Determination of the Stability Constants of Tetracycline Complexes

By LESLIE Z. BENET* and JERE E. GOYAN

The Bjerrum ΔKOH method for determining the degree of formation ($\bar{n}$) from the titration curves of a ligand with and without metals is derived without approximations. The Calvin and Wilson method is rederived in a form which allows easy comparison between the values calculated by each method. The advantages of the $\bar{n}$ comparison method are discussed. Thermodynamic dissociation constants and stoichiometric biligand stability constants are calculated for five tetracycline analogs. Contrary to previous work, the biologically inactive tetracycline derivatives, 4-epi-chlortetracycline and 4-epi-anhydrotetracycline, were found to form 2:1 complexes with cupric ions. The choice of methods for the calculation of the stability constants from $\bar{n}$ and [HT⁻] potentiometric data is discussed.

THERE ARE two common methods of determining biligand stability constants from potentiometric data. Bjerrum (1) introduced the method of titrating a solution of the ligand with and without metal and showed that at any given pH the difference in the amount of base consumed in each of the two titrations was a measure of the amount of ligand complexed at that pH. However, this method is only an approximation and, as will be shown, is not valid when complexation takes place in a pH range near the first acid dissociation constant of the ligand or when the product of the first and second dissociation constants (K_1K_2) is a significant value when compared with the product of the hydrogen ion concentration and the first acid dissociation constant ($K_1[H^+]$).

The second method is that of Calvin and Wilson (2), where calculations are made using only the titration curve of the combined ligand and metal solution. Albert (3–5) also has derived this method in a slightly different form and used it to calculate the stability constants of tetracycline, chlortetracycline, and oxytetracycline.

Doluisio and Martin (6) used the Bjerrum method in calculating the stability constants of tetracycline and some of its active and inactive derivatives. Their results showed that the therapeutically active tetracycline derivatives formed 2:1 complexes with cupric, nickel, and zinc ions, while the inactive tetracycline analogs formed only 1:1 complexes.

In the present paper, the Bjerrum method is derived in a form which takes into account all species present and therefore allows $\bar{n}$ (the degree of formation) to be calculated without approximations over the entire titration range.

The Calvin and Wilson method is rederived in a form whereby $\bar{n}$, at any given pH, is easily comparable with the $\bar{n}$ values calculated by the Bjerrum method at the same pH. This comparison gives an estimate of the accuracy of the data and also serves as a measure of the reliability of the dissociation constants and the stoichiometric concentration of the tetracycline used.

THEORY

The previous workers (4–6) have shown that the complexing species of the tetracycline analogs is the HT^- form, which complexes in a stepwise manner with a divalent metal M^{+2} in the following manner:

$$M(HT)_n + (HT) \rightleftharpoons M(HT)_{n+1} \quad \text{(Eq. 1)}$$

where n takes the values 0 and 1. Therefore the first stoichiometric stability constant, β_1, and the product of the first and second stoichiometric stability constants, β_2, are defined as

$$\beta_1 = \frac{[M(HT)^+]}{[M^{+2}]\,[HT^-]} \quad \text{(Eq. 2)}$$

$$\beta_2 = \frac{[M(HT)_2]}{[M^{+2}][HT^-]^2} \quad \text{(Eq. 3)}$$

The degree of formation of the system, $\bar{n}$, is the average number of ligands bound to each central metal ion.

$$\bar{n} = \frac{[M(HT)^+] + 2[M(HT)_2]}{M^\circ} \quad \text{(Eq. 4)}$$

The total concentrations of metal, M°, and tetracycline, T°, are given by

$$M^\circ = [M^{+2}] + [M(HT)^+] + [M(HT)_2] \quad \text{(Eq. 5)}$$

$$T^\circ = [H_2T^+] + [H_2T] + [HT^-] + [M(HT)^+] + 2[M(HT)_2] \quad \text{(Eq. 6)}$$

The term $[T^{-2}]$ is omitted from the T° equation and all following derivations since all significant data occurred below a pH of 5.5 and calculations show that the $[T^{-2}]$ term is insignificant. Calculations at a higher pH than 5.5 would tend to be inaccurate since complexes having a dissociable hydrogen

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remote from the site of metal coordination will not have the same dissociation constant for that hydrogen as the uncomplexed acid (7). In such instances the presence of the metal inductively enhances the acid strength of the particular proton from 1 to 5 pK units. Due to the insolubility of the tetracycline chelates this third K could not be calculated.

