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Kinetic studies on the decomposition of erythromycin A in aqueous acidic and neutral buffers

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Summary

The results of a study are presented on the effect of pH, buffer concentration and temperature on the decomposition of erythromycin A in aqueous solution. The decomposition proceeds via the known intermediate erythromycin A-6,9-hemiketal and observed rate coefficients (k_{obs}) for both erythromycin A and hemiketal degradation are reported. Both reactions are subject to specific acid catalysis and catalysis by buffer species. Buffer independent rate coefficients have been obtained and these show a linear increase with decreasing pH. The mechanistic implications of these observations are discussed.

Introduction

Erythromycin is an antibiotic of the macrolide class which was first isolated more than thirty years ago. It is either bacteriostatic or bactericidal depending on the organism and the concentration used and functions by inhibiting protein synthesis. It is particularly effective against gram-positive cocci, with very low minimal inhibitory concentrations. Few strains of bacteria are resistant to erythromycin, although it is not active against aerobic gram-negative bacilli. Erythromycin is the preferred therapeutic treatment in a variety of diseases, e.g. Pneumonia, Streptococcoe and Staphylococcoe infections and Tetanus. It is also used as an alternative to penicillin for the treatment of infections in penicillin-sensitive patients (Goodman and Gillman, 1980; Martindale, 1982).

Erythromycin is administered orally as either a tablet or suspension, in several forms. The free base is given as a tablet, whilst both the water insoluble salt (erythromycin stearate) and the ester prodrug (erythromycin ethylsuccinate) are given either as a tablet or suspension. Erythromycin is also administered parenterally via intravenous infusion as a water soluble salt (erythromycin lactobionate or erythromycin gluceptate) or intramuscularly as erythromycin ethylsuccinate in macrogel 400. This method of administration is extremely painful and is only used for the treatment of acute infections (Martindale, 1982).

It has been known for many years that erythromycins are susceptible to acid deactivation giving two degradation products, depending on the

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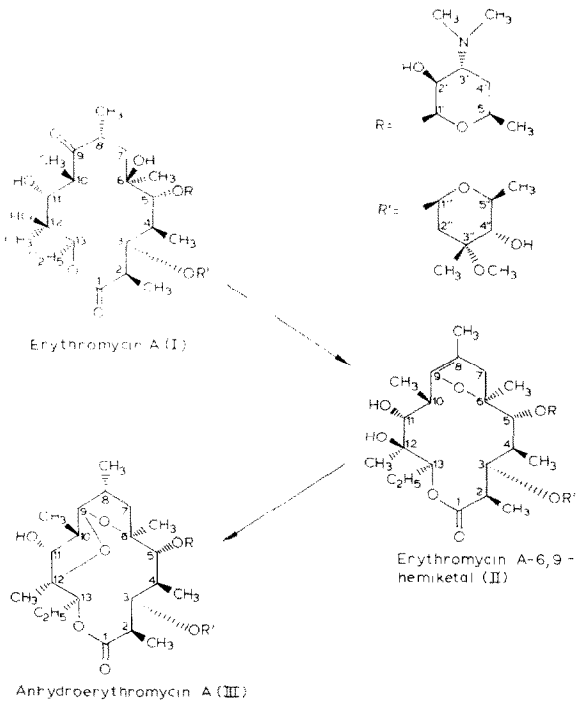


Scheme 1.

conditions of the reaction. Mild acid treatment (glacial acetic acid, 2 h) of erythromycin A yields erythromycin A-6,9-hemiketal (II) (Scheme 1). Further treatment in acid (methanolic hydrochloric acid, 1 h) gives the "spiroketal" anhydroerythromycin A (III). Alternatively, if erythromycin A is treated in strong acid (hydrochloric acid, pH 2.0, 30 min) only the "spiroketal" is obtained. Both these compounds have been prepared previously (Stephens and Conine, 1958; Wiley et al., 1957; Kurath et al., 1971). Under all conditions reported, no cleavage of the glycosidic linkages has been observed. More recently the structures of these compounds have been fully assigned by the use of ^{13}C-NMR (Terui et al., 1975).

