Some Observations on the Kinetics of the C·4 Epimerization of Tetracycline

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The C·4 epimerization of tetracycline has been shown to follow the kinetics of a first-order reversible reaction. Phosphate and citrate ions increased the rate of C·4 epimerization. The reaction rate in either 1.00 molar phosphate or 1.00 molar citrate buffer at pH 4.0 and 23° was approximately 70 times the rate observed in distilled water under the same conditions of temperature and pH. An Arrhenius plot indicated that the apparent energy of activation for the C·4 epimerization of tetracycline at pH 4.0 and 0.10 molar phosphate buffer was approximately 20 kilocalories/gram-mole. The equilibrium concentration and the rate at which equilibrium was approached were dependent on the pH. Of the five pH levels studied, a maximum C·4 epimer level of 55 per cent at equilibrium occurred at pH 3.2. Of the five pH levels studied, the rate at which equilibrium was approached was highest at pH 3.2, 4.0, and 5.0.

IN 1955 DOERSCHUK, *et al.* (1), observed a reversible isomerization in members of the tetracycline family. This observation was confirmed by Stephens, *et al.* (2), in 1956 and by Kaplan, *et al.* (3), in 1957. The initial observations reported by Doerschuk, *et al.* (1), were extended by McCormick, *et al.* (4, 5), in a series of papers in which the preparation, properties, and proof of structure of several 4-epi-tetracyclines were described. In the paper by McCormick, *et al.* (5), a number of experimental conditions permitting the epimerization of members of the tetracycline family was presented with values of the equilibrium concentration of C·4 epimer in a variety of solvent systems.

The purpose of this paper is to describe the kinetics of the C·4 epimerization in solutions displaying pharmaceutical utility. A study of this type provides useful background information in formulating stable solutions of tetracycline.

EXPERIMENTAL

Analytical Procedure.—The analytical procedure used in this study for measuring 4-epi-tetracycline was a slight modification of the absorbancy-ratio assay described by McCormick, *et al.* (5). The assay is based on the observation that the ratio of absorbancies at 254 and 267 mμ differs for tetracycline and its C·4 epimer. To measure small amounts of C·4 epimer precisely, the original absorbancy ratio assay was modified by F. S. Chiccarelli (private communication) so that it might be run as a differential assay. Samples of the standards for tetracycline·HCl and 4-epi-tetracycline (ammonium salt) were obtained from F. S. Chiccarelli, Assay Development Department.

Experimental Procedure.—Solutions of various buffers were prepared and adjusted to the desired pH. The buffers were allowed to equilibrate at the desired temperature in a volumetric flask before

tetracycline·HCl or its C·4 epimer was dissolved in the buffer. At frequent intervals aliquots were withdrawn to determine the C·4 epimer content by the method described above.

Estimation of Rate Constants.—The reaction rate constants for the C·4 epimerization were estimated from the equation describing the kinetics of a first-order reversible reaction

$$ln \frac{Ao - Ae}{A - Ae} = (k_1 + k_{-1}) t \qquad \text{(Eq. 1)}$$

where Ao = per cent tetracycline·HCl at $t = o$; A = per cent tetracycline·HCL at $t = t$; Ae = per cent tetracycline·HCl at equilibrium; t = time, hours; k_1 = forward reaction rate constant, hours^{-1}; and k_{-1} = backward reaction rate constant, hours^{-1}.

Throughout this paper the symbol k_1 is used for the reaction rate constant for the forward reaction of tetracycline epimerizing to 4-epi-tetracycline. The symbol k_{-1} is used for the backward reaction of 4-epi-tetracycline to tetracycline. The symbols for the two reaction rates are used for the directions of the reactions as indicated above even though the principal starting material in the reaction may be 4-epi-tetracycline in some experiments.

Equation 1 indicates that if the logarithm of the reciprocal of the fraction of equilibrium attained at time t is plotted as a function of time, a straight line should be obtained whose slope is equal to $(k_1 + k_{-1})$. The method of least squares was used to estimate the slope of the straight line passing through the experimental points, thereby providing an estimate of $(k_1 + k_{-1})$. The individual rate constants k_1 and k_{-1} are readily determined from the equilibrium concentration of tetracycline using the relationship

$$\frac{1 - Ae}{Ae} = \frac{k_1}{k_{-1}}$$

The 95% confidence limits for k_1 and k_{-1} were determined from the least squares calculations presented in standard statistical texts. For all other computations of slopes and intercepts the method of least squares was used.

