facetamide and sulfabenzamide were estimated to be 13.4 and 56.8 M^{-1}, respectively. The I molecules appear to have 1.28 binding sites for these compounds under the described conditions.

In conclusion, the fluorescent probe technique can provide a simple, sensitive, and rapid determination of the extent and nature of binding between sulfonamides and I. The technique, however, may not be applicable for some drugs such as sulfanilamide.

REFERENCES

(1) G. Weber, *Biochem. J.*, **51**, 155 (1952).

(2) R. F. Chen, in "Fluorescent Theory, Instrumentation and Practice," G. C. Guilblaut, Ed., Dekker, New York, N.Y., 1967, p. 443.

(3) H. F. Fischer, *Proc. Natl. Acad. Sci. USA*, **51**, 1285 (1964).

(4) S. F. Velick, *J. Biol. Chem.*, **223**, 1455 (1958).

(5) S. F. Velick, S. W. Parker, and H. N. Eisen, *Proc. Natl. Acad. Sci. USA*, **46**, 1470 (1960).

(6) D. J. R. Laurence, *Biochem. J.*, **51**, 168 (1952).

(7) K. Kono, T. Nagai, and H. Nogami, *Chem. Pharm. Bull.*, **21**, 366 (1973).

(8) T. Kinoshita, F. Linumd, I. Moriguchi, and A. Tsuji, *ibid.*, **19**, 861 (1971).

(9) H. W. Jun, R. T. Mayer, C. M. Himel, and L. A. Luzzi, *J. Pharm. Sci.*, **60**, 1821 (1971).

(10) H. W. Jun, L. A. Luzzi, and P. L. Hsu, *ibid.*, **61**, 1835 (1972).

(11) P. L. Hsu, J. K. H. Ma, H. W. Jun, and L. A. Luzzi, *ibid.*, **63**, 27 (1974).

(12) H. W. Jun, L. A. Luzzi, and J. K. H. Ma, *ibid.*, **64**, 493 (1975).

(13) L. Stryer, *J. Mol. Biol.*, **13**, 482 (1965).

(14) G. M. Edelman and W. O. McClure, *Acc. Chem. Res.*, **1**, 65 (1968).

(15) "Scientific Tables," K. Diem and C. Lenter, Eds., 7th ed., Ciba-Geigy, Basle, Switzerland, 1971, p. 281.

(16) D. V. Naik, W. L. Paul, R. M. Threatte, and S. G. Schulman, *Anal. Chem.*, **47**, 267 (1975).

(17) G. Weber and L. B. Young, *J. Biol. Chem.*, **239**, 1415 (1964).

(18) C. T. Dollery, D. Emslie-Smith, and D. F. Muggleton, *Br. J. Pharmacol. Chemother.*, **17**, 488 (1941).

(19) I. M. Klotz, H. Triwush, and F. M. Walker, *J. Am. Chem. Soc.*, **70**, 2935 (1948).

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Rate and Proposed Mechanism of Anhydrotetracycline Epimerization in Acid Solution

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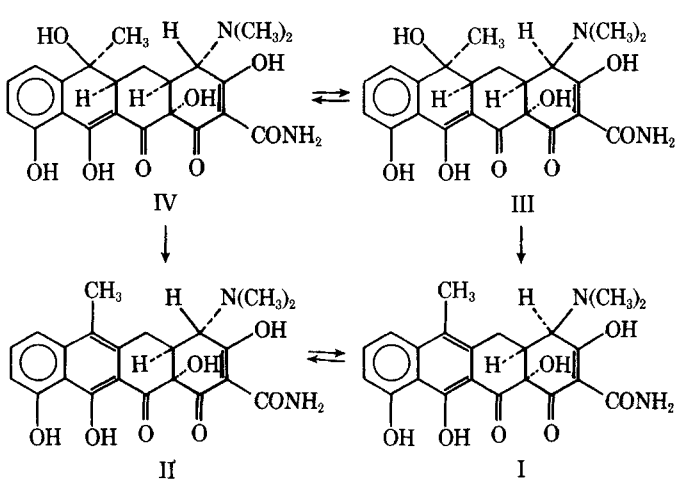
Abstract □ The pathway through which the toxic tetracycline degradation product epianhydrotetracycline forms in solution was studied using high-performance liquid chromatography and circular dichroism, taking advantage of the large difference in ellipticity between the reactant and the product at 285 nm. The epimerization of anhydrotetracycline followed a reversible first-order process, and both analytical methods yielded the same rate constants. The rate constants indicate that anhydrotetracycline epimerization is faster than tetracycline epimerization. The equilibrium favored anhydrotetracycline, and the activation energies for the forward and reverse rates were almost the same as those for tetracycline epimerization. The epimerization was catalyzed by phosphate. Activation energies in 0.1 and 1 *M* phosphate were essentially the same. The equilibrium constants for both anhydrotetracycline and tetracycline favored the natural configuration rather than the epi series. Possible rationalization based on conformational and hydrogen bonding effects is presented.

Keyphrases □ Anhydrotetracycline—epimerization, kinetics and mechanism in acid solution, high-performance liquid chromatographic and circular dichroism study □ Tetracycline degradation—kinetics and mechanism of anhydrotetracycline epimerization in acid solution, high-performance liquid chromatographic and circular dichroism study □ Epimerization—anhydrotetracycline in acid solution, kinetics and mechanism, high-performance liquid chromatographic and circular dichroism study □ High-performance liquid chromatography—study of epimerization of anhydrotetracycline in acid solution □ Circular dichroism—study of epimerization of anhydrotetracycline in acid solution □ Antibacterials—tetracycline degradation, epimerization of anhydrotetracycline in acid solution

Among the degradative pathways of the tetracyclines, those leading to the toxic epianhydrotetracycline by epimerization and dehydration are of special concern. Formation of epianhydrotetracycline (I) from tetracycline (IV) occurs along two routes (Scheme I). Epimerization of tetracycline occurs *via* a reversible first-order process between pH 3 and 5 (1–4). Dehydration of tetracycline to anhydrotetracycline (II) and of epitetracycline (III) to epianhydrotetracycline occurs *via* a pseudo-first-order process at low pH (5–7).