There are very few reports in the literature on the stability of erythromycin A in aqueous solution (Kondratieva and Bruns, 1962; Amer and Takla, 1978; Tsuji and Goetz, 1978). Of these, an early report (Kondratieva and Bruns, 1962) in the Russian biological literature gives both pH and temperature effects on the rates of deactivation of erythromycin A. These workers concluded that the pH of maximum stability was approximately 8.0, and this decreased with increasing temperature.

More recently Amer and Takla (1978) have published an extensive study on the stability of erythromycin, its salts, and some 2′-esters, in the presence of added metal ions and other drugs. They concluded that the pH of optimum stability of erythromycin base is in the region of pH 7.0 to 7.5. This study, although extremely detailed, is not particularly rigorous. No details are given of how the erythromycin content of the reaction mixtures was measured and no account has been made of buffer effects. However, this report does show that metal ions, in particular Al^{+++}, Fe^{+++} and Cu^{++} have a detrimental effect on the activity of erythromycin.

Tsuji and Goetz (1978), whilst developing an HPLC assay for erythromycin and its degradation products, also studied the effect of pH on the degradation. At acidic pH's they observed the expected acid degradation product, erythromycin A-6,9-hemiketal (II), however at pH 8.8 and above, another product, dihydroerythromycin A, was the major degradation product. They concluded that the pH of maximum stability was about 8.8.

The present work represents the first systematic study of the kinetics of the decomposition of erythromycin A in acidic aqueous solution. We have investigated the affect of a variety of buffer species in the pH range 3.06–7.46 on the rates of decomposition of both erythromycin A and erythromycin A-hemiketal (II). The affects of temperature and buffer concentration on both these processes are also reported.

Materials and Methods

Materials

Erythromycin A-6,9-hemiketal (II) was prepared from erythromycin A according to the method of Stephens and Conine (1958). The crude product was recrystallised from carbon tetrachloride to give a white solid, mp. 128–131°C (lit., 133–135°C) (Terui et al., 1975), and had a ^{13}C nmr spectrum consistent with the expected structure. The compound was 95% pure by HPLC (see below for conditions).

Anhydroerythromycin A (III) was prepared by treating erythromycin A with dilute hydrochloric acid according to the method of Wiley et al. (1957). The product was recrystallised from dichloromethane/hexane, mp 135–139°C (lit., 130–140°C) (Terui et al., 1975). The ^{13}C nmr spectrum was consistent with the postulated structure and was significantly different from that of erythromycin A and erythromycin A-6,9-hemiketal in the C_6–C_{12} region.

Erythromycin A was used as received (The Upjohn Co.,). All other reagents were of analytical grade (Fisons Scientific Apparatus Ltd.) and were used without further purification.

Equipment

UV absorption spectra were recorded on a Kontron Uvikon 810 Spectrophotometer fitted with a Uvikon 21 recorder, and interfaced to a Hewlett-Packard 85A computer. The cell housing was thermostatted by a water circulating bath (Thermomix 1420, B. Braun) to a precision of ±0.1°C.

HPLC chromatograms were run on a system consisting of the following components:

Laboratory Data Control, Constametric IIG pump,

Rheodyne 7125 injection port,

Lichrosorb RP-8 5 μm column (4.9 mm × 25 cm) (HPLC Technology),

Laboratory Data Control, Spectromonitor III variable wavelength UV detector,

Servoscribe 750 Heat sensitive recorder,

Pye-Unicam DP-88 integrator.

The mobile phase was prepared by dissolving 2.5 g of sodium dihydrogen orthophosphate in water (400 ml), making up to 1000 ml with acetonitrile (Far UV grade) and then adding 100 μl of N,N-dimethyloctylamine (Lancaster Synthesis Ltd). The mixture was then degassed with helium and filtered through a Whatman glass microfibre filter.

Typical operating conditions:

Flow rate:	1 ml/min
Injection volume:	20 μl
Detector wavelength:	214 nm
Integrator:	1 V output, attn. 16.

