KINETICS OF THE HYDROLYSIS OF CEPHALOSPORIN C

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The decomposition rates of cephalosporin C in aqueous buffers and in fermentation broth at 25°C were determined by high pressure liquid chromatography and were found to obey the rate law \(-\frac{dC}{dt} = [0.27 (H^+) + 0.005 + 500 (OH^-)] C \text{ mole.}^{-1}\cdot \text{h}^{-1}\). In the acidity range from pH 3 to 7 the principal reaction is the slow attack of water on the nucleus. In alkaline and in strongly acidic solutions hydrolysis of the acetyl side chain is sufficiently fast relative to the base or acid catalyzed destruction of the nucleus to produce desacetyl cephalosporin C or its lactone in appreciable yields. In a typical fermentation non-enzymatic hydrolysis destroys about 15% of the product.

The decomposition of aqueous cephalosporin C has been discussed in two reviews of the chemistry of cephalosporin C and its biosynthesis\(^1^2\). The original papers, mainly by NEWTON, ABRAHAM and coworkers, deal primarily with the nature of the identifiable end products and intermediates formed under acid, neutral and basic conditions of hydrolysis. Except for the work of HUBER and coworkers\(^3\), who measured the decomposition rate in sterilized and incubated broth at neutral pH, no rate studies of the reaction have been reported.

Since the decomposition rates are of interest in the synthesis of cephalosporin C derivatives, in the processing of fermentation broth and in fermentation studies, we have investigated the kinetics of the reaction in aqueous buffers representing a wide range of acidity.

The molecule of cephalosporin C has at least three sites more or less susceptible to attack by hydrogen ions, hydroxide ions and water: the ester group of the acetyl side chain, the amide group of \(\alpha\)-amino adipyl side chain and the \(\beta\)-lactam ring. Due to the complexity of the reaction system, the instability of the intermediates and the associated analytical problems, the present knowledge of the hydrolytic reactions is still incomplete. It is known that the decomposition at neutral pH is slow and leads to D-4-(2-amino-4-carboxybutyl)-thiazole-4-carboxylic acid as one of the products\(^4\). Although acids catalyze the removal of the acetyl and the amino adipic side chains\(^5\), cephalosporin C is still quite stable at room temperature at pH 2.5\(^6\). Acids catalyze the conversion of desacetyl cephalosporin C to the lactone\(^7\) and cleave the nucleus as well\(^8\). Mild acid hydrolysis leads to the formation of the lactones of desacetyl cephalosporin C and desacetyl-7-amino cephalosporanic acid, and also to 7-amino cephalosporanic acid in very low yields\(^9\). The end products of acid hydrolysis are carbon dioxide and \(\alpha\)-amino adipic acid\(^10\). Alkaline hydrolysis at pH 9.5 or 11 leads to the formation of desacetyl cephalosporin C as an intermediate together with
uncharacterized ninhydrin-positive material\textsuperscript{7}. In weak alkali, the 260 nm UV band, characteristic of the cephalosporin nucleus\textsuperscript{2}, disappears rapidly\textsuperscript{5,6}.

In the present work we have followed the decomposition by high pressure liquid chromatography. The method, which is also suitable for the assay of cephalosporin C in fermentation broths, takes advantage of the 260 nm UV band of cephalosporin C and detects the parent compound as well as the intermediates with the intact nucleus. We also report reaction rates as measured by the differential spectrophotometric method of Claridge and coworkers\textsuperscript{8}, which utilizes the large change in optical density at 260 nm, associated with the destruction of the cephalosporin nucleus by weak alkali.

The rate measurements, carried out at 25°C, span the acidity range from pH 1 to 12.5 and furnish information about the overall reaction and the relative rates of desacetylation and of the destruction of the nucleus in alkaline, neutral and weakly acid solutions. The decomposition rates in neutral aqueous buffers are compared with the decomposition rates in broths and are used to estimate the loss of product in fermentation due to hydrolysis.