The concentrations of the ionic forms of tetracycline can be derived by solving the following equations:

$$K_1^c[H_3T^+] - [H^+][H_2T] = 0 \quad \text{(Eq. 7)}$$

$$K_2^c[H_2T] - [H^+][HT^-] = 0 \quad \text{(Eq. 8)}$$

$$[H_3T^+] + [H_2T] + [HT^-] = T^\circ \text{ or } (T^\circ - \bar{n}M^\circ) \quad \text{(Eq. 9)}$$

Here, T°, the stoichiometric concentration of tetracycline is used when no metal is present; however, when a metal is added the ionic species of Eq. 9 will be equal to the stoichiometric concentration of tetracycline less that amount which is bound to the metal.

When metal is present

$$[H_3T^+] = \frac{[H^+]^2(T^\circ - \bar{n}M^\circ)}{[H^+]^2 + [H^+]K_1^c + K_1^cK_2^c} = \alpha(T^\circ - \bar{n}M^\circ) \quad \text{(Eq. 10}a\text{)}$$

$$[H_2T] = \frac{[H^+]K_1^c(T^\circ - \bar{n}M^\circ)}{[H^+]^2 + [H^+]K_1^c + K_1^cK_2^c} = \beta(T^\circ - \bar{n}M^\circ) \quad \text{(Eq. 10}b\text{)}$$

$$[HT^-] = \frac{K_1^cK_2^c(T^\circ - \bar{n}M^\circ)}{[H^+]^2 + [H^+]K_1^c + K_1^cK_2^c} = \gamma(T^\circ - \bar{n}M^\circ) \quad \text{(Eq. 10}c\text{)}$$

When metal is not present in the titration the $(\bar{n}M^\circ)$ term in Eq. 10 is dropped.

Calvin and Wilson Method.—Consider the titration with potassium hydroxide of a solution of tetracycline hydrochloride and bivalent metal chloride to which hydrochloric acid, concentration A°, was added. Using the principle of electroneutrality the following equation is written:

$$[H_3T^+] + [M(HT)^+] + 2[M^{+2}] + [K^+] + [H^+] = [Cl^-] + [OH^-] + [HT^-] \quad \text{(Eq. 11)}$$

$$\text{where } [Cl^-] = T^\circ + A^\circ + 2M^\circ \quad \text{(Eq. 12)}$$

Substitution of Eqs. 4, 5, 10, and 12 into Eq. 11 gives

$$\alpha(T^\circ - \bar{n}M^\circ) - \gamma(T^\circ - \bar{n}M^\circ) + [K^+] + [H^+] - A^\circ - T^\circ - [OH^-] = \bar{n}M^\circ \quad \text{(Eq. 13)}$$

Letting $\bullet = [K^+] + [H^+] - A^\circ - T^\circ - [OH^-]$ and recalling that $\alpha + \beta + \gamma = 1$, Eq. 13 rearranges to

$$\bar{n} = \frac{(\alpha - \gamma)T^\circ + \epsilon}{(2\alpha + \beta)M^\circ} \quad \text{(Eq. 14)}$$

which is the equation for calculating $\bar{n}$ when only one titration is made, that of a solution of a metal and a ligand with two dissociable hydrogens.

Bjerrum or ΔKOH Method.—To derive the ΔKOH method we must set up an electroneutrality equation for the titration of pure ligand.

$$[H_3T^+] + [K^+] + [H^+] = [Cl^-] + [OH^-] + [HT^-] \quad \text{(Eq. 15)}$$

In this case $[Cl^-]$ is equal to the stoichiometric concentration of tetracycline·HCl plus the amount of acid initially added. Substituting Eq. 10 without metal terms into Eq. 15 and rearranging gives

$$[K^+_{T^\circ}] = T^\circ + A^\circ + [OH^-] - [H^+] - \alpha T^\circ + \gamma T^\circ \quad \text{(Eq. 16)}$$

Rearrangement of Eq. 13 gives

$$[K^+_{T^\circ + M^\circ}] = T^\circ + A^\circ + [OH^-] - [H^+] - \alpha(T^\circ - \bar{n}M^\circ) + \gamma(T^\circ - \bar{n}M^\circ) + \bar{n}M^\circ \quad \text{(Eq. 13)}$$

Therefore at any pH, when T° and A° are the same in both the titrations of ligand with and without metal, we may substract Eq. 16 from Eq. 13 and at that pH

$$\bar{n} = \frac{\Delta KOH}{(2\alpha + \beta)M^\circ} \quad \text{(Eq. 17)}$$

Formation Function.—The formation function relating $\bar{n}$ to the stability constants is derived by substituting Eqs. 2, 3, and 5 into Eq. 4.

$$\bar{n} = \frac{\beta_1[HT^-] + 2\beta_2[HT^-]^2}{1 + \beta_1[HT^-] + \beta_2[HT^-]^2} \quad \text{(Eq. 18)}$$

The $[HT^-]$ term is calculated from Eq. 10c.