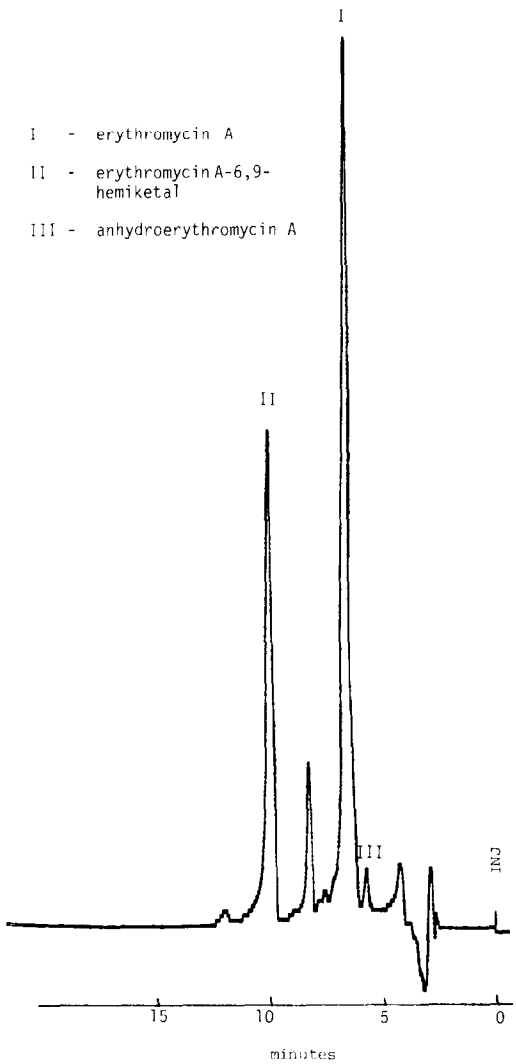


Fig. 1. HPLC chromatogram of the reaction of erythromycin A in 0.1 M phosphate buffer, pH 7.0, at 37°C after 20 h.

Recorder speed: 2 min/cm.

A chromatogram showing the separation of erythromycin A, erythromycin A-6,9-hemiketal and anhydroerythromycin A is given in Fig. 1. ^{13}C-NMR spectra were obtained on a Bruker WM 360 instrument operating at 90.55 MHz, located at the School of Molecular Sciences, University of Sussex.

Melting points were obtained on a Koeffler hot stage microscope (Reichert, Austria) and were uncorrected.

Stock buffer solutions (0.5 M) were prepared by dissolving the appropriate acid (0.05 moles) in water (50 ml). To this was then added a sufficient volume of sodium hydroxide solution (1.0 M) to give the desired pH, and the solutions were then made up to 100 ml with water. Sodium chloride (0.5 M) was used to dilute stock buffer solutions to a given molarity, maintaining the ionic strength at 0.5 M throughout.

For those reactions which were monitored by UV spectroscopy, stock solutions of substrate (20 → 25 mg) in methanol (1 ml) were prepared daily. The reactions were monitored at 230 nm or, in the cases where the buffer gave excessive absorbance at this wavelength (succinate and the higher concentrations of acetate and formate) at 235 nm, under pseudo first-order conditions (at least 40 : 1 excess buffer).

Buffer solutions were pipetted into pairs of 10 mm path length cells (2.5 ml in each), placed in the cell housing of the spectrophotometer, and allowed to reach thermal equilibrium at 37°C. The reactions were initiated by adding the substrate stock solution (50 μl) and rapidly mixing. The change in absorbance was recorded at preset time intervals, and this was output to an HP 85A, and printed.

For studies on the decomposition of erythromycin A, the appearance of the "hemiketal" (II) was monitored and the initial rates method was used to determine observed rate coefficients. This method evaluates the rate coefficient from the gradient of a graph of absorbance against time. The gradient was computed by linear regression analysis using the HP 85A Standard Pac routine, and was used in conjunction with the extinction coefficient of the product at the appropriate wavelength, and the initial concentration of the substrate to determine the observed rate, according to equation 1 (Connors, 1981),

$$\left(\frac{d[II]}{dt}\right)_{t=0} = k[I]_o \qquad (1)$$

which holds over the first 2–3% of a reaction.