The epimerization of anhydrotetracycline is the only reaction of Scheme I whose kinetics have not yet been reported, probably because of a lack of a suitable method of



Scheme I

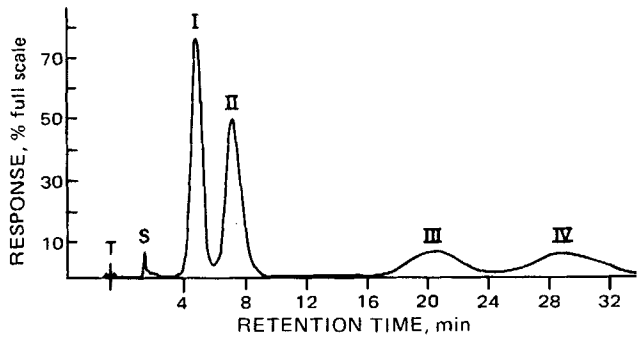


Figure 1—*High-performance liquid chromatogram for a mixture of tetracycline (IV), epitetracycline (III), anhydrotetracycline (II), and epianhydrotetracycline (I). The T is the time of injection, and S is the solvent peak. Four microliters of a solution containing 0.466 mg/ml of IV, 0.346 mg/ml of III, 0.436 mg/ml of II, and 0.512 mg/ml of I was injected with instrument attenuation at 0.08 absorbance unit. Flow rate was 1.08 ml/min (2000 psi).*

analysis for the reactant and the product. However, separation of tetracycline from its degradation products and from rolitetracycline by liquid chromatography recently was described (8–11). The present study utilized a different liquid chromatographic system, as suggested previously[1]. The other potential method to study the reaction is circular dichroism, as suggested by previous results which showed large differences in the ellipticity of anhydrotetracycline and epianhydrotetracycline at 285 nm in 0.03 *N* HCl (12). It thus seems feasible to follow the rate of epimerization by following the rate of change in ellipticity at this wavelength.

EXPERIMENTAL

Materials—Tetracycline hydrochloride[2], 4-epitetracycline ammonium salt[3], anhydrotetracycline hydrochloride[4], and 4-epianhydrotetracycline[5] were used as obtained. All other chemicals were reagent grade.

Apparatus—High-performance liquid chromatographic[6] (HPLC) studies were made using a UV detector at 254 nm with a 1-m × 2.1-mm i.d. column[7]. Circular dichroism measurements[8] were made using a jacketed 1-cm pathlength cell at constant temperature[9]. Visible and UV light[10] absorption measurements were made using a thermostated 1-cm cell.

Chromatographic Methods—For the separation and quantification of anhydrotetracycline and epianhydrotetracycline, the following method was used. The mobile phase consisted of 0.07 *M* sodium phosphate and 0.008 *M* ethylenediaminetetraacetic acid adjusted to pH 7. The degassed solvent was stored in the solvent reservoir of the instrument[6]. The instrument, with a 1-m cation-exchange column[7], was operated at a flow rate of 1 ml/min (1800 psi) at ambient temperature. Known amounts of anhydrotetracycline and epianhydrotetracycline dissolved in 0.03 *N* HCl were placed in an ice bath, and known volumes (2–5 μl) were withdrawn and injected into the chromatographic column using a 5-μl high-pressure syringe. The resulting areas under the chromatograms were measured using a planimeter.

The procedure used in the kinetic studies was as follows. Monobasic potassium phosphate solution at a given concentration was adjusted to pH 4.2 with concentrated hydrochloric acid. The solution was equilibrated at the desired temperature. Appropriate amounts of anhydrotetracycline were weighed into flasks and brought to volume with the phosphate solution. Final concentrations ranged from 7.7 to 13 × 10^{-4} *M*. The flask was immediately placed in a water bath, and a fixed volume (3.5–5 μl) was injected onto the chromatographic column at about 10-min intervals for the first 2 hr and at 30-min intervals thereafter until equilibrium was established. The area under the response (0.08 absorbance unit full scale) *versus* time chromatograms for anhydrotetracycline (and epianhydrotetracycline) were measured and equated to the concentration through a standard curve.

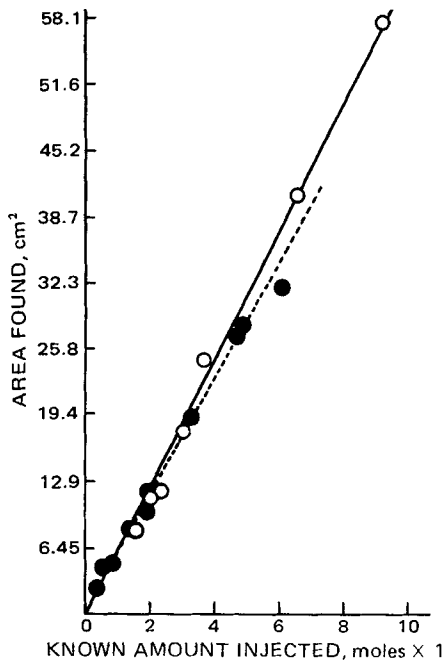


Figure 2—*Relation between the area under the HPLC peak and the amount injected for anhydrotetracycline (●, - - -) and epianhydrotetracycline (○, —).*

Spectropolarimetric Methods—In the rate studies, stock solutions of anhydrotetracycline or epianhydrotetracycline were prepared by weighing each into separate 10-ml volumetric flasks. The materials were dissolved and brought to volume with 0.03 *N* HCl at room temperature, and the solutions were immediately refrigerated. One or 2 ml of the stock solution was diluted to 50 ml with phosphate solution (pH 4.2). The ellipticity of the various concentrations of pure anhydrotetracycline or epianhydrotetracycline was then measured at 285 nm.

Phosphate solution was prepared by adding concentrated hydrochloric acid to several different concentrations of monobasic potassium phosphate to give pH 4.2. Salt effects were studied by adjusting ionic strength with potassium chloride. The phosphate solution was equilibrated at the temperature to be used before mixing with a known volume of stock solution of anhydrotetracycline, resulting in solutions having a concentration of approximately 3 × 10^{-5} *M*. The solution was immediately transferred to a thermostated circular dichroism cell, and the change in ellipticity was measured continuously for about 5–6 hr at 285 nm. An equilibrium ellipticity was determined about 15 hr later.