RESULTS AND DISCUSSION

Determination of Reaction Order for the Epimerization of Tetracycline to 4-epi-Tetracycline.—

TABLE I.—EXPERIMENTAL DISAPPEARANCE RATES AND AVERAGE CONCENTRATIONS FOR TETRACYCLINE DURING FIRST 8.5 HOURS OF EPIMERIZATION

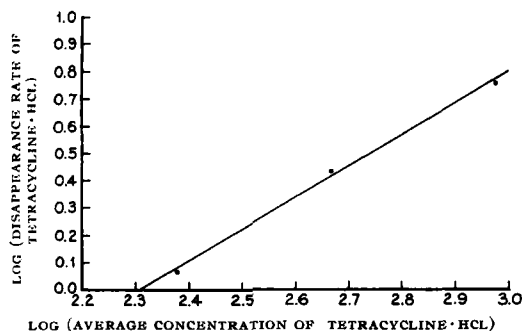| Initial Concn. of Tetracycline and C·4 Epimer	Tetracycline, Initial %	Tetracycline after 8.5 Hr., %	Rate of Disappearance $-\dfrac{\Delta \text{ (Tetracycline)}}{\Delta t}$	Average Concn. of Tetracycline During First 8.5 Hr. of Reaction
250 mcg./ml.	96.80	92.78	1.182 mcg./ml.-hr.	237.0 mcg./ml.
500 mcg./ml.	96.80	92.20	2.706 mcg./ml.-hr.	472.5 mcg./ml.
1,000 mcg./ml.	96.80	91.95	5.706 mcg./ml.-hr.	943.8 mcg./ml.

Fig. 1.—The rate of disappearance of tetracycline·HCl as a function of its average concentration at pH 4.0 and 23°C.

Three solutions of tetracycline were prepared in 0.10 M phosphate buffer at pH 4.0 and 23° at total tetracycline levels of 250, 500, and 1000 mcg./ml. Immediately after solution and 8.5 hours later, aliquots of the solutions were withdrawn and assayed for C·4 epimer. The experimental results are presented in Table I.

The reaction order for the C·4 epimerization was determined from the data in Table I using the differential method described by Laidler (6). In this method the logarithm of the rate of disappearance of tetracycline is plotted as a function of the logarithm of the average concentration of tetracycline as shown in Fig. 1. Such a plot should give a straight line, whose slope and intercept are equal to the reaction order and reaction rate constant, respectively.

In order to state that a reaction follows first-order reversible kinetics, (a) the slope of the line of the plot in Fig. 1 should be equal to 1.00, and (b) the experimental data when plotted according to Eq. 1 should give a straight line whose slope is equal to $k_1 + k_{-1}$.

Inspection of Fig. 1 indicates that the experimental observations appear to result in a straight line. The value obtained for the slope of the line was 1.134 with 95% confidence limits of 0.890 and 1.374. Since the value of 1.00 is within the confidence limits, the C·4 epimerization of tetracycline may be safely considered to satisfy condition (a)

above for first-order reactions in the concentration range studied. The antilogarithm of the intercept gives a value of 2.443 $\times$ 10^{-3} hours^{-1} for the forward reaction rate constant. This value compares favorably with other values obtained in later experiments under the same conditions.

Determination of the Reaction Order for the Epimerization of 4-epi-Tetracycline to Tetracycline.—The experiment described above was repeated using 4-epi-tetracycline instead of tetracycline as the starting material. Three solutions of 4-epi-tetracycline were prepared in 0.10 M phosphate buffer at pH 4.0 and 23° at total tetracycline levels of 250, 500, and 1000 mcg./ml. Immediately after solution and at 7.5 hours later, aliquots of the solutions were withdrawn and assayed for C·4 epimer. The experimental results appear in Table II and are plotted in Fig. 2 in the same manner as for the previous experiment.