When the disappearance of the "hemiketal" was studied, the method of analysis depended on the rate of reaction. Whenever it was feasible the reaction was monitored over 6 half lives (98% reaction) but for very slow reactions, the initial rates method was used. For those reactions that were studied over their full time course, once the linearity of plots of $\ln(A_t - A_\infty)$ against time had been established (i.e. the reaction obeyed first order kinetics), the Guggenheim method (Connors, 1981) was then used to compute the observed rate coefficients, with the aid of a routine written for the HP 85A.

Rate data for the decomposition of both erythromycin A and erythromycin A-6,9-hemiketal (II) are given in Tables 1 and 2. Good reproducibility was obtained between runs studied in duplicate (c.f. pH 3.82, formate buffer, "hemiketal" disappearance Table 2) so the confidence limits on the observed rate coefficients can be set quite high. Due to inherent inaccuracies in the measurement

TABLE 1

RATE DATA (10^4 k_{obs}) [a] FOR THE DECOMPOSITION OF ERYTHROMYCIN A AT 37°C IN AQUEOUS BUFFERS

10^4 k_{obs} (min^{-1})		Buffer concentration (M)					
pH	Buffer	0.05	0.10	0.15	0.20	0.30	0.40
3.82	Formate	–	53.5	72.0	89.4	–	–
4.10	Formate	–	56.0	65.0	78.7	–	–
4.75	Acetate	–	20.3	23.9	25.2	29.8	–
5.20	Acetate	5.92	7.50	9.02	10.22	12.42	–
6.00	Succinate	1.46	2.00	3.03	3.89	4.91	–
6.51	Phosphate	–	0.57	0.79	1.03	1.33	1.43
7.00	Phosphate	0.14	0.21	0.27	0.34	0.50	0.64
7.46	Phosphate	–	0.12	0.14	0.18	0.23	0.28

[a] All rate coefficients were derived by the initial rates method.

TABLE 2

RATE DATA (10^3 k_{obs}) FOR THE DECOMPOSITION OF ERYTHROMYCIN A-6,9-HEMIKETAL AT 37°C IN AQUEOUS BUFFERS

10^3 k_{obs} (min^{-1})		Buffer concentration (M)			
pH	Buffer	0.05	0.10	0.15	0.20
3.06	Formate	95.4	101.4	104.6	107.8
3.82 [a]	Formate	20.0 ± 0.8	23.8 ± 0.3	27.3 ± 0.3	29.5 ± 0.7
4.10 [a]	Formate	13.9 ± 0.3	16.8 ± 0.1	19.6 ± 0.1	21.3 ± 0.3
4.75	Acetate	–	4.22	4.63	4.90
5.20	Acetate	–	1.66	1.89	2.25
6.00 [b]	Succinate	–	0.43	0.61	0.73
6.51 [b]	Phosphate	–	0.13	0.16	0.20

[a] Reactions performed in duplicate. Average values are given with variance limits.
[b] Rate data obtained by the initial rates method.

of rate coefficients by the initial rates method (because of small absorbance changes) no attempt has been made to measure these rates in duplicate. However some measure of consistency can be obtained by comparing rate data obtained by UV and HPLC methods, under the same conditions of buffer concentration and pH (c.f. 0.1 M phosphate, pH 7.0, Tables 1 and 3).

For those reactions which were also monitored by HPLC, the chromatography system and the operating conditions are described above. A chromatogram of a typical reaction mixture is shown in Fig. 1. The linearity of peak integral with concentration was established for both erythromycin A and erythromycin A-6,9-hemiketal by dissolving an accurately determined amount of substrate in mobile phase and immediately assaying. Good linearity and standard deviations on reproducibility were obtained for each substrate.