This same procedure was repeated using tetracycline or epitetracycline as the starting material. The ellipticity of the solutions was measured as a function of time at 270 nm, and standard curves for pure materials were also determined at this wavelength. At a wavelength of 270 nm and pH

Table I—Rate Constants for the C-4 Epimerization of Anhydrotetracycline in 0.1 *M* Phosphate at pH 4.2 and Several Temperatures Studied Using HPLC

Temperature ±2.0°	Forward Reaction Rate Constant, $k_1 \times 10$ hr^{-1}	Reverse Reaction Rate Constant, $k_{-1} \times 10$ hr	Equilibrium Constant
45°	0.89 (0.07)[a]	1.82 (0.13)	0.489
50°	1.35 (0.10)	2.67 (0.21)	0.506
55°	2.38 (0.66)	4.85 (1.38)	0.491
60°	3.55 (0.23)	7.27 (0.50)	0.488

[a] Parentheses contain root mean square error of two determinations.

[1] G. Manning, Alza Corp., Palo Alto, CA 94304, July 1972, personal communication.
[2] Lot 420433, Parke, Davis and Co.
[3] Batch 430, British Pharmacopoeia Commission.
[4] Batch 428, British Pharmacopoeia Commission.
[5] GS-6659, Lot 3339-99-1, Pfizer Inc., and Batch 429, British Pharmacopoeia Commission.
[6] DuPont model 830 high-performance liquid chromatograph.
[7] DuPont SCX column.
[8] Durrum Jasco ORD/UV-5 spectropolarimeter with the Sprowl Scientific SS-20-2 modification.
[9] MGM Lauda K2 water bath.
[10] Cary model 16 spectrophotometer.

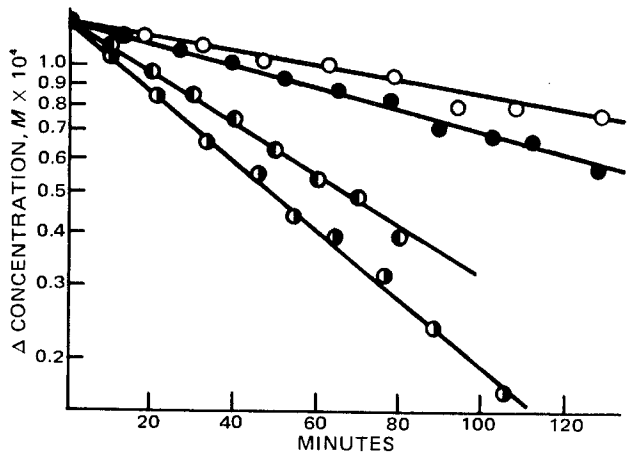


Figure 3—*Relation between the logarithm of the difference in concentration of anhydrotetracycline at time* t *and its equilibrium concentration and the time for anhydrotetracycline epimerization at pH 4.2 in 0.1* M *phosphate. Anhydrotetracycline and its epimer were separated by HPLC and detected using UV spectrophotometry at 45° (○), 50° (●), 55° (◐), and 60° (◑).*

4.2, there was a large difference in the circular dichroism spectra of tetracycline and epitetracycline, similar to those found in 0.03 *N* HCl (12). Visible and UV absorbances at 427 and 269 nm for epianhydrotetracycline and anhydrotetracycline in 0.1 *M* phosphate at 60° were measured as a function of time by withdrawing samples from solutions at 30-min intervals.

Circular dichroism studies of the anhydrotetracycline epimers at several pH values were conducted by making stock solutions at 0.003% in 0.03 *N* HCl. The materials were dissolved in approximately 2 ml of methanol and diluted to 100 ml with 0.03 *N* HCl. To 5.0 ml of stock solution was added 5.0 ml of 0.001 *N* HCl, and the pH was adjusted to its final value (1.00, 3.25, 7.25, and 9.25) with a few drops of strong hydrochloric acid or sodium hydroxide. The circular dichroism spectrum of each sample was determined immediately. A solvent blank was made in the same manner but without drug.

RESULTS

The HPLC procedure provided adequate separation of anhydrotetracycline and epianhydrotetracycline within 10 min and also of tetracycline and epitetracycline after 30 min (Fig. 1). When the area under the chromatograms for anhydrotetracycline and its epimer was measured for known amounts of material injected, it accurately represented the amount present (Fig. 2). The sensitivity achievable at 0.08 absorbance unit full scale permits analysis of compounds in concentrations of 10^{-5}–10^{-4} *M*.

With the HPLC procedure, solutions of anhydrotetracycline kept at various temperatures and phosphate concentrations were analyzed for anhydrotetracycline (and epianhydrotetracycline) content by the measurement of areas under the chromatograms. It was assumed that, like tetracycline, the epimerization followed a reversible first-order process where:

$$\ln\left(\frac{C_0 - C_\infty}{C_t - C_\infty}\right) = (k_1 + k_{-1})t \qquad \text{(Eq. 1)}$$

Table II—Activation Energies for the C-4 Epimerization of Anhydrotetracycline at pH 4.2 in Phosphate

Buffer Concentration, *M*	Reaction	Activation Energy[a], kcal/mole: HPLC Results	Activation Energy[a], kcal/mole: Circular Dichroism Results
0.1	Forward	19.9 (15.8–24.1)	18.6 (15.7–21.5)
0.1	Reverse	20.0 (14.6–25.4)	18.6 (15.7–21.6)
1	Forward		17.9 (17.3–18.6)
1	Reverse		17.5 (17.1–17.9)

[a] Parentheses contain the upper and lower 95% confidence limits obtained from linear regression analysis.

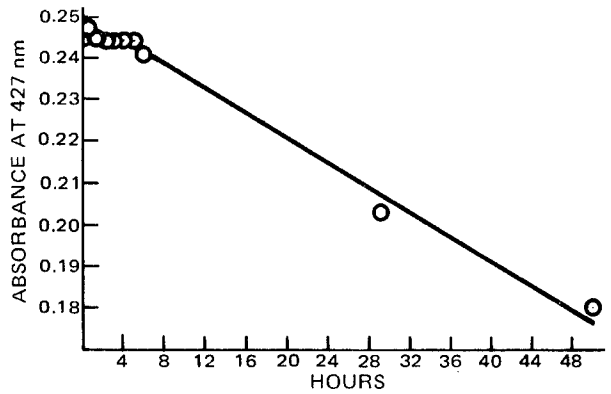


Figure 4—*Absorbance of a solution of anhydrotetracycline at 427 nm as a function of time at 60° in 0.1* M *phosphate.*

describes the kinetic reaction where C_0, C_t, and C_∞ are the concentrations of anhydrotetracycline at times 0, t, and equilibrium, respectively; k_1 and k_{-1} are the forward and reverse rate constants, respectively; and t is time. Based on Eq. 1, a semilogarithmic plot of the difference in concentration ($C_t - C_\infty$) *versus* time should be linear with a slope of ($k_1 + k_{-1}$).

