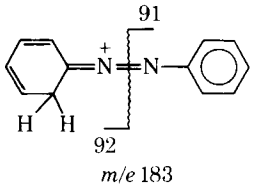


m/e 183

Scheme VI—Fragmentation of the m/e *183 ion*

the spectrum of oxyphenbutazone, analogous peaks were seen at *m/e* 108 (7.6%), $(C_6H_6NO)^+$, and at *m/e* 107 (11.2%), $(C_6H_5NO)^+$.

A characteristic fragmentation of all pyrazolidinediones studied was the loss of the elements of phenyl isocyanate either to form the radical ion $(C_6H_5NCO)^+$, *m/e* 119 [$(HOC_6H_5NCO)^+$, *m/e* 135 in the case of II] or as a neutral molecule. These pathways have been observed in the mass spectrum of aminopyrine (12). Fragmentations involving the loss of phenyl isocyanate as a neutral molecule are listed in Table II.

The ions (X, Table II) resulting from the loss of the elements of phenyl isocyanate as a neutral molecule may then lose CO to give ions $C_6H_5NCR_1R_2$ (Table III).

The remaining peaks in the low mass range, below *m/e* 100, are common to substituted aromatic systems, and the fragmentations observed are illustrated in Figs. 1–6.

REFERENCES

(1) J. Slingsby and D. A. Zuck, *Can. J. Pharm. Sci.*, **7**, 115(1972).

(2) D. V. C. Awang, A. Vincent, and F. Matsui, *J. Pharm. Sci.*, **62**, 1673(1973).

(3) B. Unterhalt, *Arch. Pharm.*, **305**, 334(1972).

(4) V. G. Pesin, A. M. Khaletskii, and T. Jun-Hsiang, *J. Gen. Chem. USSR*, **28**, 2841(1958).

(5) J. Büchi, J. Ammann, R. Lieberherr, and E. Eichenberger, *Helv. Chim. Acta*, **36**, 75(1953).

(6) A. M. Khaletskii, V. G. Pesin, and T. Jun-Hsiang. *J. Gen. Chem. USSR*, **28**, 2393(1958).

(7) H. Stenzl, U.S. pat. 2,562,830; through *Chem. Abstr.*, **47**, 1191(1953).

(8) J. H. Bowie, G. E. Lewis, and R. G. Cooks, *J. Chem. Soc., B*, **1967**, 621.

(9) J. M. Desmarchelier and R. B. Johns, *Org. Mass. Spectrom.*, **2**, 697(1969).

(10) D. P. Maier, G. P. Happ, and T. H. Regan, *ibid.*, **2**, 1289(1969).

(11) E. Larsen, I. H. Qureshi, and J. Møller, *ibid.*, **7**, 89(1973).

(12) W. Arnold and H. F. Grutzmacher, *Z. Anal. Chem.*, **247**, 179(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 30, 1974, from the *Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta, Canada, T6G 2H7*

Accepted for publication June 21, 1974.

Presented at the 21st Annual Canadian Conference on Pharmaceutical Research, Ottawa, Canada, 1974.

Supported in part by Grant MA 3078 from the Medical Research Council of Canada.

* Recipient of a Medical Research Council of Canada Summer Scholarship.

[x] To whom inquiries should be directed.

Kinetics of Dehydration of Epitetracycline in Solution

BETTY-ANN HOENER, THEODORE D. SOKOLOSKI [x], LESTER A. MITSCHER, and LOUIS MALSPEIS

Abstract □ The dehydration kinetics of epitetracycline in solution to form epianhydrotetracycline were studied using UV and visible spectrophotometry. The reaction was found to be first order with respect to epitetracycline and hydronium-ion concentrations. The activation energy for the reaction was 28.3 kcal/mole at pH 2.0 in 0.1 *M* potassium chloride solutions. Dehydration of epitetracycline at pH 2.0 and 70° was found to be slower than that for tetracycline under similar solution conditions, although the activation energy for both reactants is essentially the same. This result is explicable on the basis of conformational differences in the molecules. This paper represents a portion of studies of the rates of various degradation reactions of tetracycline that lead to the toxic material epianhydrotetracycline.