These reactions were initiated by adding buffer (5 ml), previously incubated at 37°C, to a solution of substrate (9 → 12 mg) in methanol (100 μl) and mixing well. The solutions were immediately returned to the water bath and aliquots were taken at various time points and assayed by HPLC using the conditions described previously. The concentrations of both erythromycin A and "hemiketal" (II) were determined, and depending on the rate of reaction, rate coefficients were calculated by either the initial rates method, or by plots of ln(concentration) against time. Rate data obtained by this method are given in Table 3.

Rate data were also obtained over a 25°C temperature range for both substrates. These are given in Table 4.

TABLE 3

RATE DATA OBTAINED BY HPLC ANALYSIS OF THE REACTIONS OF ERYTHROMYCIN A AND ERYTHROMYCIN A-6,9-HEMIKETAL IN AQUEOUS ACIDIC BUFFERS AT 37°C

Buffer	pH	10^2 k_{obs} (min^{-1})
Erythromycin A-6,9-hemiketal		
0.1 M formate	4.1	1.69
0.2 M formate	4.1	1.96 ± 0.09 [a]

Buffer	pH	10^5 k_{obs} (min^{-1}) [b]	
		Erythromycin disappearance	"Hemiketal" appearance
Erythromycin A			
0.1 M phosphate	6.5	9.11	6.25
	7.0	2.37	2.42
0.2 M phosphate	7.0	6.59	2.74
0.2 M succinate	6.0	81.8	36.9

[a] Average values of duplicate runs.
[b] Obtained by the initial rates method.

Results and Discussion

The decomposition of erythromycin A in aqueous acidic buffers initially shows the appearance

TABLE 4

RATE AND ARRHENIUS DATA FOR THE DECOMPOSITION OF ERYTHROMYCIN A AND ERYTHROMYCIN A-6,9-HEMIKETAL UNDER THE CONDITIONS INDICATED

Buffer molarity (M)	10^4 k_{obs} (min^{-1})				
	Temperature (°C)				
	25	37	50	ΔH^{310} ($kJ \cdot mol^{-1}$)	ΔS^{310} ($J \cdot mol^{-1} \cdot K$)
Erythromycin A (Acetate buffer pH 5.20)					
0.1	1.87	7.50	19.77	72.80	−70.2
0.2	2.77	10.29	26.95	70.12	−76.2
0.3	3.51	12.42	33.60	69.62	−76.2
Buffer molarity (M)	10^3 k_{obs} (min^{-1})				
	Temperature (°C)				
	25	37	50	ΔH^{310} ($kJ \cdot mol^{-1}$)	ΔS^{310} ($J \cdot mol^{-1} \cdot K$)
Erythromycin A-6,9-hemiketal (Formate buffer, pH 4.10)					
0.1	7.01	16.85	40.80	53.7	−105.9
0.25	7.58	19.60	46.26	54.6	−101.8
0.2	8.56	21.35	52.27	55.3	−98.8

of the expected product, erythromycin A-6,9-hemiketal (II). This undergoes a subsequent reaction to form anhydroerythromycin A (III) at appreciable rates, thus, only the initial formation of (II) can be observed. We have used this to determine rates of erythromycin A decomposition.

When the reactions were monitored by HPLC no build up of other intermediates was detected. The rates of erythromycin A disappearance and "hemiketal" appearance were of a similar order of magnitude (see Table 3). Thus reliable estimates of the rate of erythromycin A decomposition can be obtained. In formate buffer (pH 4.1, 0.05 M) the full extent of this complex reaction can be seen (Fig. 2). The concentration of erythromycin A decreases exponentially, and the concentration of "hemiketal" shows a rapid increase, to approximately 10% of the expected value. The reaction to form anhydroerythromycin A then takes over and the concentration of (II) slowly decreases. The degradation of erythromycin A under these conditions follows pseudo first order kinetics.

