcal./mole as explained above.

The actual heat evolved in each determination of ΔH°_1 is about 0.5 cal., and the ΔH°_1 values calculated from these results are accurate to about ±5%. The ΔH°_1 values are approximately equivalent for all five tetracycline analogs, as is reflected in the agreement of the ΔH°_6 values in Table III. The epi-derivatives have ΔS°_6 values which are slightly more negative, indicating that the change in pK_1 (1) is an entropy effect as would be expected for a stereochemical change (compare chlortetracycline with 4-epi-chlortetracycline).

The actual heat evolved in each determination of ΔH°_2 is about 0.25 cal. Due to the overlap of pK_2 and pK_3 there are fewer determinations for ΔH°_2, and consequently, these values are not as accurate as the ΔH°_1 values. Due to the low solubility of the isoionic form of 4-epi-anhydrotetracycline, no accurate values of ΔH°_2 could be obtained for this analog.

Once ΔH°_1 and ΔH°_2 had been determined, tetracycline–metal titrations were run to find ΔH°_3, the heat of 1:1 chelation, and ΔH°_4, the heat of 2:1 chelation. These values are presented in Table IV. Particular notice should be directed toward the values determined for tetracycline·HCl where the exothermic heat of chelation, ΔH°_3, is significantly less than those values determined for the chlor-derivatives. Since the ΔG°_3's for the four analogs are reasonably similar, the large difference in ΔH°_3 for tetracycline·HCl, appears as an entropy effect causing ΔS°_3 to be +10.5 for tetracycline and about −4 for the chlor-analogs.

Before attempting to explain this difference, let us take a further look at ΔH°_4, the heat of complexation for the second ligand. The actual heat given off for each addition of titrant in ΔH°_4 determinations was in many cases below 0.2 cal. Also the number of determinations for ΔH°_4 in each titration was limited to the lower pH ranges to

TABLE V.—THERMODYNAMIC DATA FOR THE COMBINED REACTION *2 + 3*

$$Cu^{++} + H_2T + OH^- \rightleftharpoons Cu(HT)^+ + H_2O$$
$$\Delta G^\circ_{2+3} = -1.364 (\log K_1 + \log \beta_1 - \log Kw)$$

Analog	ΔG°_{2+3} (Kcal./mole)	ΔH°_{2+3} (Kcal./mole)	ΔS°_{2+3} (e.u.)
Chlortetracycline·HCl	−18.90	−18.92	−0.01
Demethylchlortetracycline·HCl	−20.00	−18.70	+4.4
Tetracycline·HCl	−19.23	−13.3	+19.9
4-Epi-chlortetracycline·HCl	−19.11	−18.91	+0.7
4-Epi-anhydrotetracycline·HCl	−19.90	−14.0	+19.8

insure that the third dissociable hydrogen from the chelated tetracycline was not present to a significant extent. For 4-epi-chlortetracycline only one value of ΔH°_4 could be calculated per titration before precipitation began in the calorimeter. This could easily be seen on the recorder as irregular bursts of heating replaced a smooth drift curve. Even with all the errors inherent in measuring such a small heat change and the pyramiding of effects of inaccurate ΔH°_1 and ΔH°_2 values, it is possible to get some idea of ΔH°_4, at least qualitatively. The ΔH°_4 values listed in Table IV show a reasonable degree of consistency, as do the ΔS°_4 values. In this case all four derivatives, the chlor-analogs and tetracycline itself, show a high positive ΔS°_4.

As mentioned previously, ΔH°_1 is the most accurate enthalpy determined in this work. Therefore, to prove that the difference in ligational entropy was not just a factor of an incorrect ΔH°_2, it was decided to examine the entropy of the over-all Reaction *2 + 3*.

$$Cu^{++} + H_2T + OH^- \rightleftharpoons Cu(HT)^+ + H_2O$$
$$\Delta H^\circ_{2+3} \quad \text{Reaction } 2 + 3$$

$$\Delta G^\circ_{2+3} = -1.364 (\log K_1 + \log \beta_1 - \log K_w) \quad \text{(Eq. 7)}$$

The ΔH°_{2+3} can be calculated, using only one predetermined value, ΔH°_1. In addition, Reaction *2 + 3* is a good approximation to what is actually happening in solution, since below $\bar{n} = 1$ [*i.e.*, where $Cu(HT)^+$ is forming] the concentration of free ligand (HT^-) is negligible and the addition of titrant (OH^-) results in pH changes (Reaction *5*), neutralization of H_3T^+ (Reaction *1*), and the conversion of H_2T to $Cu(HT)^+$. Therefore,

$$\Delta H^\circ_{2+3} = \frac{Q - \Delta[H^+](\Delta H^\circ_2) - \Delta[H_3T^+](\Delta H^\circ_1)}{\Delta[Cu(HT)^+]} \quad \text{(Eq. 8)}$$

The thermodynamic values obtained from such a calculation are illustrated in Table V. Since ΔH°_{2+3} can be calculated without knowledge of ΔH°_2, this calculation may also be made for 4-epi-anhydrotetracycline. Calculating ΔS°_{2+3}, a value of +19.8 is obtained for 4-epi-anhydrotetracycline, +19.9 for tetracycline, and much lower values for the three chlor-analogs.