Figure 3 plots the results of such studies at four temperatures. The sum of the rate constants together with the equilibrium constants obtained after about 10 half-lives permitted the calculation of the forward and reverse rate constants (Table I). Based on the data of Table I, the activation energies for the reaction were calculated (Table II). A precipitate formed in the solutions upon storage after epimerization, particularly at higher temperatures after standing for a few days. A study of the absorbance of anhydrotetracycline at 427 nm at 60° in 0.1 *M* phosphate showed that the content of anhydrotetracyclines decreased after a lag phase of about 6 hr (Fig. 4). The wavelength chosen should reflect total anhydro solution content (12).

Although the HPLC procedure is acceptable for following epimerization kinetics, the reaction was also studied utilizing the large difference in ellipticity of anhydrotetracycline and epianhydrotetracycline at around 285 nm (Fig. 5). The results obtained when ellipticity at 285 nm was measured as a function of time at various temperatures and phosphate

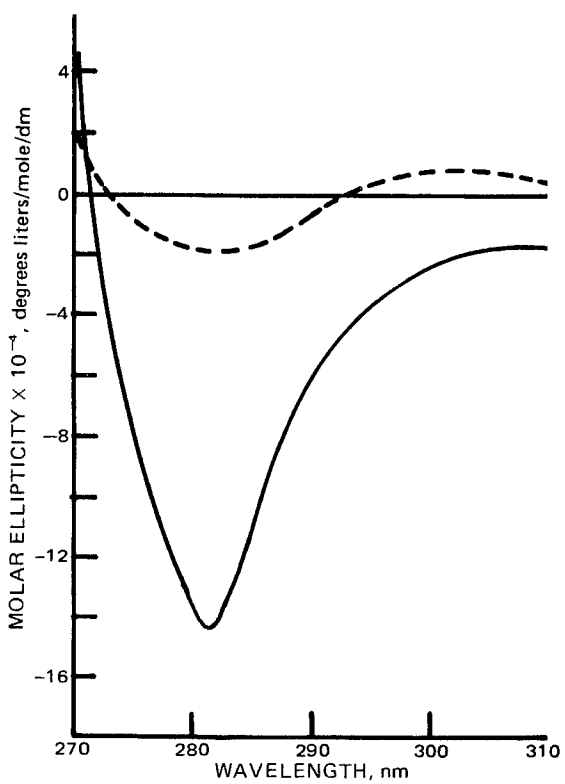


Figure 5—*Molar ellipticity of epianhydrotetracycline (—) and anhydrotetracycline (- - -) as a function of wavelength at pH 4.2 in 0.1* M *phosphate at ambient temperature (29°).*

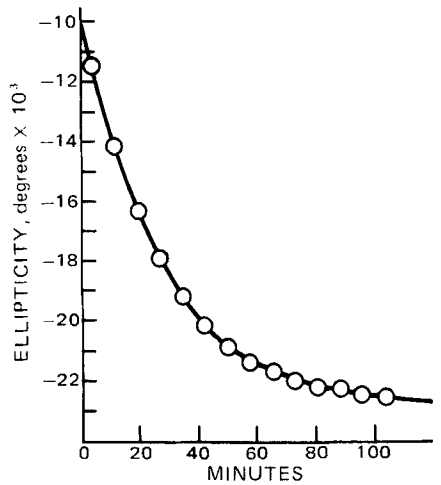


Figure 6—*Relation between ellipticity at 285 nm and time for anhydrotetracycline epimerization at pH 4.2 in 1* M *phosphate at 50°.*

concentrations are typified by those given in Fig. 6. When such data are treated assuming a reversible first-order process following the relationship:

$$\ln \frac{\psi_0 - \psi_\infty}{\psi_t - \psi_\infty} = (k_1 + k_{-1})t \qquad \text{(Eq. 2)}$$

for the reaction anhydrotetracycline ⇄ epianhydrotetracycline, where the ψ values are the ellipticities in degrees with subscripts 0, t, and ∞ referring to initial, time t, and equilibrium values, respectively, and k_1, k_{-1}, and t are as defined for Eq. 1, a linear relationship should be obtained when $\ln (\psi_t - \psi_\infty)$ is plotted as a function of time. The ψ_∞ is obtained after about 10 half-lives. A linear relationship was found for studies made at five different temperatures in 1 *M* phosphate solution (Fig. 7). The slopes of these lines provide the sum of the rate constants. The equilibrium constant, K, can be obtained from:

$$K = \frac{k_1}{k_{-1}} = \frac{\psi_0 - \psi_\infty}{\psi_\infty - bC_0} \qquad \text{(Eq. 3)}$$

where b relates the concentration of epianhydrotetracycline to its ellipticity, C_0 is the initial concentration of anhydrotetracycline, and the other terms are as defined previously. By using Eqs. 2 and 3, it is possible to get the forward and reverse rate constants. The constant b was obtained from a linear relationship found for ellipticity as a function of epianhydrotetracycline concentration; b has a value of $-975°/M$. The similar constant for anhydrotetracycline is $-186°/M$. The forward and reverse rate constants for studies made in 1 *M* phosphate using Eqs. 2 and 3 are given in Table III, and the activation energy values calculated using these data are found in Table II.