By the method outlined above, a value for the slope of 1.141 was obtained with 95% confidence limits of 0.823 and 1.449. Since the value of 1.00 is within the confidence limits, the epimerization of 4-epi-tetracycline to tetracycline may be considered to satisfy conditions (a) for first-order reactions in the concentration range studied. The antilogarithm of the intercept gives a value of 3.963 $\times$ 10^{-3} hours^{-1} for the backward reaction rate constant for the epimerization of tetracycline to 4-epi-tetracycline. This value compares favorably with
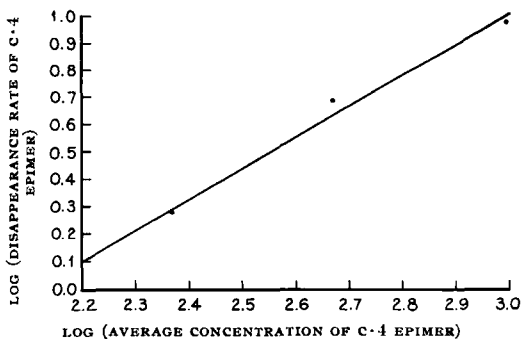

Fig. 2.—The rate of disappearance of C·4 epimer as a function of its average concentration at pH 4.0 and 23°.

TABLE II.—EXPERIMENTAL DISAPPEARANCE RATES AND AVERAGE CONCENTRATIONS FOR 4-EPI-TETRACYCLINE DURING FIRST 7.5 HOURS OF EPIMERIZATION

Initial Concn. of Tetracycline and C·4 Epimer	C·4 Epimer, Initial %	C·4 Epimer after 7.5 Hr., %	Rate of Disappearance $-\dfrac{\Delta \text{ (C·4 Epimer)}}{\Delta t}$	Av. Concn. of C·4 Epimer During First 7.5 Hr. of Reaction
250 mcg./ml.	96.62	90.86	1.920 mcg./ml.-hr.	234.4 mcg./ml.
500 mcg./ml.	96.62	89.43	4.793 mcg./ml.-hr.	465.1 mcg./ml.
1,000 mcg./ml.	96.62	89.67	9.267 mcg./ml.-hr.	931.5 mcg./ml.

TABLE III.—REACTION RATE CONSTANTS AS A FUNCTION OF PHOSPHATE BUFFER CONCENTRATION

Molar Concn. of Phosphate Buffer, pH 4.0 at 23°C.	Forward Reaction Rate Constant, $k_1 \times 10^3$, hr.$^{-1}$	Backward Reaction Rate Constant, $k_{-1} \times 10^3$, hr.$^{-1}$
0.01	0.406 (0.294–0.518)	0.662 (0.550–0.774)
0.10	2.13 (2.02–2.24)	3.47 (3.36–3.59)
1.00	15.5 (14.9–16.1)	25.3 (24.7–25.9)

TABLE IV.—REACTION RATE CONSTANTS AS A FUNCTION OF PHOSPHATE BUFFER CONCENTRATION

Molar Concn. of Phosphate Buffer, pH 4.0 at 23°C.	Forward Reaction Rate Constant, $k_1 \times 10^3$, hr.$^{-1}$	Backward Reaction Rate Constant, $k_{-1} \times 10^3$, hr.$^{-1}$
0.01	0.680 (0.670–0.690)	1.11 (1.10–1.12)
0.10	2.48 (1.83–3.13)	4.05 (3.40–4.70)
1.00	15.7 (6.71–24.6)	25.5 (16.6–34.5)

subsequent values obtained in later experiments under identical conditions.

The second condition for first-order reactions is demonstrated in the following experiments.

Effect of Phosphate Ion Concentration on the Epimerization of Tetracycline to 4-epi-Tetracycline.—Three solutions of tetracycline were prepared at a total tetracycline level of 1500 mcg./ml. in 0.01, 0.10, and 1.00 M phosphate buffer at pH 4.0 and 23°. Aliquots of the solutions were withdrawn periodically and assayed for C·4 epimer to determine the reaction rate constants under these conditions. Table III presents the reaction rate constants with their 95% confidence limits in parentheses.