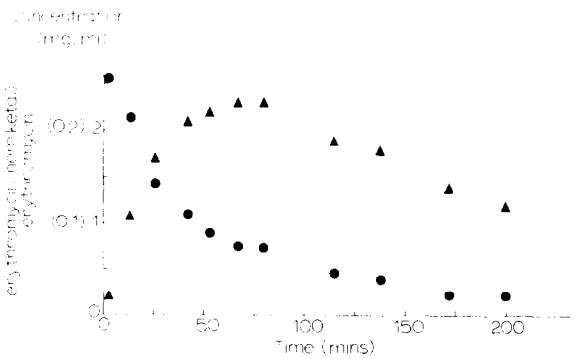


Fig. 2. Plot of concentration against time for erythromycin A (●) and erythromycin A-6,9-hemiketal (▲) in 0.05 M formate buffer, pH 4.1, 37°C.

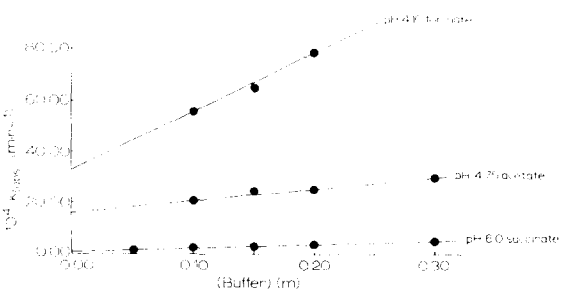
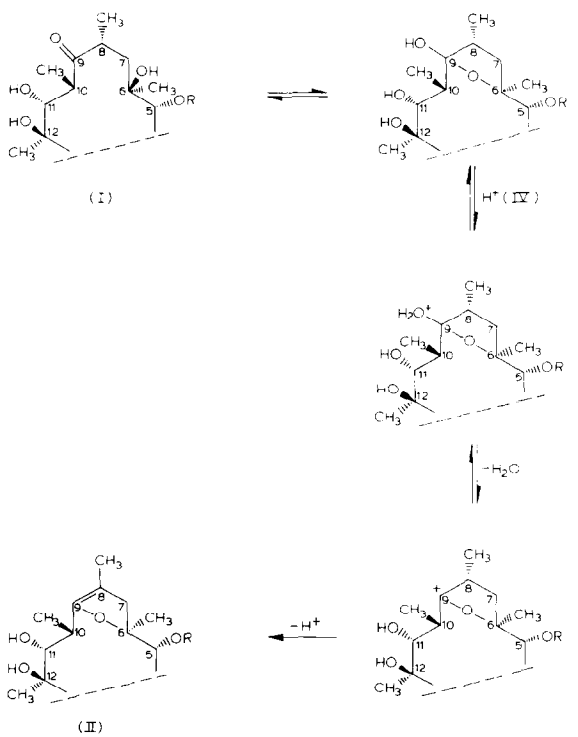


Fig. 3. Plot of k_{obs} against total buffer concentration for the decomposition of erythromycin A in aqueous buffers at 37°C.

The observed rate coefficients, k_{obs}, for the appearance of "hemiketal" (II) show a linear dependence on the total buffer concentration (Fig. 3). The slopes of these plots increase with decreasing pH, indicating catalysis by the acidic component of the buffer. Extrapolation of these plots to zero buffer concentration gives the intercept k_o, which is the buffer independent rate coefficient. These values are given in Table 5, they show a good linear dependence with pH and a very small (negligible) intercept on the abscissa. Thus the reaction of erythromycin A to give erythromycin A-6,9-hemiketal in aqueous solution appears to be subject to both general and specific acid catalysis. No catalytic effect of the solvent could be detected. Plotting the individual rate coefficient (k_{HA}) obtained for each catalytic species, against the acidity of the buffer, as determined by the dissociation constant of the acid (pKa), (see Table 5) in a conventional Brønstead treatment, gives an α value of 0.71 clearly showing the greater catalytic activity of the solvated proton in this reaction.



Scheme 2.