EXPERIMENTAL

Materials.—Tetracycline·HCl, chlortetracycline·HCl, demethylchlortetracycline·HCl, 4-epi-chlortetracycline·HCl, and 4-epi-anhydrotetracycline·HCl were donated by Lederle Laboratories. Carbonate-free potassium hydroxide solution was prepared by the method of Albert and Serjeant (8). Triple distilled water was boiled for approximately 15 minutes to remove carbon dioxide and was stored under nitrogen. The water had a specific conductance less than 2 μmhos. The solution being titrated was maintained oxygen and carbon dioxide free by nitrogen, which had been passed through a pyrogallol–potassium hydroxide solution and then through water. Reagent grade cupric chloride dihydrate, potassium chloride, and hydrochloric acid were used.

Procedure.—Solutions of a pure tetracycline·HCl derivative (about $4.75 \times 10^{-4}M$) or the tetracycline·HCl and cupric chloride ($2.374 \times 10^{-4}M$) and containing 3.854×10^{-4} M HCl (making up to a 158 ml. total volume) were placed in a thermostated vessel (25 ± 0.05°) under an atmosphere of nitrogen. Ionic strength was adjusted to 0.01 with KCl. Carbonate-free KOH solution was added from a 1-ml. microburet.[1] A Beckman research pH meter[2] standardized with 0.05 M potassium hydrogen phthalate (pH 4.010) was used. Due to the degradation of tetracycline·HCl solutions all titrations were run within 6 hr. after preparation of the solution.

Calculations.—The tetracycline·HCl samples were assumed to be pure, but the exact number of waters of hydration (especially for demethylchlortetracycline and the 4-epi derivatives) was unclear. Therefore, since the first and second dissociation constants were sufficiently separated the following

[1] Gilmont Micropipet-Buret, Scientific Products, Evanston, Ill.

[2] Beckman Instruments, Inc., Fullerton, Calif.

equation was derived by rearrangement of the buffer equation.[3]

$$Z = T^\circ - \frac{1}{K_1^c} Z[H^+] \qquad \text{(Eq. 19)}$$

where $Z = [K^+] + [H^+] - A^\circ$.

A plot of Z *versus* $Z[H^+]$ will give a straight line with slope $-1/K_1^c$ and intercept T° as shown in Fig. 1. The accuracy of the method was confirmed by the constant values calculated for K_2^c and K_3^c over wide pH ranges and also by the agreement of $\bar{n}$ values calculated by the two different methods. Since the second and third dissociation constants were not widely separated they were determined by the method of Noyes (9).

The acid dissociation constants were first calculated in concentration terms. Thus, using the Kielland (10) individual activity coefficients for ions in water, pH values were converted to hydrogen ion concentrations by assuming an activity coefficient of 0.914. The stoichiometric dissociation constants were converted to thermodynamic values assuming the activity coefficients for $[H_3T]^+$ $[HT^-]$ to be 0.900 and that for $[T^{-2}]$ to be 0.663.[4]

The arrangement of a typical $\bar{n}$ calculation is shown below:

$$D = [H^+]^2 + [H^+]K_1^c + K_1^cK_2^c$$

$$2\alpha + \beta = \frac{2[H^+]^2 + [H^+]K_1^c}{D}$$

$$\epsilon = [KOH] + [H^+] - A^\circ - T^\circ$$

$$\bar{n} = \frac{\Delta KOH}{(2\alpha + \beta)M^\circ} \qquad \text{(Eq. 17)}$$

$$\bar{n} = \frac{(\alpha - \gamma)T^\circ + \epsilon}{(2\alpha + \beta)M^\circ} \qquad \text{(Eq. 14)}$$

$$[HT^-] = \frac{K_1^cK_2^c}{D}(T^\circ - \bar{n}M^\circ) \qquad \text{(Eq. 10}c\text{)}$$

The stoichiometric stability constants were determined by plotting $(\bar{n} - 1)[HT^-]/\bar{n}$ *versus* $(2 - \bar{n})[HT^-]^2/\bar{n}$ according to the method of Speakman (11). The stability constants for tetracycline and chlortetracycline were calculated also by the Bjerrum half $\bar{n}$ method (1) and were found to be similar. The demethylchlor-, 4-epi-chlor-, and 4-epi-anhydro derivatives (at 4.75×10^{-4} M) gave initial results just at or above $\bar{n} = {}^1/_2$ and therefore the half $\bar{n}$ method was not applicable.