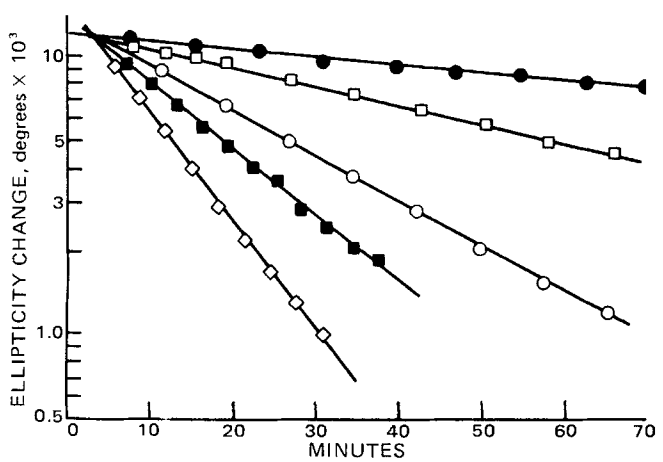


Figure 7—*Semilogarithmic relation between the change in ellipticity (time* t *minus equilibrium value) and the time for anhydrotetracycline epimerization at 30° (●), 40° (□), 50° (○), 55° (■), and 60° (◇). Ellipticity is measured at 285 nm in solutions at pH 4.2 (1* M *phosphate).*

Table III—Rate Constants for the C-4 Epimerization of Anhydrotetracycline in 1 *M* Phosphate at pH 4.2 Studied Using Circular Dichroism at Several Temperatures

Temperature ±2°	Forward Reaction Rate Constant, hr^{-1}	Reverse Reaction Rate Constant, hr^{-1}	Equilibrium Constant
30°	0.118 (0.111–0.125)[a]	0.244 (0.226–0.255)	0.484
40°	0.303 (0.301–0.311)	0.616 (0.605–0.625)	0.492
50°	0.754 (0.735–0.760)	1.50 (1.48–1.52)	0.503
55°	1.10 (1.09–1.12)	2.18 (2.15–2.21)	0.505
60°	1.76 (1.74–1.80)	3.57 (3.49–3.63)	0.493

[a] Parentheses contain the upper and lower 95% confidence limits obtained from linear regression analysis.

Studies made at lower phosphate concentrations, where the reaction was slower, gave ellipticity *versus* time plots for which it was difficult to estimate an infinity ellipticity, probably because of interference from the secondary reaction resulting in precipitation, as observed in the HPLC studies. As a consequence, the epimerization reactions occurring in 0.1 *M* phosphate were analyzed differently than those previously presented. In these cases, an equilibrium ellipticity was calculated from the equilibrium constant found in Tables I and III. The equilibrium constant did not change with temperature, and values obtained in lower temperature studies were considered reliable since the precipitation reaction was minimal. By using such calculated values and only ellipticities at times shorter than 2 hr, semilogarithmic plots of $\psi_t - \psi_\infty$ *versus* time were constructed (Fig. 8). The plots showed curvature at longer times, but rate constants calculated from the slopes of the linear portion of such lines together with the equilibrium constant (*via* Eq. 3) were in excellent agreement with the rate constants calculated from initial rate data. Initial rates were obtained from plots of ellipticity *versus* time data (Table IV). Activation energies calculated from the data of Table IV are given in Table II.

In addition to the rate studies made at 0.1 and 1.0 *M* phosphate at pH 4.2, studies using circular dichroism were made at 50° and at a constant

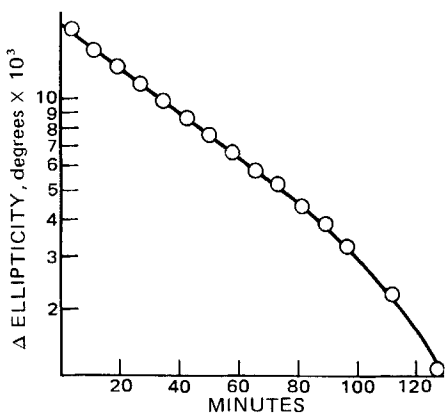


Figure 8—*Relation between the logarithm of the change in ellipticity for solutions of anhydrotetracycline at time* t *and that estimated for equilibrium and the time at 60°, pH 4.2 (0.1* M *phosphate).*

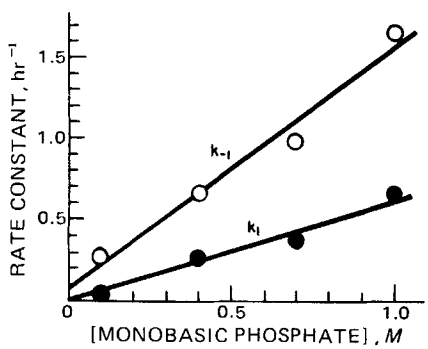


Figure 9—*Forward (●) and reverse (○) rate constants for anhydrotetracycline epimerization as a function of phosphate concentration at pH 4.2 at 50° and constant ionic strength (1* M*).*

Table IV—Rate Constants for the C-4 Epimerization of Anhydrotetracycline in 0.1 *M* Phosphate at pH 4.2 Studied Using Circular Dichroism at Several Temperatures

Temperature ±2°	Forward Reaction Rate Constant, $k_1 \times 10\ hr^{-1}$ ± Average Error[a]		Reverse Reaction Rate Constant, $k_{-1} \times 10\ hr^{-1}$ ± Average Error[a]	
	From Semilog Data	From Initial Rates	From Semilog Data	From Initial Rates
43.5°	0.945 ± 0.001[b]	0.91 ± 0.02[b]	1.93 ± 0.001[b]	1.89 ± 0.03[b]
48.5°	1.59	1.56	3.25	3.18
53°	2.40 ± 0.008[b]	2.44 ± 0.03[b]	4.89 ± 0.018[b]	4.98 ± 0.08[b]
58°	3.58 ± 0.022[c]	3.66 ± 0.25[c]	7.32 ± 0.050[c]	7.47 ± 0.98[c]

[a] Calculated as the root mean square. [b] Average of two values. [c] Average of three values.

ionic strength (1 *M*) at various total phosphate concentrations (0.1–1 *M*). A plot of the rate constants obtained as a function of monobasic phosphate concentration (essentially the total phosphate) gave the results shown in Fig. 9.

For comparative purposes, the epimerization of tetracycline was studied making use of the difference in spectra between tetracycline and epitetracycline at 270 nm. This difference was previously reported in 0.03 *N* HCl and substantiated at pH 4.2 (12). When starting with either tetracycline or epitetracycline, the change in ellipticity was treated as a reversible first-order process as reported in several studies (1–4). With Eqs. 2 and 3, modified for the reaction tetracycline $\rightleftarrows$ epitetracycline, where b for tetracycline and for epitetracycline is -521.6 and $376.6°/M$, respectively, excellent conformance with the reversible first-order process was obtained (Fig. 10), giving the rate constants listed in Table V.