**Experimental**

All solutions were prepared with analytical grade reagents. The sodium salt of cephalosporin C $\text{NaC}_{14}\text{H}_{20}\text{N}_{3}\text{O}_{8}\text{S}$ and the sodium salt of desacetyl cephalosporin $\text{NaC}_{14}\text{H}_{18}\text{N}_{3}\text{O}_{7}\text{S}$ were chromatographically pure and their molar extinction coefficients were 8470 and 7900 at 260 nm. The reaction mixtures in which the decomposition was very slow (pH 3 to 9) were sterilized by Millipore filtration. The alkaline solutions were kept under nitrogen to exclude atmospheric carbon dioxide. The pH of the reaction mixtures was measured at 20°C with the Metrohm pH meter E 500, using Merck “Titrisol” buffers as standards up to pH 12 and a Metrohm buffer at pH 13.

The runs were carried out in a constant temperature bath maintained at 25.00±0.05°C, samples being withdrawn at suitable time intervals. Generally, 2-ml aliquots of the reaction mixture were pipetted into 1 ml of 1 M phosphate buffer pH 7 and were then analysed at once or stored over night at −20°C prior to analysis by liquid chromatography.

The liquid chromatography was carried out under the following conditions.

**Apparatus:** VARIAN Liquid Chromatography, Series 4000

**Column:** Stainless steel, 125×2.3 mm i.D

**Static phase:** SAX-ZIPAX DU PONT (strong anion-exchanger)

**Mobile phase:** 0.35 M boric acid, adjusted to pH 9.6 with sodium hydroxide

**Pressure:** 55 atm

**Temperature:** ambiet

**Flow rate:** 0.45 ml/min.

**Detector:** UV\textsubscript{254} (VARIAN)

**Sample size:** 5 μl, corresponding to a total amount of 0.5 to 5.0 μg of cephalosporins (optimum concentration range)

**Fermentation samples:** Aliquots of the samples were generally diluted to approx. 0.01 % active substance (this prolongs column life).

The identity of cephalosporin C peaks was tested using the sodium hydroxide treatment: aliquots of the sample solutions were held for 30 minutes at 25°C the pH being 12.5 to 13. A complete destruction of all known cephalosporin C derivatives was observed after this treatment. This method has been applied also for differential analysis, when unresolved peaks of cephalosporins together with ingredients of certain fermentation media appeared.

The test conditions in the differential spectrophotometric assay were as described\textsuperscript{8}. 
except for the length of treatment of the samples with alkali (pH 12.5), which was 100 minutes in place of 15 minutes, the destruction of cephalosporin C being complete in about one half of the time.

The broth came from a fermentation with a mutant derived from Cephalosporium acremonium, CMI 49137 mutant 8650, the sample being taken at the point where the concentration of cephalosporin C reached the maximum. It was sterilized by Millipore filtration.

**Results**

Chromatograms of neutral, basic and acidic reaction mixtures, sampled at about 50% conversion of cephalosporin C, and of a fermentation broth are shown in Fig. 1. The peaks designated 1, 2 and 3 are the parent compound, desacetyl cephalosporin C and the lactone of desacetyl cephalosporin C. The peaks designated U have not been identified*. The shoulder on the left side of the lactone peak indicated poor resolution of the lactone and another compound. The desacetyl peak in the broth did not disappear completely on treatment of the sample with alkali.

The course of the reaction at pH 10.1 is shown graphically in Fig. 2, where the concentrations of the substrate, of desacetyl cephalosporin C, and the sum of the two concentrations, expressed as mole percent of the initial concentration of the substrate, are plotted against the reaction time. The decomposition rate of cephalosporin C may be represented by the first order equation

\[ \ln \frac{C}{C_0} = -k_1 t \]

where \( C_0 \) is the initial concentration of the substrate and \( C \) is concentration at time \( t \). A first order plot of the data from the runs at pH 1.5 and pH 10.1 is shown in Fig. 3 (curves 1 and 2). The third curve represents the sum of the molar concentrations of the substrate (\( C \)) and desacetyl cephalosporin C (\( D \)) for the run at pH 10.1. When no appreciable concentrations of other cephalosporin derivatives are formed the rate constant \( k_2 \), defined by

\[ \ln \frac{C+D}{C_0} = -k_2 t \]

represents the rate of destruction of the nucleus. The values of \( k_1 \) and \( k_2 \) are summarized in Table 1 and plotted as a function of pH in Fig. 4.