Keyphrases □ Epitetracycline—dehydration kinetics in solution, activation energy, UV and visible spectrophotometry □ Dehydration—epitetracycline to epianhydrotetracycline kinetics in solution, activation energy, UV and visible spectrophotometry □ Epianhydrotetracycline—formation from epitetracycline, dehydration kinetics in solution, activation energy, UV and visible spectrophotometry □ Kinetics, dehydration—epitetracycline in solution

Studies show that commercially available tetracycline products contain significant amounts of degradation products of the antibiotic (1–3). This might be expected because tetracyclines can degrade through at least four different pathways: epimerization, dehydration, hydrolysis, and oxidation. Since the first two reactions are the most commonly encountered, they have been of specific interest for study. In solution at acid pH, two pathways connect tetracycline and 4-epianhydrotetracycline, as shown in Scheme I.

Epimerization about carbon-4 in tetracycline leads to inactive, nontoxic epitetracycline (4). The kinetics of this reaction were studied by other workers (4–7). Epimerization, which is a reversible first-order process, occurs most rapidly between pH 3 and 5. Dehydration and aromatization of the C-ring of tetracycline follow pseudo-first-order kinetics, leading to anhydrotetracycline, which is inactive *in vivo* and nontoxic. This reaction occurs in solution at very low pH (8) and in the solid state under thermal conditions (9).

There are two important steps in the overall degradation of tetracycline, whose kinetic characteristics have not as yet been studied in solution or in the solid state. These are the epimerization of anhydrotetracycline and the dehydration of epitetracycline. Both reactions lead to the inactive, but toxic, epi-

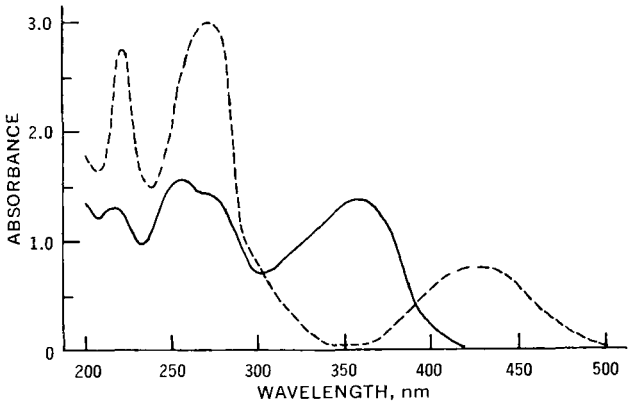


Figure 1—*UV and visible spectra of epitetracycline (——) and epianhydrotetracycline (– – –) at a concentration of 1 × 10^{-4} M in 0.1 M KCl, pH 2.0, using a 1-cm cell.*

anhydrotetracycline. To supply some of the missing kinetic parameters, studies on the effects of pH and temperature on the dehydration of epitetracycline solutions were conducted.

Epianhydrotetracycline formation may be conveniently and accurately studied by the appearance of an absorption peak at 427 nm or through the loss of epitetracycline, as reflected by the decrease in the absorption peak at 356 nm (Fig. 1). Analogous to the dehydration of tetracycline, epianhydrotetracycline formation is the only reaction occurring at pH 2 or less (8). In addition to the effects of pH and temperature, the influence of citric acid on the rate of dehydration of epitetracycline is reported, since citric acid has been shown to enhance markedly the degradation of tetracycline powder (1).

EXPERIMENTAL

Authentic samples of 4-epitetracycline[1], 4-epianhydrotetracycline[2], and tetracycline[3] were used as obtained. All other chemicals were reagent grade. Solutions were prepared in demineralized, double-distilled water, again distilled over ethylenediaminetetraacetic acid. All reaction solutions were 0.1 *M* in potassium chloride, and hydrochloric acid was added to adjust the pH[4]. In one experiment, citric acid at a concentration of 0.001 *M* was added to the 0.1 *M* KCl solution before adjusting the pH.