The relations between molar ellipticity and wavelength found for anhydrotetracycline and epianhydrotetracycline at four pH values (1.0, 3.25, 7.25, and 9.25) are presented in Figs. 11 and 12.

DISCUSSION

The HPLC method used to separate tetracycline and its degradation products is different from that reported previously (8, 9, 11). The separation achievable for anhydrotetracycline and epianhydrotetracycline allows a valid and convenient study of the epimerization, since peaks are sharp and retention times are small. The procedure used may not be as convenient in a study of tetracycline epimerization, since bandwidths are broad and retention times are longer for tetracycline and its epimer.

With HPLC, epimerization of anhydrotetracycline complies with a reversible first-order process just as does the epimerization of tetracycline (2–4, Fig. 10). A reaction other than epimerization occurs for anhydrotetracycline at pH 4.2 and results in precipitation. Such a reaction occurs slowly at high temperatures but occurs even at lower temperatures at apparently slower rates. Identification of this product(s) is being undertaken.

Circular dichroism provides an especially convenient way to study the epimerization reaction. It is only necessary to place a solution of either anhydrotetracycline or its epimer in the instrument cell and to follow the change of ellipticity as a function of time at a fixed temperature. The results obtained for the rate constants using circular dichroism were in very close agreement with those obtained using HPLC (Tables I and IV), showing that the circular dichroism method is perfectly valid. The difference between the methods averaged 7% (2–12% range), which was the expected error limit with either method.

The secondary reaction especially observable at high temperatures complicated analysis of circular dichroism data for slower reactions (lower phosphate concentrations). However, this reaction is sufficiently slow and has an induction period (Fig. 4) that permits analysis of ellipticity changes at early times (40–60 min) as an uncomplicated reversible first-order process. It is valid to use early data points because it is possible to calculate expected infinity ellipticity values. The reason for this approach is that the equilibrium constant for this reaction was found to be independent of temperature (Table I), and reliable equilibrium constants can be obtained at lower temperatures where the precipitation reaction is slow.

In comparison with tetracycline epimerization, anhydrotetracycline is apparently faster by an average of 28% (Tables I, III, and V), and the energy of activation is perhaps 2 kcal/mole less than that of tetracycline when compared with reported values (2–4). Whether significant differences are present, however, is questionable because of the uncertainties in estimating energies of activation (Table II).

Just like tetracycline, phosphate catalyzes the epimerization of anhydrotetracycline (2, Fig. 9). Neither intercept obtained was significantly different from zero. These results support the hypotheses that anhydrotetracycline epimerization may be general base catalyzed and that, in the case of phosphate, the monobasic form is a likely candidate for the catalytic species.

The special role phosphate plays in these reactions is possibly a consequence of its interesting ability to act as a molecular proton conductor (Scheme II).

One interesting result of these studies and those of others (1–4) involving tetracycline is that the equilibrium favors the solution species (tetracycline or anhydrotetracycline) that would not be conformationally favored at acid pH, providing hydrogen bond formation is ignored. The results can be rationalized using the following argument.

Because of the flexible *cis*-A/B ring juncture, anhydrotetracyclines may adopt two opposite solution conformations in which both the A-ring

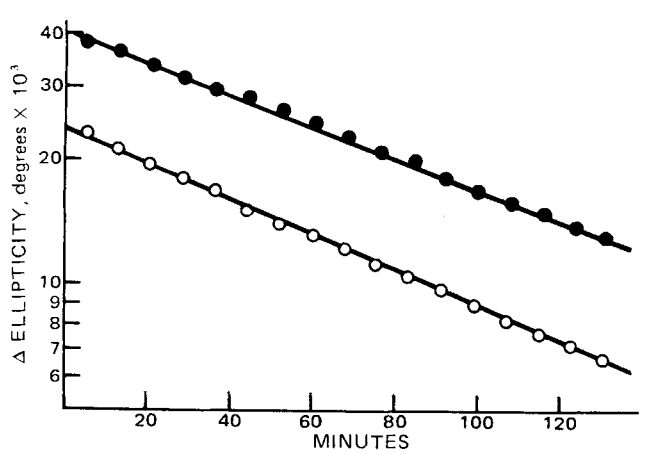
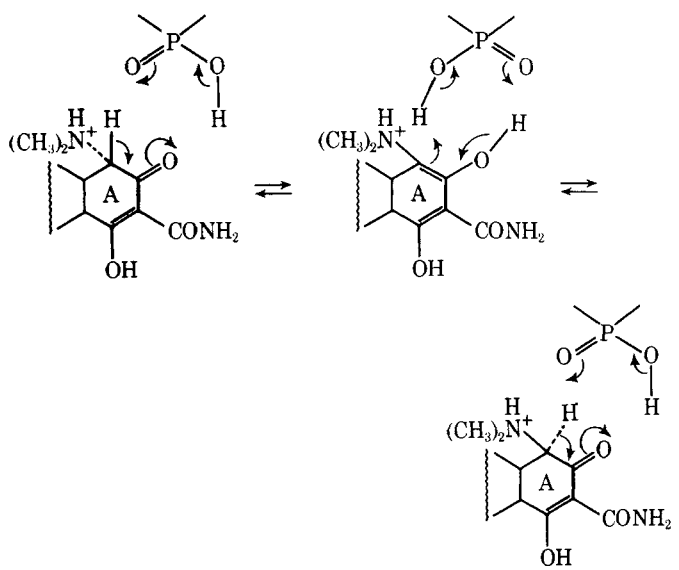
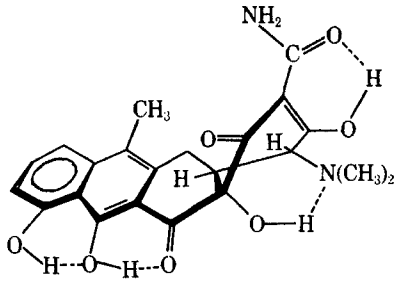


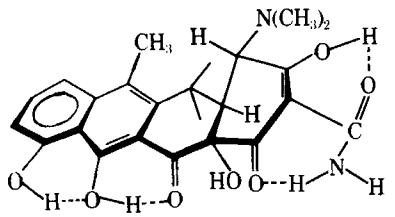
Figure 10—*Semilogarithmic plot of the difference in ellipticity (at time t and equilibrium) at 270 nm and the time using tetracycline (○) and epitetracycline (●) as the starting material. Study made at 40°, pH 4.2, and 1 M phosphate.*



Scheme II



IIa



IIb

and the BCD-ring chromophores are planar and in which there is no readily apparent bond angle strain (IIa and IIb). Conformation IIa is the same as that previously assigned to the parent antibiotic, tetracycline, as fitting best the bulk of the available data collected in neutral to moderately acidic solutions (12–14). Conformation IIb is that attributed to 5,12a-diacetyloxytetracycline in the crystal (15) and to 5-hydroxytetracyclines in alkaline solutions (12–14).