Figure 3 shows that when the reaction rate constants are plotted on log-log paper as a function of the molarity of the phosphate buffer, a linear relationship is obtained which may be represented by

$$k_1 \times 10^3 = 22.5 \, (M)^{0.791}$$
$$k_{-1} \times 10^3 = 23.9 \, (M)^{0.791}$$

where M is the molarity of the phosphate buffer. The 95% confidence limits for the exponent were 0.728 and 0.854. Since the confidence limits do not include zero, the phosphate ion concentration ex-
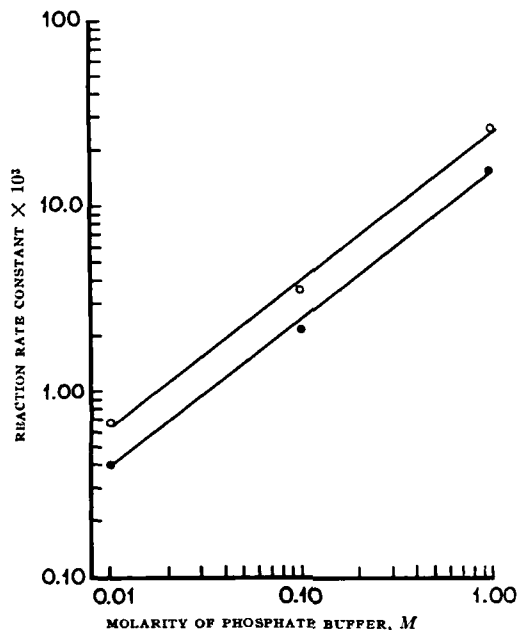
erted a significant effect on the rate of epimerization of tetracycline to its C·4 epimer at pH 4.0.

The above experiment was repeated using 4-epitetracycline as the principal starting material to determine the reaction rate constants under these conditions. Table IV presents the reaction rate constants with their 95% confidence limits in parentheses.

Using the reaction rate constants presented in Tables III and IV, one can construct a family of curves showing the C·4 epimerization as a function of time in phosphate buffer starting with either member of the epimeric pair. Such a family of curves is presented in Fig. 4. This figure shows that the equilibrium between epimeric pairs may be approached from either direction.

The rate constants in this experiment were determined by the method described earlier in this paper. When the experimental data from this and subsequent experiments were plotted according to Eq. 1, a straight line resulted, thereby satisfying the second condition for a first-order reversible reaction.

Effect of Citrate Ion Concentration on Epimerization of Tetracycline to 4-epi-Tetracycline.—The previous experiment was repeated using citrate buffers at pH 4.0 and 23° in the place of phosphate buffers. Aliquots of the solutions were withdrawn frequently and assayed for C·4 epimer to determine the reaction rate constants under these conditions. Table V summarizes the reaction rate constants with the 95% confidence limits in parentheses.

The reaction rate constants when plotted on log-log paper as a function of citrate molarity resulted in straight lines which may be represented by

$$k_1 \times 10^3 = 16.3 \, (M)^{0.723}$$
$$k_{-1} \times 10^3 = 26.7 \, (M)^{0.723}$$

where M is the molarity of the citrate buffer. The 95% confidence limits for the exponent are 0.714

Fig. 3.—Reaction rate constants in phosphate buffers of various molarities at pH 4.0 and 23°: ●— forward reaction rate constant, ○—backward reaction rate constant.
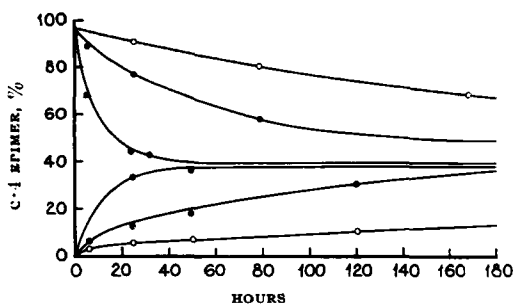

Fig. 4.—Equilibrium of tetracycline and its C·4 epimer in phosphate buffers of various molarities at 23°: ●—1.00M phosphate, pH, 4.0; ⊙—0.10M phosphate, pH 4.0; ○—0.01M phosphate, pH 4.0.