TABLE 5

RATE COEFFICIENTS FOR THE DECOMPOSITION OF ERYTHROMYCIN A IN AQUEOUS BUFFERS AT 37°C

pH	Buffer	linearity [a]	$10^5 k_o$ (min.$^{-1}$) [b]	$10^3 k_{HA}$ (M^{-1} min.$^{-1}$)
3.82	Formate	0.999	178.0	79.2 ± 2.9
4.10	Formate	0.993	325.0	
4.75	Acetate	0.989	163.0	9.81 ± 1.05
5.20	Acetate	0.993	48.8	
6.00	Succinate	0.991	7.6	5.82 ± 0.51
6.51	Phosphate	0.970	3.6	0.28 ± 0.07
7.00	Phosphate	0.999	0.62	
7.46	Phosphate	0.997	0.61	

[a] Correlation coefficients for linear regression analysis of plots of k_{obs} against total buffer concentrations.
[b] A plot of k_o against $[H^+]$ gives $k_o = 40.25 \pm 2.1\ [H^+]$.

The proposed mechanism for the reaction is given in Scheme 2. Rate limiting acid-catalysed internal acetal formation (IV), is followed by rapid dehydration of the internal acetal to give the enol ether, erythromycin A-6,9-hemiketal (II). The conformation of the erythronolides is such that the hydroxyl at C-6 is in close proximity to the C-9 carbonyl (Perun, 1971), and therefore there is a minimum energy requirement for the formation of the internal acetal. Hence this process quite facile, indeed many polyene macrolide antibiotics are thought to exist naturally in this form in solution (Pandey and Rinehart, 1976). However, this intermediate, being a relatively labile tertiary alcohol readily undergoes dehydration to give the enol ether (II). This type of intermediate has been previously postulated in the acid-catalysed degradation of erythronolide B, when, in the case of the triacetate of erythronolide B an equilibrium was established between the hydroxy-ketone structure and enol ether. (Perun, 1971.)

In acidic buffers the enol ether (II) is itself relatively unstable and readily rearranges to give the spiro ketal, anhydroerythromycin (III). The observed rate coefficients for this reaction are given in Table 2. They show a linear dependence on the total buffer concentration, and when extrapolated to zero buffer concentration, give values of k_o which vary linearly with $[H^+]$ (Table 6, Fig. 4). The slopes of the plots of k_{obs} against total

TABLE 6

BUFFER INDEPENDENT RATE COEFFICIENTS (k_o) AND BUFFER CATALYSIS RATE COEFFICIENTS (k_{cat}) FOR THE DECOMPOSITION OF ERYTHROMYCIN A-6,9-HEMIKETAL IN AQUEOUS BUFFERS AT 37°C.

pH	Buffer	linearity [a]	$10^3\ k_{cat}$ ($M^{-1}\cdot min^{-1}$)	$10^3\ k_o$ (min^{-1})
3.06	Formate	0.986	80.80	92.20
3.82	Formate	0.993	63.70	17.20
4.10	Formate	0.994	50.20	11.60
4.75	Acetate	0.994	6.80	3.56
5.20	Acetate	0.992	5.90	1.05
6.00	Succinate	0.994	2.97	0.14
6.50	Phosphate	0.991	0.71	0.06

[a] Correlation coefficients for linear regression analysis of plots of k_{obs} against total buffer concentration.

buffer concentration yield the buffer catalysis rate coefficients (k_{cat}). The rate coefficients for the individual components of the buffer can be determined by plotting the catalytic rate coefficients (k_{cat}) against the fraction of free base buffer (Fig. 5). The intercept at zero α is the catalytic rate coefficient for the acidic component of the buffer (k_{HA}) and when α equals unity the intercept is the catalytic rate coefficient for the basic component of the buffer ($k_{\bar{A}}$). The data derived from these plots are given in Table 7.

Thus the rearrangements appear to be subject to general acid/base as well as specific acid catalysis. Vinyl ethers are known to undergo rapid acid-catalysed hydrolysis in weakly acidic solution (Chiang et al., 1979; Kresge and Chwang, 1978).