Neither conformation is the same as that adopted by various tetracycline antibiotic salts, as shown by X-ray studies (16–21) and circular dichroism studies in strongly acidic solutions (12–14). Under these conditions, a twisted form of conformation IIa is favored in which the $N(CH_3)_2$ group rotates slightly away from its interaction with the 12a-OH group and toward ring B. It does not, however, go all the way to conformation IIb. The only corresponding X-ray study of an anhydrotetracycline salt showed essentially conformation IIa (22). The reason why this conformation should be favored in the crystal of anhydrotetracycline hydrobromide is not readily apparent, because it is the only member of this family that clearly has conformation IIa.

In fact, conformation IIb would appear to be intuitively more satisfactory based on relief of the C-4–C-12a interaction and the resulting equatorial orientation of the dimethylamino group. Other independent evidence, however, rules this out. Dimethyl sulfoxide-d_6 solutions of various anhydrotetracyclines show a small H-4–H-4a coupling constant (5 Hz), which is consistent with conformation IIa or the twisted tetracycline conformation but not with conformation IIb. The circular dichroism spectra of anhydrotetracycline in aqueous methanol solutions at various pH's (Fig. 11) show that there is no dramatic conformational change below pH 7 and can be rationalized best by invoking either conformation IIa or, better, the twist form (16–21). The situation differs somewhat with 4-epianhydrotetracycline. The PMR spectrum of chelocardin (23), for example, shows a small (4.5 Hz) coupling constant.

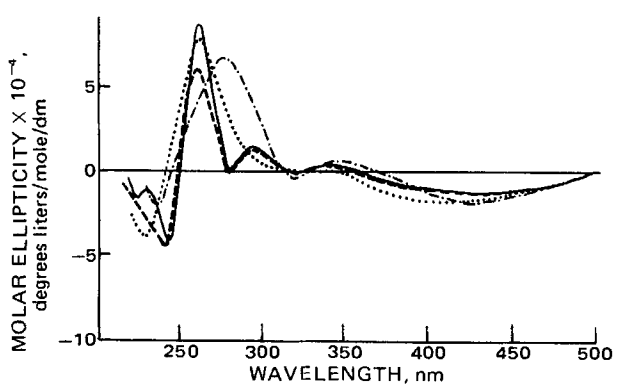


Figure 11—*Molar ellipticity of anhydrotetracycline as a function of wavelength at various pH values. Key: —, 1.0; - - -, 3.25; . . . . , 7.25; and . - . - ., 9.25.*

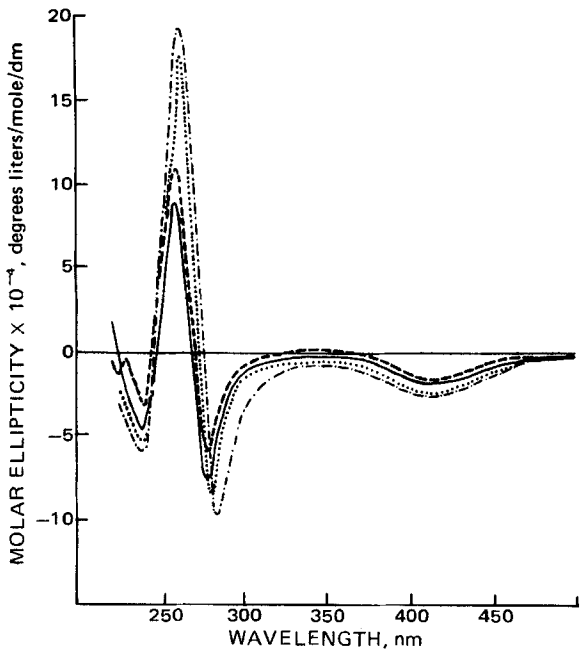


Figure 12—*Molar ellipticity of epianhydrotetracycline as a function of wavelength at various pH values. Key: —, 1.0; - - -, 3.25, ... , 7.25; and . - . - ., 9.25.*

However, either conformation IIa or IIb gives an intermediate sized J value, so PMR measurements of this type do not help.

The circular dichroism spectra of 4-epianhydrotetracycline do not change significantly with rising pH (Fig. 12), indicating a stable conformation, but differ significantly from that of anhydrotetracycline itself under the same conditions. In particular, at all pH levels measured, strong exciton coupling is evident (24, 25). This finding would be expected with conformation IIa, in which the A-ring chromophore is very close in space to the BCD chromophore so that such coupling is possible. Furthermore, the A-ring peak (λ_{max} 275 nm) is of the correct energy content to couple with the middle BCD-ring transition occurring at about the same wavelength. Such circular dichroism bands characteristically are biphasic and bilaterally symmetrical about the zero ellipticity line and have their amplitude median at approximately the same wavelength as the UV transition from which they are derived. Deviation from ideality is expected when they are superimposed on a noncoupled band.

Inspection of Fig. 12 shows that these relationships are obeyed and that the peaks in question are those at approximately 255 and 285 nm. Lack of such coupling with anhydrotetracycline is strong evidence for different conformations for the two series. Conformation IIb would be expected to allow exciton coupling also but with opposite signs. This lack, along with the other data described, suggests that anhydrotetracyclines possess the twist tetracycline conformation (16–21) in which the chromophores are fixed farther apart in space.