TABLE V.—REACTION RATE CONSTANTS AS A
FUNCTION OF CITRATE BUFFER CONCENTRATION

Molar Concn. of Citrate Buffer, pH 4.0 at 23°C.	Forward Reaction Rate Constant, $k_1 \times 10^3$, hr.$^{-1}$	Backward Reaction Rate Constant, $k_{-1} \times 10^3$, hr.$^{-1}$
0.01	0.586 (0.439–0.733)	0.956 (0.717–1.195)
0.10	3.09 (2.23–3.87)	5.05 (3.79–6.31)
1.00	16.3 (12.3–20.4)	26.7 (20.0–33.3)

TABLE VI.—EFFECT OF TEMPERATURE ON THE C·4
EPIMERIZATION OF TETRACYCLINE

Temp., °C.	Forward Reaction Rate Constant, $k_1 \times 10^3$, hr.$^{-1}$	Backward Reaction Rate Constant, $k_{-1} \times 10^3$, hr.$^{-1}$
4	0.193 (0.165–0.221)	0.316 (0.288–0.344)
23	2.61 (2.20–3.02)	4.26 (3.85–4.67)
37	8.96 (6.86–11.0)	14.6 (12.5–16.7)
42	15.4 (8.62–2.22)	25.0 (18.2–31.8)
56	78.5 (20.5–178.0)	128.2 (29.2–227.0)

and 0.732. Since the confidence limits do not include zero, the citrate ion concentration exerted a significant effect on the rate of epimerization of tetracycline to its C·4 epimer at pH 4.0.

Rate of Epimerization of Tetracycline in Water.— A solution of tetracycline was prepared in distilled water at a total tetracycline level of 1500 mcg./ml. The solution was titrated to pH 4.0 with 0.1 N NaOH and allowed to epimerize at 23°. Aliquots of the solution were withdrawn periodically and assayed for C·4 epimer. During the aging of the unbuffered solution, the pH gradually fell from 4.0 to 3.5 in the first 10 days after which it remained constant at 3.5 for the remainder of the reaction. The reaction rate constants for this condition (with the 95% confidence limits in parentheses) can thus be summarized.

Forward Reaction-Rate
Constants, $k_1 \times 10^3$, hr.$^{-1}$
0.234 (0.014–0.454)

Backward Reaction-Rate
Constant, $k_{-1} \times 10^3$, hr.$^{-1}$
0.381 (0.161–0.601)

A comparison of the epimerization rates of tetracycline in 1.00 M phosphate and 1.0 M citrate buffer with that observed in distilled water under closely similar conditions of temperature and pH reveals that the epimerization rate in 1.00 M phosphate or citrate buffers proceeds approximately 70 times more rapidly than in distilled water.

Effect of Temperature on Epimerization of Tetracycline.—Solutions of tetracycline were prepared at a total tetracycline level of 1500 mcg./ml. in 0.10 M phosphate buffer at pH 4.0 that had been previously equilibrated at 4, 23, 37, 42, and 56°. Aliquots of the solutions were withdrawn frequently and assayed for C·4 epimer. Table VI presents the
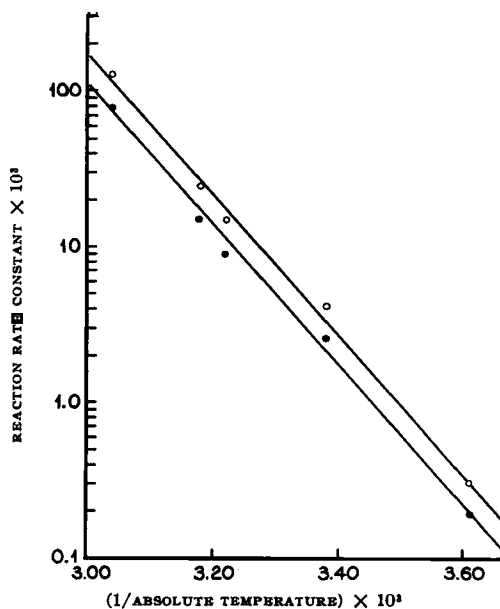

Fig. 5.—Arrhenius plot for the forward and backward reaction rates for the C·4 epimerization of tetracycline: ○—backward rate, ●—forward rate.

reaction rate constants with their 95% confidence limits in parentheses.

The above reaction rate constants were then plotted to give the Arrhenius plot presented in Fig. 5. Inspection of the figure reveals that the reaction rate constants fall rather closely on a straight line, the slope of which is equal to $-E/2.303\,R$ where E is the apparent energy of activation, and R is the gas constant, 1.986 calories per degree-mole.