Solutions of 4-epitetracycline were sealed in ampuls for the studies carried out at 70.7° and pH 2.00. All other reactions were carried out in a sealed reaction vessel (Fig. 2). The 98.5° reaction was carried out using a boiling water bath, and those at 89.8 and 70.7° were carried out using an oil bath[5]. For those reactions carried out in the reaction vessel (Fig. 2), samples of about 4 ml were withdrawn *via* the syringe and placed in test tubes immersed in ice to stop the reaction. The time of the sample was taken as the time of placement in the test tube.

The absorbances of the samples were determined[6] after warming to room temperature. When ampuls were used, they were removed from the constant-temperature bath at appropriate time intervals and chilled in an ice bath. The ampuls were then opened, 5 ml was withdrawn and diluted to 10 ml with cold 0.1 *M* KCl–HCl solution, and the absorbance was measured at room temperature.

The absorbances of all epitetracycline solutions at concentrations ranging from 8.3×10^{-5} to 1.82×10^{-4} *M* were read at ambient temperatures at 356 and 427 nm, using a 1-cm cell. These absorbances measure the content of epitetracycline (ϵ_{356} = 13,800, ϵ_{427} = 0) and epianhydrotetracycline (ϵ_{427} = 5190, ϵ_{356} = 736). The absorption spectra for these compounds are given in Fig. 1. For the tetracycline solutions, absorbances at 356 nm measure the content of tetracycline and those at 433 nm measure the content of anhydrotetracycline (8).

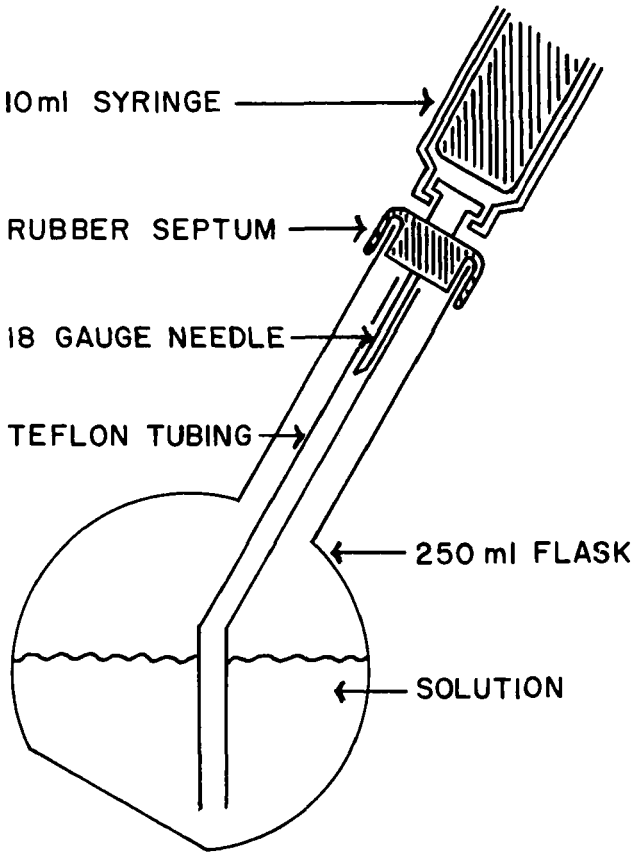
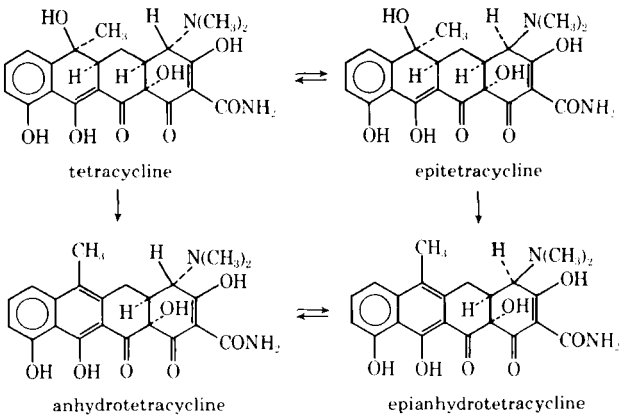


Figure 2—*Schematic representation of the reaction vessel used to study the dehydration of epitetracycline and tetracycline. The flask is immersed in a constant-temperature bath.*

RESULTS

Semilogarithmic plots of the difference between absorbance at a given time and absorbance at infinite time (determined after about 10 half-lives) at 356 nm and vice versa at 427 nm of the epitetracycline solutions gave an excellent linear relation. Figure 3 shows the results obtained at pH 2.00 and 70.7° for both wavelengths.