In the early phase of this work, the reaction was followed by the differential spectrophotometric method*, generally up to 60% conversion, as indicated by the spectrophotometric titer. The data in this range may also be represented by a first order equation and the rate constants \( k_3 \) are summarized in Table 2. Insofar as the extinction coefficients of cephalosporin C and the intermediates with an intact nucleus do not differ appreciably, and the differential optical density of the other products is negligible, \( k_3 \) is also a measure of the rate of destruction of the nucleus.

The decomposition rate of cephalosporin C in the broth sample, sterilized by

* Further experiments concerning the decomposition products formed in 0.05 M HCl were carried out after the completion of this work. In addition to the lactone of desacetyl cephalosporin C, 7-aminocephalosporanic acid and its decomposition products were detected. On a freshly packed column the relative retention times were: cephalosporin C 1.00, desacetyl cephalosporin C 0.80, 7-aminocephalosporanic acid 0.59, desacetyl-7-aminocephalosporanic acid 0.52, desacetyl cephalosporin C lactone 0.49 and desacetyl-7-aminocephalosporanic acid lactone 0.42.
Millipore filtration, was $k_1 = 0.0054$ h$^{-1}$ at 25$^\circ$ and pH 6.5 and agreed, within experimental error, with the value obtained by biological assay.

The decomposition rate of 7-aminocephalosporanic acid in 0.05 M HCl, $k_1 = 0.022$ h$^{-1}$, had practically the same value as that of cephalosporin C.

**Table 1. Reaction rate at 25$^\circ$C as a function of pH$^a$**

<table>
<thead>
<tr>
<th>pH</th>
<th>$10^b k_1$, h$^{-1}$</th>
<th>$10^b k_2$, h$^{-1}$</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>51</td>
<td>—</td>
<td>0.20 M HCl</td>
</tr>
<tr>
<td>1.5</td>
<td>20</td>
<td>—</td>
<td>0.050 M HCl</td>
</tr>
<tr>
<td>2.9$^b$</td>
<td>6.6</td>
<td>—</td>
<td>0.20 M CH$_3$COOH/0.10 M NaOH</td>
</tr>
<tr>
<td>6.8$^b$</td>
<td>5.2</td>
<td>—</td>
<td>0.20 M KH$_2$PO$_4$/0.10 M NaOH</td>
</tr>
<tr>
<td>7.0</td>
<td>5.2</td>
<td>4.7</td>
<td>0.040 M KH$_2$PO$_4$/0.020 M NaOH</td>
</tr>
<tr>
<td>8.1$^b$</td>
<td>6.2</td>
<td>—</td>
<td>0.049 M Tris/0.029 M HCl</td>
</tr>
<tr>
<td>8.3</td>
<td>7.4</td>
<td>5.2</td>
<td>0.021 M Tris/0.010 M HCl</td>
</tr>
<tr>
<td>8.9</td>
<td>13.7</td>
<td>7.0</td>
<td>0.055 M Tris/0.010 M HCl</td>
</tr>
<tr>
<td>10.1</td>
<td>76</td>
<td>19</td>
<td>0.040 M NaHCO$_3$/0.020 M NaOH</td>
</tr>
<tr>
<td>12.5</td>
<td>13700</td>
<td>2700</td>
<td>0.030 M NaOH</td>
</tr>
</tbody>
</table>

$^a$ Rates measured by liquid chromatography; initial concentration of cephalosporin C 0.5 g/liter unless indicated otherwise; $^b$ 4 g/liter cephalosporin C.

**Discussion**

The solid U-shaped curve in Fig. 4 representing the overall rate of cephalosporin C decomposition is characteristic of reactions which are susceptible to specific acid-base catalysis and which obey the rate law

$$k_1 = k_a(H^+) + k_w + k_b(OH^-)$$

The curve was calculated with the following values of the rate constants: $k_a = 0.27$ l.mole$^{-1}$.h$^{-1}$, $k_w = 0.005$ h$^{-1}$ and $k_b = 5 \times 10^2$ l.mole$^{-1}$.h$^{-1}$, the pH of the strongly acid solutions...
being based on the activities of hydrochloric acid.