DISCUSSION

Rossotti (10) has suggested that from a simple viewpoint, the entropy change on complex formation would be expected to be negative (about −25 e.u.) due to the conversion of translational to vibrational and rotational entropy, accompanying the decrease in the number of particles in solution. However, the role of the solvent must also be estimated in rationalizing ligational entropy changes. Association of a metal ion with a charged ligand in aqueous solution will bring about a decrease in the numbers of ions, (partial) neutralization of electrical charge, attenuation of the remaining charge, and displacement of water from the hydration spheres of the reactant (10). The last term is a major factor and has brought about a differentiation between inner and outer sphere complexes. An inner sphere complex is formed when a ligand is able to replace a water molecule from the hydration sphere of the metal. An outer sphere complex does not replace water from the metal hydration sphere but is rather an ion pair complex. A less favorable entropy change will be expected from the formation of an outer sphere complex than from the corresponding inner sphere complex.

However, it should be noted that the magnitude of the enthalpy of complexation must also be considered, and that judgment concerning the sphere of complexation cannot be made strictly from an entropy viewpoint. Outer sphere complexes with Cl^- or SO_4^{--} as ligands usually show a slight *endothermic* heat of formation (11), whereas inner sphere complexes presuppose the formation of strong covalent bonds, which show large exothermic heats of formation. It should also be expected that a ligand of the size and complexity of tetracycline would show large conformational changes during chelation which would be reflected in the entropy changes.

Therefore, in considering what type of complexation takes place, no set rule for entropy values may be established, but rather an over-all view of all the processes listed above by Rossotti must be taken, with special emphasis for tetracycline on the conformational changes expected. On the basis of the high negative ΔH°_3 values obtained for the 1:1 complexes, it would seem that the two tetracycline analogs and the three chlortetracycline analogs all form inner sphere complexes with cupric ions. However, there is a significant difference between the ΔH°_3 values obtained for tetracycline and 4-epi-anhydrotetracycline[5] as opposed to the ΔH°_3 values for chlortetracycline, demethylchlortetracycline, and 4-epi-chlortetracycline. Since the ΔG°_3 values for tetracycline and the three chlor-derivatives are approximately the same, the difference in enthalpy is probably a function of the conformational changes required for tetracycline and the chlortetracyclines to reach a favorable complexing configuration.

As a system becomes more ordered, the entropy

[5] Although ΔH°_3 has not actually been determined for 4-epi-anhydrotetracycline, it seems reasonable to assume, on the basis of the data presented in Table V, that ΔH°_3 for 4-epi-anhydrotetracycline will be very close to ΔH°_3 for tetracycline.

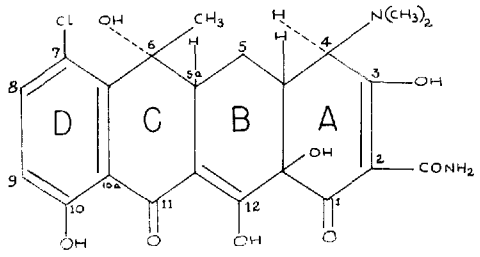

Fig. 2.—Configuration for chlortetracycline after Donohue *et al.* (12). Other authors (15) show the configuration for the enantiomorph.

of the system decreases. Therefore, the chlortetracycline analogs with negative $\Delta S°_3$ values seem to be required to undergo a more severe conformational change than tetracycline in order to reach a favorable configuration for chelation. From the data presented in Tables IV and V it would be expected that the requirement for a more severe conformational change would be dependent on the presence of the chlorine atom at carbon 7 (see Fig. 2). These ideas were tested by constructing framework molecular models[6] of the various tetracyclines using the bond lengths and configuration for chlortetracycline as determined by Donohue *et al.* (12) from X-ray data. The hydrogens on 6-methyl and 6-hydroxyl and the chlorine at carbon 7 were so constructed that the van der Waals radii for these atoms (13) were physically represented. From the model it is seen that the C ring can readily flip from a "boat" conformation where the 6-methyl is axial to the ring, to a "boat" conformation where the 6-methyl is equatorial to the C ring (while the 6-hydroxyl group goes from equatorial to axial positions) and that this flipping would be influenced greatly by the steric hindrance occurring between the chlorine at carbon 7 and either the 6-methyl or 6-hydroxyl.