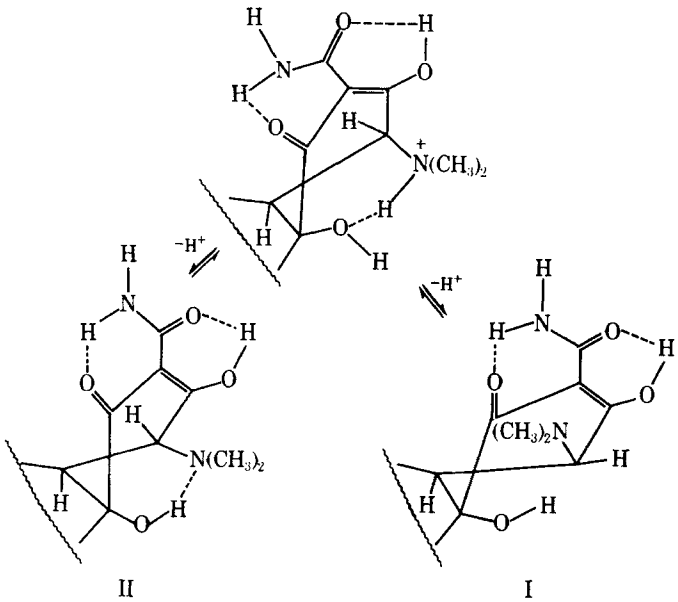
With anhydrotetracycline and 4-epianhydrotetracycline apparently possessing different ground-state conformations, the kinetic expression should ultimately contain contributions from conformational as well as configurational components. This situation involves a balance of subtle factors of presently indeterminable magnitude.

These two conformations allow an acceptable rationale for the long known, but unexplained, observation that the 4-epi series are stronger bases than the naturally occurring series (26). Intramolecular hydrogen bonding in the natural series would reduce the external availability of

Table V—Rate Constants for the C-4 Epimerization of Tetracycline in Phosphate at pH 4.2

Buffer Concentration, M	Temperature	Forward Rate Constant, hr^{-1} ± Average Error[a]	Reverse Rate Constant, hr^{-1} ± Average Error[a]
0.1	60°	0.311 ± 0.008[b]	0.508 ± 0.013[b]
1	40°	0.216 ± 0.012[c]	0.352 ± 0.019[c]

[a] Calculated as the root mean square. [b] Average of two values. [c] Average of three values.



Scheme III

the lone-pair electrons of the $N(CH_3)_2$ group to acids. It may be presumed that the molecule gains sufficient stabilization from this effect to overcome in part the destabilization induced by 1,3-diaxial steric bulk interactions, and this stabilization explains the small but definite preference for the natural configuration when at equilibrium (Scheme III). The picture is, however, somewhat further complicated by the superposition of a conformational equilibrium, since the two epimers do not have the same ground-state conformation.

REFERENCES

(1) J. R. D. McCormick, S. M. Fox, L. L. Smith, B. A. Bitler, J. Reichenthal, V. E. Origoni, W. H. Muller, R. Winterbottom, and A. P. Doerschuk, *J. Am. Chem. Soc.*, **79,** 2849 (1957).

(2) E. G. Remmers, G. M. Sieger, and A. P. Doerschuk, *J. Pharm. Sci.*, **52,** 752 (1963).

(3) D. A. Hussar, P. J. Niebergall, E. T. Sugita, and J. T. Doluisio, *J. Pharm. Pharmacol.*, **20,** 239 (1968).

(4) K. D. Schlecht and C. W. Frank, *J. Pharm. Sci.*, **62,** 258 (1973).

(5) H. J. Pryves, M.S. thesis, Columbia University, New York, N.Y., 1957.

(6) B. Hoener, T. D. Sokoloski, L. A. Mitscher, and L. Malspeis, *J. Pharm. Sci.*, **63,** 1901 (1974).

(7) K. D. Schlecht and C. W. Frank, *ibid.*, **64,** 352 (1975).

(8) A. G. Butterfield, D. W. Hughes, N. J. Pound, and W. L. Wilson, *Antimicrob. Ag. Chemother.*, **4,** 11 (1973).

(9) K. T. Tsuji, J. H. Robertson, and W. F. Beyer, *Anal. Chem.*, **46,** 593 (1974).

(10) A. G. Butterfield, D. W. Hughes, W. L. Wilson, and N. J. Pound, *J. Pharm. Sci.*, **64,** 316 (1975).

(11) K. Tsuji and J. H. Robertson, *ibid.*, **65,** 400 (1976).

(12) R. F. Miller, T. D. Sokoloski, L. A. Mitscher, A. C. Bonacci, and B. Hoener, *ibid.*, **62,** 1143 (1973).

(13) L. A. Mitscher, A. C. Bonacci, B. Slater-Eng, A. K. Hacker, and T. D. Sokoloski, *Antimicrob. Ag. Chemother.*, **1969,** 111.

(14) L. A. Mitscher, B. Slater-Eng, and T. D. Sokoloski, *ibid.*, **2,** 66 (1972).

(15) R. B. VonDreele and R. E. Hughes, *J. Am. Chem. Soc.*, **93,** 7290 (1971).

(16) J. Donahue, J. D. Dunitz, K. N. Trueblood, and M. S. Webster, *ibid.*, **85,** 851 (1963).

(17) Y. Takeuchi and M. J. Buerger, *Proc. Natl. Acad. Sci. USA*, **46,** 1366 (1960).

(18) S. Hirokawa, Y. Okaya, F. M. Lovell, and R. Pepinsky, *Acta Crystallogr.*, **12,** 811 (1959).

(19) S. Hirokawa, Y. Okaya, F. M. Lovell, and R. Pepinsky, *Z. Kristallogr.*, **112,** 439 (1959).

(20) H. Cid-Dresdner, *ibid.*, **121,** 170 (1965).

(21) K. Kamiya, M. Asai, Y. Wada, and M. Nishikawa, *Experientia*, **27,** 363 (1971).

(22) R. Restivo and G. J. Palantik, *Biochem. Biophys. Res. Commun.*, **36,** 621 (1969).

(23) L. A. Mitscher, J. V. Juvarkar, W. Rosenbrook, Jr., W. W. Andres, J. Schenck, and R. S. Egan, *J. Am. Chem. Soc.*, **92,** 6070 (1970).

(24) A. D. Buckingham and P. J. Stiles, *Acc. Chem. Res.*, **7,** 258 (1974).

(25) N. Harada and K. Nakanishi, *ibid.*, **5,** 257 (1972).

(26) N. E. Rigler, S. P. Bag, D. E. Leyden, J. L. Sudmeier, and C. N. Reilley, *Anal. Chem.*, **37,** 872 (1975).

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