RESULTS AND DISCUSSION

When the two equations for calculating $\bar{n}$ are placed side by side as above, the advantage of using both methods becomes apparent. Since the denominators of Eqs. 14 and 17 are identical, the numerators of Eqs. 14 and 17 must be equal in order to obtain the same result by each method at any given pH. The numerator of Eq. 17 is independent of the numerical values obtained for T°, A°, $[H^+]$, and the dissociation constants K_1^c and K_2^c, while the numerator of Eq. 14 is dependent on the values of all these terms. The significance of each term fluctuates over the entire titration. For example at pH values of 3 to 4, $[H^+]$ is very significant in ϵ, $(\alpha - \gamma)T^\circ$, D, and $(2\alpha + \beta)$, whereas γ ($\gamma = K_1^cK_2^c/D$) is completely insignificant. However at a pH of 5, the $[H^+]$ effect is greatly diminished in all the above terms except D, whereas γ begins to take on importance, especially in the case of 4-epi-anhydrotetracycline, which has a low pK_2. The above method is also valuable in checking the concentration of T° where water of hydration or other nonionizing impurities may be present. A 1% error in determining T° will cause the two $\bar{n}$ values which were identical at a given pH to differ by up to 1.5%. Thus the reliability of the experimental data is confirmed by obtaining similar values for the $\bar{n}$'s at each pH over the titration range, and conversely, invalid data or just one faulty point is easily spotted.

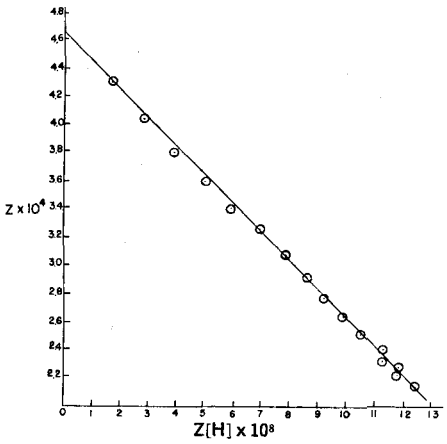

Fig. 1.—Determination of k_1^c and T° for 4-epi-chlortetracycline · HCl by means of Eq. 19.

Equation 17 shows that the Bjerrum *approximation* method ($\bar{n} = \Delta KOH/M^\circ$) becomes very inexact at hydrogen ion concentrations which are very near or greater than the first acid dissociation constant, since the greater the hydrogen ion concentration, the closer $(2\alpha + \beta)$ approaches 2. All the tetracycline derivatives investigated in this work did show significant $\bar{n}$ values at a pH equal to the first dissociation constant. This would be especially telling in determining stability constants by the Bjerrum half $\bar{n}$ method [*e.g.*, for chlortetracycline·HCl ($5.01 \times 10^{-4}M$), $\bar{n} = 0.5$ at pH = 3.68 and where $(2\alpha + \beta) = 1.30$]. Conversely, when the pH approaches pK_2, the $K_1^cK_2^c$ term becomes significantly greater than $[H^+]^2$ and then $(2\alpha + \beta)$ becomes less than 1.

It is interesting to note that the above equations also apply for the calculation of the stability constants of glycine, but because complexation takes place in a range intermediate between two widely separated dissociation constants, $[H^+]K_1^c$ is the only significant term in D. Therefore $(2\alpha + \beta)$ is approximately 1 and $K_1^cK_2^c/D$ in Eq. 10c reduces to $K_2^c/[H^+]$.

[3] The buffer equation without approximations is

$$[H_3O^+] = K_1^c \frac{C_{acid} - [H_3O^+] + [OH^-]}{C_{base} + [H_3O^+] - [OH^-]}$$

For the titrations in this work $A^\circ - [K^+]$ must be added to the numerator and subtracted from the denominator. $C_{acid} = T^\circ$ and $C_{base} = 0$.