The overall decomposition is a result of at least three independent primary reactions—ester, amide and β-lactam hydrolysis—with different pH-rate profiles. The curves for the hydrolysis of ethyl acetate, and the slower hydrolysis of acetamide, are V-shaped with a minimum near pH 3.9. (Rate data have been reported10,11, for the hydrolysis of γ-, δ- and ε-lactams, but not of β-lactams). As a consequence of the different profiles, the ratio of the products changes with pH.

The principal reaction in the flat region extending from pH 3 to 7 is the attack of water on the nucleus. This is evident from the virtual absence of products with appreciable absorptivity at 254 nm (Fig. 1 A) and the agreement of the decomposition rates measured by the differential spectrophotometric method and by

![Fig. 2. Course of the reaction at pH 10.1: (1) cephalosporin C, (2) desacetyl cephalosporin C, (3) sum of the two.](image)

![Fig. 3. First order plots of rate data: (1) cephalosporin C at pH 1.5 (upper time scale), (2) same at pH 10.1 (lower time scale), (3) cephalosporin C+desacetyl at pH 10.1.](image)

![Fig. 4. The rate constants as a function of pH.](image)
liquid chromatography.

In the acid region desacetylation proceeds at a significant rate relative to the destruction of the nucleus. The low concentrations of desacetyl cephalosporin C relative to the lactone indicate that the acid-catalyzed conversion of the desacetyl intermediate to the lactone is faster than its formation.

The principal reactions in the alkaline and intermediate pH region may be represented by the scheme

$$\text{Cephalosporin C} \xrightarrow{k_1} \text{Desacetyl cephalosporin C} \xrightarrow{k_2} \text{Products} \quad \text{Products}$$

where $k_5$ is the rate of destruction of nucleus, taken to be the same for cephalosporin C and desacetyl cephalosporin C$^*$. Subject to this assumption the experimental rate constants $k_1$ and $k_2$ are simply related to $k_1$ and $k_5$, namely, $k_1 = k_4 + k_5$ and $k_2 = k_5$. In the strongly alkaline region $k_1/k_2 = 5$ and hence $k_1/k_5 = 4$. Thus the base-catalyzed desacetylation is about four times as fast as the base-catalyzed destruction of the nucleus.

There is no evidence of enzymatic decomposition of cephalosporin C in the broth tested**: The decomposition rate had practically the same value as the rate in a pure buffer of comparable pH and as the rate reported by Huber and co-workers$^3$ for sterile and inoculated broth. The quantity of cephalosporin C lost by non-enzymatic hydrolysis in a fermentation may be calculated from the production curve by the equation

$$S_t = C_t + k_1 \int_0^t C dt$$

where $C_t$ is concentration of cephalosporin C in the broth at time $t$, $k_1$ the specific rate of hydrolysis at the given temperature and pH, $S_t$, the concentration of cephalosporin C which would have been reached in time $t$ if no hydrolysis occurred, and where the integral represents the area under the production curve.

Curve 1 in Fig. 5 is quite similar to the production curve shown by Huber and co-workers and may be taken as fairly typical of the fermentation. Curve 3 shows the production curve after correction for losses by hydrolysis, and curve 2 the total amount of product which had hydrolysed.

* In view of the structure of the two compounds, this assumption is reasonable. Furthermore large differences in reactivity would manifest themselves by a curvature of the first order plots of $C+D$ (see equation (2) and Fig. 3), which is not evident.

** There is, however, evidence of a cephalosporin C destroying enzyme in broths fermented beyond the maximum yield of cephalosporin C (M. Liersch, communication).
At 110 hours the loss amounts to 14% of the substance actually produced, the result of the calculation being independent of the concentration scale. This value corresponds to the level of desacetyl cephalosporin C in fermentation broth, reported by Huber and co-workers\(^3\). However, at pH 7 the main reaction is the destruction of the nucleus. Thus desacetyl cephalosporin C concentrations of the magnitude reported must have an other origin than the hydrolysis of the product.

Acknowledgements

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References