On the basis of the data presented, it seems most likely that the first tetracycline ligand forms an inner sphere complex with cupric ions and that this complexation takes place with the formation of a chelate ring involving the cupric ion, oxygen 10, carbons 10, 10a, and 11, and oxygen 11, in which case it is necessary that all of the above atoms and ions lie in a plane (14). To accomplish this it is necessary that the C ring be essentially planar with the D ring, and in this case there would be considerable steric interaction between the chlorine atom and probably both the methyl and hydroxyl groups on carbon 6. Donohue *et al.* (12) point out that the Cl . . . CH_3 distance for this conformation is 2.99 Å. which is much less than 3.8 Å., the sum of the van der Waals radii. Obviously, if the chlorine at carbon 7 was not present as for tetracycline, the steric problem would be decreased and the entropy change would essentially depend on the replacement of water from the hydration spheres of copper.

In light of the above reasoning, it is hypothesized that the difference in the entropy of formation for 1:1 complexes of chlortetracycline and tetracycline is due to the strained steric requirement that chlortetracycline must undergo. If this hypothesis is true, it follows that there should be little difference between $\Delta S°_3$ for chlortetracycline and 4-epichlortetracycline, since the 4-dimethylammonium group would not be involved in complexation. This is found to be true in Table IV, and lends support to the idea that chelation takes place at oxygens 10 and 11.

It might seem that demethylchlortetracycline should have a $\Delta S°_3$ which is more positive than $\Delta S°_3$ for chlortetracycline, since demethylchlortetracycline has no 6-methyl group available for steric interaction with the chlorine. However, it seems reasonable to assume that for demethylchlortetracycline the 6-hydroxyl would generally be found axial to the C ring, since there would be much less steric interaction between the 6-hydrogen (equatorial) and the 7-chlorine. Therefore, it might be reasoned that it would be less favorable for the C ring in demethylchlortetracycline to become planar (bringing about steric interaction between chlorine and hydroxyl) than for the C ring in chlortetracycline, where there is no specially favored conformation for the hydroxyl (or methyl) group, and, thus, it could be hypothesized that a slightly more severe conformational change is required for demethylchlortetracycline than chlortetracycline in order to bring about complexation.[7]

In the case of 4-epi-anhydrotetracycline, it would be expected that the entropy of chelation would be similar to that of tetracycline (as is seen in Table V) for two reasons. First, there is no chlorine present at carbon 7 to cause steric interactions with the 6-methyl; and second, since chelation seems to occur at oxygens 10 and 11, entropy of chelation should not depend on whether the 4-dimethylammonium group is epimerized. In addition, for an anhydrotetracycline analog, the C ring is initially in a planar configuration due to the additional double bond at C_{5a}—C_6. Thus, it might be expected that all anhydrotetracyclines would have a $\Delta S°_3$ similar to tetracycline, regardless of whether a chlorine was present at carbon 7.

The results obtained for Reaction *4*, the formation of the 2:1 chelate from the 1:1 chelate and the free ligand, are generally similar for all four compounds, but considerably different from the results obtained for Reaction *3*. In the previous discussion it was hypothesized that the first ligand forms strong covalent bonds with the cupric ion. As a simple picture, we may consider a small cupric ion surrounded by the much larger tetracycline molecule, and although the C and D rings of the ligand are in a fixed position, the A and B rings and the bulky groups attached to them are still free to rotate. Now another large, negatively-charged tetracycline molecule complexes with the copper ion. It is very difficult to visualize a second tetracycline as being capable of approaching the cupric ion close enough to form a covalent ligand bond. This is confirmed by the low values obtained for $\Delta H°_4$ for all of the compounds studied, and thus it may reasonably be presumed that the second tetracycline forms an outer sphere or ion-pair complex.

[6] Prentice-Hall, Inc., Englewood Cliffs, N. J.

[7] An alternate hypothesis, suggested by the reviewer, proposes that the conformation in unchelated demethylchlortetracycline is the same as in chlortetracycline, perhaps due to hydrogen bonding to the 7-chlorine. Therefore, a similar conformational change occurs upon chelation leading to the observed similar entropy change. It should also be pointed out that the above explanations are only valid if chelation actually does take place at oxygens 10 and 11 as would be expected from the protonation scheme determined by Rigler *et al.* (16).

In this reaction $\Delta S°_4$ has a high value (see Table IV) for all of the analogs studied, both tetracycline and chlor-derivatives. This may be explained by the fact that when the second ligand attaches to the metal, the over-all charge of the complex becomes zero, and the number of ions in solution is decreased. This neutralization of charge and decrease in the number of ions is common to all the analogs studied and would be expected to give a positive entropy change. In addition, the conformational entropy changes between tetracycline and the chlor-analogs is not significant here, since little conformational change is needed to form an ion-pair complex bond.