The slope, activation energy, and frequency factors for the epimerization of tetracycline are presented in Table VII. The energy of activation and frequency factors from the Arrhenius plot have been obtained by a weighted regression analysis described by McBride and Villars (7). A weighted regression was used (a) to prevent equal weighting of rate constants determined with high precision with those obtained with low precision and (b) to compensate for the unequal weighting introduced by using the logarithm of the rate constant instead of the rate constant directly. McBride and Villars have shown that the above objections can be eliminated if the rate constants are weighted by a quantity directly proportional to the square of the rate constant and inversely proportional to its variance. The necessity for performing a weighted regression analysis can be seen from Table VI. At 56° the epimerization proceeded so rapidly that only three assays could be performed before the reaction was essentially complete. As a result, the reaction rate constant is estimated with low precision as indicated by the wide confidence interval. A weighted regression is used so that a rate constant determined

TABLE VII.—ACTIVATION ENERGY AND FREQUENCY FACTORS FOR THE C·4 EPIMERIZATION OF TETRACYCLINE

Reaction	Slope of Arrhenius Plot, 95% Confidence Limits	Activation Energy in Calories per Mole, 95% Confidence Limits	Frequency Factor, hr.$^{-1}$
Forward	−4.452 (−3.74)–(−5.16)	20,400 (17,100–23,600)	2.69×10^{15}
Backward	−4.452 (−3.89)–(−5.11)	20,400 (17,800–22,900)	3.61×10^{15}
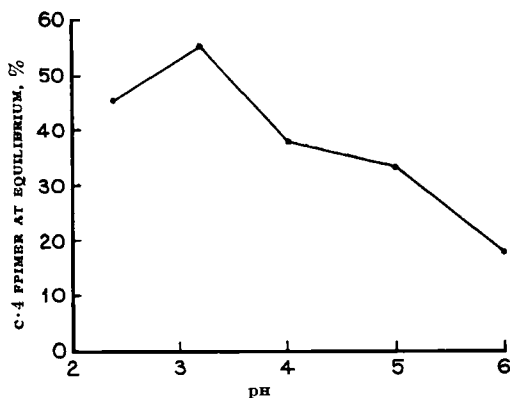
Fig. 6—Equilibrium concentrations of C·4 epimer as a function of pH.

Fig. 7.—The sum of the forward and backward rate constants as a function of pH.

with low precision is not weighted equally with one estimated with high precision.

Equilibrium Levels of C·4 Epimer at Various pH Levels.—The equilibrium concentrations of C·4 epimer and the rate at which equilibrium is approached were determined as a function of pH by aging solutions containing a total tetracycline level of 1500 mcg./ml. in 0.10 M phosphate buffer at 23° until the C·4 epimer level did not change with additional time. This concentration is considered to be the equilibrium concentration and is plotted in Fig. 6 as a function of pH. The equilibrium values employed in estimating reaction rate constants in this study are those presented in Fig. 6.

Inspection of Fig. 6 indicates that the equilibrium concentration of C·4 epimer is a function of the pH of the solution. Of the five pH levels studied, the maximum C·4 epimer concentration at equilibrium is 55% and occurs at pH 3.2.

The rate at which the epimerization occurred

was also measured and is presented in Table VIII and Fig. 7. Inspection of the table and figure reveals that at pH 3.2, 4.0, and 5.0, the rates are essentially identical. As the pH is increased or decreased from the above pH range, the reaction rate declines.

SUMMARY

The C·4 epimerization of tetracycline to 4-epi-tetracycline follows first-order reversible kinetics at pH 4.0 in phosphate buffer. At pH 4.0 phosphate and citrate ions are effective in catalyzing the rate at which the equilibrium C·4 epimer concentration is approached.

The apparent energy of activation for the C·4 epimerization is approximately 20 kilocalories/gram-mole at pH 4.0 in phosphate buffer. The equilibrium concentration and the rate at which equilibrium is approached are dependent on the pH of the solution.

TABLE VIII.—SUM OF FORWARD AND BACKWARD REACTION RATE CONSTANTS $[(k_1 + k_{-1}) \times 10^3]$ FOR TETRACYCLINE AS A FUNCTION OF pH (95% CONFIDENCE LIMITS)

pH	Rate Constants
2.4	7.45 (7.31–7.59)
3.2	8.76 (8.62–8.91)
4.0	8.90 (8.64–9.15)
5.0	8.71 (8.59–8.89)
6.0	5.39 (4.95–5.82)

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