[4] The activity coefficients were chosen arbitrarily using citrate as a model. At an ionic strength of 0.01, however, the entire range of activity coefficients corresponding to the effective size of the hydrated ions from 3 to 8 Å. will give only slightly different thermodynamic dissociation constants, which are well within the accuracy reported.

TABLE I.—COMPLEXATION OF TETRACYCLINE · HCl ANALOGS WITH CUPRIC ION

Reference	pK_1^T	pK_2^T	pK_3^T	log β_1	log β_2	Ionic Strength and Temperature	
Tetracycline·HCl							
This work[a]	3.33	7.70	9.50	7.80	13.13	0.01	25°
(5)	3.35	7.82	9.57	7.8	12.8	0.01	20°
(6)	3.69	7.63	9.24	7.5	12.8	0.01	30°
(12)	3.30	7.68	9.69	...	...	...	25°
Chlortetracycline·HCl							
This work	3.27	7.43	9.33	7.31	12.41	0.01	25°
(4)	3.30	7.44	9.27	7.6	12.6	0.01	20°
(6)	3.66	7.40	9.06	7.5	12.4	0.01	30°
(12)	3.30	7.44	9.27	...	...	...	25°
Demethylchlortetracycline·HCl							
This work	3.30	7.16	9.25	7.85	13.30	0.01	25°
(6)	3.85	7.31	9.23	6.9	12.3	0.01	30°
4-Epi-chlortetracycline·HCl							
This work	3.65	7.65	9.2[b]	7.64	12.70	0.01	25°
(6)	4.07	7.56	9.26	7.6	...	0.01	30°
4-Epi-anhydrotetracycline·HCl							
This work	3.48	5.87	8.86	6.46	10.62	0.01	25°
(6)	4.38	...	8.95[c]	9.9	...	0.01	30°

[a] $pK^T_{1 \text{ and } 2}$ ± 0.03, pK^T_3 ± 0.06, log β ± 0.06. [b] Approximate value. [c] Only two dissociation constants reported.

The results obtained using the $\bar{n}$ comparison method, together with the results of previous workers, are presented in Table I. A series of runs showed our thermodynamic pKa's to be within ±0.03, and the log stoichiometric stability constants to be within ±0.06. Since an aqueous solution of chlortetracycline tends to degrade in weakly alkaline solution (pH 8), the third dissociation constant was determined by adding sufficient base to raise the pH from a point where the second dissociable hydrogen is about $^2/_3$ neutralized to a point where the third hydrogen is half neutralized. Six minutes were required for the pH to drop to a point which would correspond to the hydrogen ion concentration required to obtain the Albert and Stephens' value. The same procedure was used for 4-epi-chlortetracycline, but degradation occurs at such a rapid rate that only an approximate value for pK_3 can be given.

Rossotti and Rossotti (13) list at least fourteen methods for computing biligand stability constants from potentiometric $\bar{n}$ and $[HT^-]$ data. The method used in our work (after Speakman) tends to minimize the effect of low and high $\bar{n}$ values, whereas another method proposed by Irving and Rossotti (14) minimizes the effect of $\bar{n}$ values near 1. However, the test of any method is to put the calculated values for β_1 and β_2 back into Eq. 18 along with the $[HT^-]$ value at any given pH and calculate $\bar{n}$. If the $\bar{n}$'s calculated from Eq. 18 agree with those originally calculated from Eqs. 14 and 17 over the titration range, then β_1 and β_2 are accurate functions of the data. In the present work $\bar{n}$'s calculated at very low pH values tended to cause the formation curve to tail off, as can be seen in Fig. 2. Therefore the method of Speakman (11) was used to calculate the stability constants.

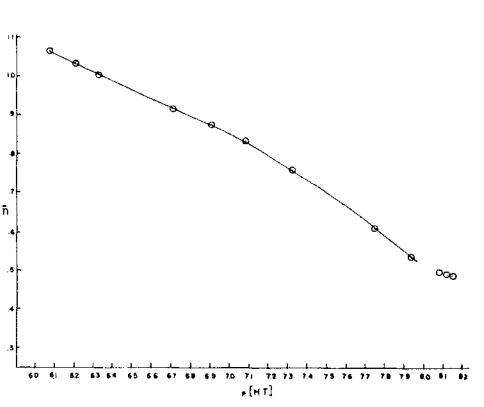

Fig. 2.—Formation curve for 4-epi-chlortetracyline · HCl.