REFERENCES

(1) Benet, L. Z., and Goyan, J. E., *J. Pharm. Sci.*, **54**, 983(1965).
(2) Rossotti, F. J. C., in "Modern Coordination Chemistry," Lewis, J., and Wilkins, R. G., eds., Interscience Publishers, Inc., New York, N. Y., 1960, p. 18.
(3) Benet, L. Z., and Goyan, J. E., *J. Pharm. Sci.*, **54**, 1179(1965).
(4) Atkinson, G., and Bauman, J. E., Jr., *Inorg. Chem.*, **1**, 900(1962).
(5) Bauman, J. E., Jr., Ph.D. Thesis, University of Michigan, Ann Arbor, Mich., 1961.
(6) Schlyter, K., and Sillen, L. G., *Acta Chem. Scand.*, **13**, 385(1959); Schlyter, K., *Trans. Roy. Inst. Technol. Stockholm*, **1959**, 132.
(7) Benet, L. Z., Ph.D. Thesis, University of California, San Francisco, Calif., 1965.
(8) Rossini, F. D., Wagman, D. D., Evans, W. W., Levine, S., and Jaffe, I., "Selected Values of Chemical Thermodynamic Properties," National Bureau of Standards, Circular 500, U. S. Government Printing Office, Washington, D. C., 1952.
(9) Papee, H. M., Canady, W. J., and Laidler, K. J., *Can. J. Chem.*, **34**, 1677(1956).
(10) Rossotti, F. J. C., *op. cit.*, p. 20.
(11) Sillen, L. G., and Martell, A. E., "Stability Constants of Metal-Ion Complexes," The Chemical Society London, London, England, 1964.
(12) Donohue, J., Dunitz, J. D., Trueblood, K. N., and Webster, M. S., *J. Am. Chem. Soc.*, **85**, 851(1963).
(13) Pauling, L., "The Nature of the Chemical Bond," 3rd ed., Cornell University Press, Ithaca, N. Y., 1960, p. 260.
(14) Martell, A. E., and Calvin, M., "Chemistry of the Metal Chelate Compounds," Prentice-Hall, Inc., New York, N. Y., 1952, p. 136.
(15) Schach von Wittenau, M., and Blackwood, R. K., *J. Org. Chem.*, **31**, 613(1966).
(16) Rigler, N. E., Bag, S. P., Leyden, D. E., Sudmeier, J. L., and Reilley, C. N., *Anal. Chem.*, **37**, 872(1965).

Ionization of Bases with Limited Solubility

Investigation of Substances with Local Anesthetic Activity

By IVO SETNIKAR

The deionization process of six local anesthetics, *i.e.*, procaine, lignocaine, cocaine, Rec 7-0518, Rec 7-0544, and Rec 7-0591, was investigated. The compounds are weak bases with a low or a very low solubility of the unionized form. The effects of this property on the deionization process were studied and an explanation of the irregularities of the deionization curve suggested. A method for plotting the deionization process as a straight line is described. Temperature markedly affects the ionization constant. A limited solubility of the unionized base influences its buffering capacity. Both phenomena may be relevant to tissue tolerance for solutions of these substances.

It is essential to know the ionization curve of a local anesthetic in aqueous solution in order to choose a pH of the injectable solution that is optimal both for pharmaceutical stability and for local tissue tolerance.

Ionization curves may be plotted by the conventional method (1) which, for monoprotic species, leads to the well-known S-shaped curve. Other expressions of the results lead to straight-lined representation of ionization, with the advantage of showing more clearly experimental errors or deviations from theory.

Methods of obtaining straight-line representation of ionization of weak acids or bases were presented by Hofstee (2), by Benet and Goyan (3, 4), and by Leeson and Brown (5). The methods involve recalculations of the results and are strongly influenced by experimental errors (4). More immediate and easier to apply is the method proposed by Druckrey (6, 7), based on the use of a specially designed scale for the titrant, which yields a straight-line expression of the law of mass action. This method may also be adapted for expressing with a straight line the ionization process of weak acids or bases in which the unionized form is sparingly soluble, a fact which limits its availability for the ionization equilibrium.

Received June 6, 1966, from the Research Division, Recordate S. A. S., Milan, Italy.
Accepted for publication July 15, 1966.

THEORY

The ionization of a proton acceptor, B, is represented by

$$B + H^+ \cdot H_2O \rightleftarrows BH^+ + H_2O \quad \text{(Eq. 1)}$$

Since in a diluted aqueous solution the concentration of H_2O remains practically constant, the equilibrium of the ionization process is expressed by Eq. 2.

$$\frac{[BH^+]}{[B][H^+ \cdot H_2O]} = K' \quad \text{(Eq. 2)}$$

where K' is the apparent ionization constant, valid