Scheme I

[1] Ammonium salt, Batch 430, British Pharmacopoeia Commission.
[2] Hydrochloride salt, Batch 429, British Pharmacopoeia Commission.
[3] Hydrochloride salt, Lot 48379-171, Lederle Laboratories.
[4] The pH was determined using a Sargent-Welch pH meter, model DR, standardized using pH 4.00 buffer, Lot 69M 388C 6471, Ohio State Reagent Laboratory.
[5] Sargent Thermonitor model ST.
[6] Cary model 16 spectrophotometer.

Table I—Pseudo-First-Order Rate Constants for Dehydration of Epitetracycline or Tetracycline at Various Temperatures at pH 2.0

Reactant[a]	Temperature	Rate Constant, $min^{-1} \times 10^2 \pm SD$[b] at 356 nm	at 427[c] or 433 nm[d]
Epitetracycline	98.5 ± 0.2°	5.59[e] ± 0.09	5.59[e] ± 0.06
Epitetracycline	89.8 ± 0.2°	3.08 ± 0.04	3.21 ± 0.06
Epitetracycline	80.0 ± 0.2°	1.18 ± 0.02	1.27 ± 0.03
Epitetracycline	70.7 ± 0.2°	0.395[e] ± 0.079	0.426[e] ± 0.084
Epitetracycline + 0.001 *M* citric acid	70.7 ± 0.2°	0.424 ± 0.023	0.460 ± 0.073
Tetracycline	70.7 ± 0.2°	0.553[e] ± 0.039	0.599 ± 0.069

[a] In 0.1 *M* KCl–HCl, pH 2.00. [b] Determined by linear regression analysis from the data of a semilogarithmic plot. [c] For epitetracycline. [d] For tetracycline. [e] Average of two determinations. The average precision obtained between all pairs of determinations was ±6.5%.

Also included in Fig. 3 are the results of an experiment under the same conditions utilizing tetracycline.

The pseudo-first-order rate constants at various temperatures at pH 2.0 are given in Table I. Also included in Table I is the study made in the presence of citric acid. An Arrhenius plot of the epitetracycline dehydration data gave an activation energy, E_a, of 28.3 ± 2.4 kcal/mole. The pseudo-first-order rate constants for the reaction at 70.7° at three different pH values are presented in Table II. A log (rate constant) *versus* pH plot gave a slope of −0.91 ± 0.1.

DISCUSSION

The dehydration of epitetracycline in solution at low pH to form epianhydrotetracycline has been shown to be first order with respect to epitetracycline. A pH–rate profile also has shown the reaction to be first order with respect to hydronium ion at below pH 2.0. The error limits found for the log rate constant–pH relation (Table II) may be due to the limited number of pH values that could be used and the change in total ionic strength over the pH range used.

Unlike the situation with tetracycline powder (1), citric acid at this low concentration had no catalytic effect on the dehydration of epitetracycline solutions. A most interesting result was that the rate of dehydration of epitetracycline was significantly less than that of tetracycline under similar conditions (Fig. 3). The rate constant obtained in this study for tetracycline is in agreement with that of Pryves (8). These epimers differ in the configuration of the dimethylamino group on carbon-4 in the A-ring of tetracycline. It seems likely that the explanation for the considerable difference in the rate for the two tetracycline epimers can be found in either the difference in electrostatic screening provided by the protonated dimethylamino function which is oriented differently in space relative to the 6β-hydroxy group in the two isomers or through the operation of the "Barton effect," *i.e.*, conformational transmission (10–12).

The transition state for dehydration is favorable in either isomer because the elements of water are located antiparallel and *trans* to each other and the 6β-hydroxy group is tertiary, benzylic, and axial. Furthermore, the canonical form of the β-diketo system at C-11, 11*a*, and 12, in which C-11 is olefinic, gives the C-ring a naphthaleneoid geometry at the outset (I).