In the present work, contrary to the results reported by Doluisio and Martin (6), the biologically inactive tetracycline derivatives were found to form 2:1 complexes (two ligands to each cupric ion). However, these complexes are highly insoluble, and precipitate after a very small amount is formed. The formation curve for 4-epi-chlortetracycline, as shown in Fig. 2, is a smooth curve in the region of $\bar{n} = 1$, and does not indicate any tendency to level off when approaching $\bar{n} = 1.0$, as would be expected for a 1:1 chelate.[5]

SUMMARY

The Bjerrum ΔKOH method for determining $\bar{n}$ from the titration curves of a ligand with and without metal was derived without approximations. The Calvin and Wilson method was rederived in a form which allows easy comparison between the

[5] In subsequent titrations run with 4-epi-chlortetracycline, using a higher ligand to metal ratio ($8T°:1M°$), $\bar{n}$ values up to 1.5 were obtained before interference by the third dissociating hydrogen occurs. The ability to obtain $\bar{n}$ values up to 1.5 dispels the ambiguities created by the insolubility of the epimer chelates, indicating that 2:1 chelates do form. In light of the work of Leeson *et al.* (15), showing that Stephens' assignment of the second and third dissociating hydrogens are reversed, it is reasonable to assume that chelation takes place at the phenolic diketone group, and therefore, there seems to be no chemical argument for epimerization interfering with 2:1 chelation.

values calculated by each method. It was shown that ΔKOH at any pH was equal to a function containing the hydrogen and hydroxide ion concentrations, dissociation constants, the stoichiometric concentrations of ligand and acid, and the concentration of potassium hydroxide in the ligand-metal titration. Comparison of $\bar{n}$ values calculated by the two methods gives a check on the validity of the data and for the concentration of ligand.

It was pointed out that calculations for tetracycline analogs at a high pH would be inaccurate since a dissociable hydrogen on a complexed molecule, but remote from the site of coordination, will have a larger dissociation constant than the same hydrogen on a free ligand.

The thermodynamic dissociation and stoichiometric stability constants were calculated for five tetracycline analogs with cupric ion and these values were compared with the data of previous workers. Contrary to the conclusions of Doluisio and Martin, the biologically inactive analogs, 4-epi-chlortetracycline and 4-epi-anhydrotetracycline, were found to form 2:1 complexes, which were quite insoluble and which precipitated at $\bar{n}$ values just above 1. Methods for the calculations of the biligand stability constants were discussed.

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Nonclassical Antimetabolites XX

Simulation of 5′-Phosphoribosyl Binding IV. Attempted Simulation with Nucleoside-5′-carbamates

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Twenty-two different ways can be envisioned for the mode of binding of the phosphate moiety of a nucleotide to an enzyme depending upon whether one, two, or three phosphate oxygens are involved and the specific groups involved on the enzyme are disregarded. A nucleoside-5′-carbamate should be able to simulate 13 of these possibilities, that is, all the modes of binding involving hydrogen bonds, but not anionic–cationic interactions. 5′-*O*-Carbamoylthioinosine (XII*a*) was synthesized, but failed to simulate the binding of thioinosinic acid (IV) to succinoadenylate kinosynthetase. Similarly, 5′-*O*-carbamoyl-2′-deoxy-5-fluorouridine (XXIV) was synthesized and failed to simulate the binding of 2′-deoxy-5-fluoro-5′-uridylic acid (VII) to thymidylate synthetase. In conjunction with some previous data, it has been concluded that the most likely mode of binding of nucleotides to these two enzymes involves one hydrogen bond and one anionic–cationic interaction of which there are four possible combinations.

IN THE FIRST paper of this series (1) on simulation of phosphate binding, the underlying biochemical reasons for the utility of this simulation were discussed; in a relatively simple assay system, that is, simulation of the inhibitory properties of 5′-adenylate on lactic dehydrogenase and glutamic dehydrogenase by 9H-adenine-9-yl alkanoic acids, the results were considered to be successful. However, later attempts to obtain phosphate simulation in binding to thymidylate synthetase with uracil-1-alkanoic acids and their derivatives were unsuccessful (2). It was concluded (2) that a more systematic study on the groups of a 5′-phosphoribosyl moiety necessary for binding to the enzyme would be required before relatively simple side chains could be used for binding in place of the 5′-phosphoribosyl binding; such a study with succinoadenylate kinosynthetase has been reported recently (3). Another approach to the simulation of phosphate binding of a nucleotide would be the replacement of the phosphate group on a known nucleotide

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Previous paper: Baker, B. R., and Tanna, P. M., *J. Pharm. Sci.*, **54**, 845(1965).