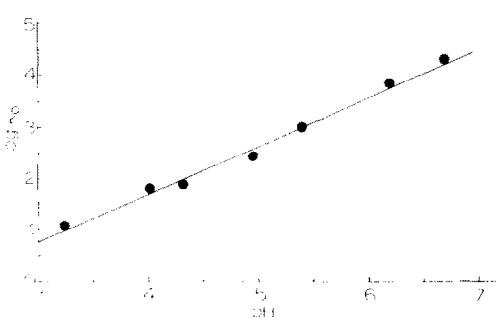


Fig. 4. Plot of log k_o against pH for the decomposition of erythromycin A-6,9-hemiketal in aqueous buffers at 37°C.

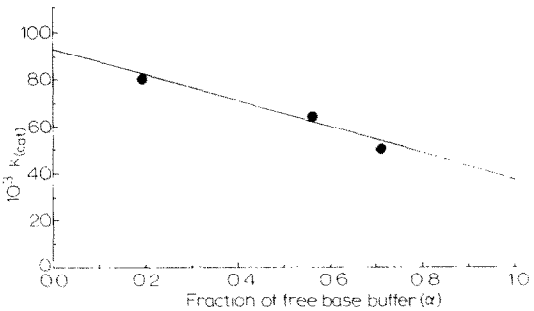
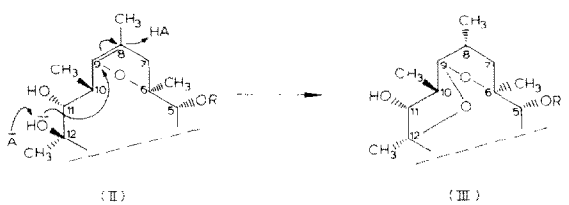


Fig. 5. Plot of k_{cat} against fraction of free base for the decomposition of erythromycin A-6,9-hemiketal in formate buffer at 37°C.

The majority of these reactions proceed via rate determining proton transfer to carbon, with no build-up of the alkoxy carbonium ion. In the rearrangement of hemiketal to anhydroerythromycin A, which can be regarded as an internal hydration, significant general base catalysis has been detected, with no detectable build-up of an intermediate. We therefore suggest that this process is concerted, as depicted in Scheme 3.



Scheme 3.

TABLE 7

CATALYTIC RATE COEFFICIENTS FOR THE DECOMPOSITION OF ERYTHROMYCIN A-6,9-HEMIKETAL IN AQUEOUS BUFFERS AT 37°C.

k_{H^+}	$104.9\ M^{-1}\cdot min^{-1}$
Formic acid	
k_{HA}	$9.31 \pm 0.04 \times 10^{-2}\ M^{-1}\cdot min^{-1}$
k_{A^-}	$3.53 \pm 0.05 \times 10^{-2}\ M^{-1}\cdot min^{-1}$
Acetic acid	
k_{HA}	$8.83 \pm 0.05 \times 10^{-3}\ M^{-1}\cdot min^{-1}$
k_{A^-}	$4.92 \pm 0.02 \times 10^{-3}\ M^{-1}\cdot min^{-1}$

The reaction appears to be essentially irreversible as anhydroerythromycin A shows no degradation under the conditions employed in these experiments. Furthermore, the relative lability of erythromycin A-6,9-hemiketal can be seen when compared with the corresponding hemiketal of erythromycin B (which does not contain a 12-hydroxyl). This has to be heated under acidic conditions before hydrolysis will take place (Kurath et al. 1971).

Observed rate coefficients for both erythromycin A and erythromycin A-6,9-hemiketal decomposition were determined over a 25°C temperature range. Linear Arrhenius plots were obtained and the activation parameters derived from these, along with the rate data, are given in Table 4. Both reactions have high negative entropies of activation. This is to be expected for processes that involve cyclisations to give five membered rings. Presumably no product of cyclisation from the 11-hydroxyl is observed because this would involve the formation of the much less favourable 4 membered ring internal acetal.

From the results presented above it is clear that erythromycin A is susceptible to degradation at both low pH and high concentrations of general acid. We have derived rate coefficients that show that, although the concentration of buffer does affect the rate of degradation, pH is the critical factor in determining the stability of erythromycin A in acidic aqueous solutions.

Acknowledgements

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