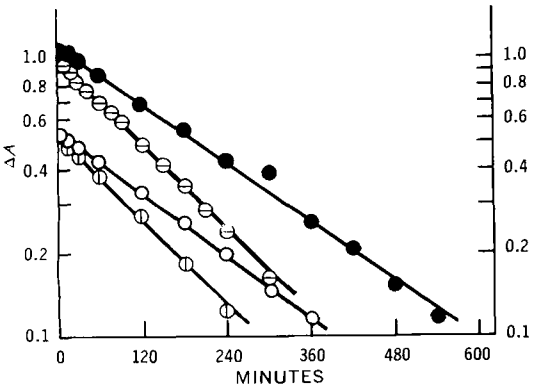
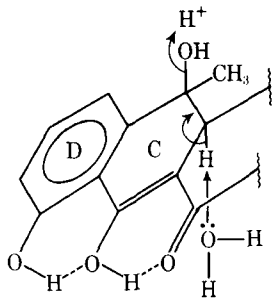


Figure 3—*Semilogarithmic plot of absorbance change* versus *time for epitetracycline* (● *at 356 nm and* ○ *at 427 nm*) *and tetracycline* (⊖ *at 356 nm and* ⊙ *at 433 nm*) *at 70° and pH 2.0.*

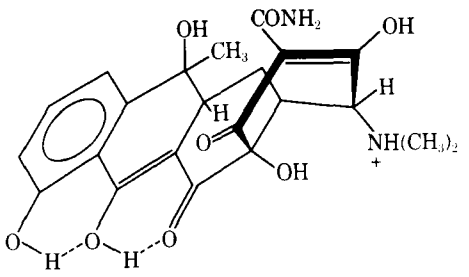
As in the conformation adopted by chlortetracycline hydrochloride in the crystal and supported by solution NMR and circular dichroism studies, the dimethylamino function in tetracycline (II) is axial and it is located on the side of the molecule opposite the 6β-hydroxy group and screened from it by the sigma framework (13, 14).

In 4-epitetracycline (III), the equitorial dimethylamino group is much closer to the 6β-hydroxy group (14) and would be expected to inhibit the approach of a hydronium ion more successfully than would tetracycline itself. Depending on the extent to which this effect would be operative, it would decrease the rate of dehydration of 4-epitetracycline relative to tetracycline and thus be in accord with the experimental findings.

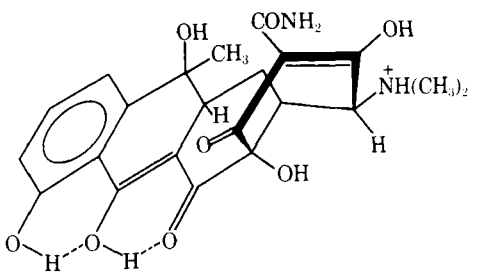
The Barton, or conformational transmission, effect has been thoroughly substantiated over the years with numerous examples (10–12). It has been found in numerous steroids that, in addition



I



II



III

Table II—Pseudo-First-Order Rate Constants for Dehydration of Epitetracycline at Several pH Values at 70.7°

pH[a]	Rate Constant, $min^{-1} \times 10^2 \pm SD$[b] at 356 nm	Rate Constant, $min^{-1} \times 10^2 \pm SD$[b] at 427 nm
2.00	0.395 ± 0.079[c]	0.426 ± 0.084[c]
1.53	1.03 ± 0.01	1.04 ± 0.01
1.04	3.19 ± 0.03	3.15 ± 0.01

[a] In 0.1 *M* KCl. [b] Determined by linear regression analysis from the data of a semilogarithmic plot. [c] Average of two determinations. The average precision obtained for these data sets was ±1.5%.

to expected electronic effects, conformational distortion in remote portions of a molecule, caused by epimeric constituents and the like, can be transmitted through the sigma framework of as many as four rings and significantly affect the rate and even steric outcome of reactions occurring at relatively great distances from the site of isomerization (15–25).

Detailed X-ray, NMR, and circular dichroism studies (13, 14) have shown that the A-ring of tetracyclines is somewhat distorted in acidic solutions because of the necessity of relieving a transannular 1,3-diaxial interaction between the 12*a*-hydroxy and protonated dimethylamino group at C-4. This apparently provides the driving force for epimerization at C-4, since epimerization relieves the interaction and releases the strain distortion of ring A. Transmission of the different strain in these two isomers from the A-ring through three to four carbon atoms to the 5*α*-hydrogen and 6*β*-hydroxy groups, whose geometry relative to one another plays a dominant role in the dehydration reaction, could conceivably result in rate differences of the magnitude observed in this study (Table I).

The involvement of steric effects in rationalizing the differential rates of dehydration of tetracycline and epitetracycline is further supported by noting that the activation energy for epitetracycline dehydration (28 kcal/mole) is essentially the same as that for the dehydration of tetracycline (27 kcal/mole) (8).

These studies reporting the solution kinetics of the dehydration of epitetracycline represent one factor in the overall degradation processes available to tetracycline in dilute aqueous solutions and in the solid state. Studies are underway to determine the rate of epimerization of anhydrotetracycline in solution to complete the solution phase picture.

REFERENCES

(1) V. C. Walton, M. R. Howlett, and G. B. Selzer, *J. Pharm. Sci.*, **59,** 1160(1970).

(2) W. W. Fike and N. W. Blake, *ibid.*, **61,** 615(1972).

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

(4) 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. Doershuk, *J. Amer. Chem. Soc.*, **79,** 2849(1957).

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

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

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

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

(9) E. R. Garrett, *J. Pharm. Sci.*, **51,** 1036(1962).

(10) D. H. R. Barton and A. J. Head, *J. Chem. Soc.*, **1956,** 932.

(11) D. H. R. Barton, A. J. Head, and P. J. May, *ibid.*, **1957,** 935.

(12) D. H. R. Barton, J. McCapra, P. J. May, and J. Thudium, *ibid.*, **1960,** 1297.

(13) J. Donohue, J. D. Dunitz, K. N. Trueblood, and M. S. Webster, *J. Amer. Chem. Soc.*, **85,** 851(1963).

(14) L. A. Mitscher, A. C. Bonacci, and T. D. Sokoloski, *Antimicrob. Ag. Chemother.*, **1968,** 78(1969).

(15) H. J. Geise, C. Altona, and C. Romers, *Tetrahedron*, **23,** 439(1967).

(16) H. B. Henbest and W. R. Jackson, *J. Chem. Soc.*, **1967,** 2459.

(17) *Ibid.*, **1967,** 2465.

(18) M. G. McCombe, H. B. Henbest, and W. R. Jackson, *J. Chem. Soc.*, **1967,** 2467.

(19) R. T. Blickenstaff, K. Atkinson, D. Beraux, E. Foster, Y. Kim, and G. C. Wolf, *J. Org. Chem.*, **36,** 1271(1971).

(20) M. B. Rubin, E. C. Blossey, A. P. Brown, and J. E. Vaux, *J. Chem. Soc.*, **1970,** 57.

(21) V. V. Egorova, A. V. Zakharychev, and S. N. Ananchenko, *Tetrahedron*, **29,** 301(1973).

(22) M. J. T. Robinson and W. B. Whalley, *ibid.*, **19,** 2123(1963).

(23) R. Bucourt, *Bull. Chim. Soc. Fr.*, **62,** 1983(1963).

(24) R. Baker and J. Hudec, *Chem. Commun.*, **1967,** 891.

(25) *Ibid.*, **1967,** 479.

ACKNOWLEDGMENTS AND ADDRESSES

Received March 5, 1974, from the *College of Pharmacy, Ohio State University, Columbus, OH 43210*

Accepted for publication June 28, 1974.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Chicago meeting, August 1974.

Supported in part by Research Grant 1RO1 FD-00689-01A1 from the Food and Drug Administration, U.S. Department of Health, Education, and Welfare, Rockville, MD 20852

[x] To whom inquiries should be directed.