ALKALIMETRIC MICROASSAY OF CEPHALOSPORINS

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(Received for publication May 1, 1978)

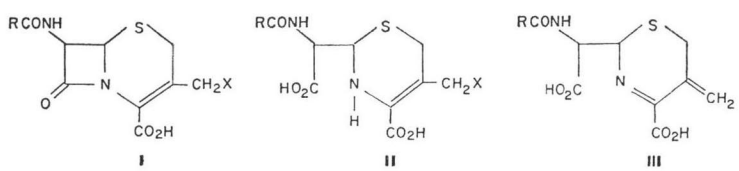
Alkalimetric pH-stat titrations of cephalosporin C, cephacetril and their deacetyl derivatives using an acetyl esterase and β-lactamase are described. The esterase was used to assay highly purified samples of cephalosporin C and cephacetril, and also to prepare analytically defined solutions of the deacetyl cephalosporins. Lactamase-catalyzed hydrolysis of the parent compounds was then found to generate exactly 2 equivalents of acid per mole; that of the deacetyl derivatives exactly 1 equivalent.

Quantitative analysis of cephalosporins by liquid chromatography[1,2,3] and other methods requires well characterized samples of the principal component and the impurities for calibration. The purification, handling and adequate characterization of these reference cephalosporins often presents considerable problems. The materials are labile, may decompose slowly on prolonged storage even at low temperatures, and conventional physical criteria of purity such as melting points are inapplicable. The variable amounts of chemically or physically bound water, like any other optically transparent impurities, are not registered in liquid chromatography by UV detectors. Direct methods for the assay of the anhydrous substances in such reference samples are therefore desirable.

The present work describes an alkalimetric assay of four cephalosporins involving the use of a non-specific acetyl esterase[4] from *Bacillus subtilis* and of β-lactamase P-99[5] from *Enterobacter cloacae* to generate stoichiometric quantities of acid.

Under proper enzymatic conditions, cephalosporins having the substituent $X=CH_3CO_2$ in their molecule (see Fig. 1, structure **I**) are hydrolyzed quantitatively to deacetyl cephalosporins and acetic acid as well defined products[4]. Lactamases, which have a much wider range of applicability, produce labile[6] acids having the proposed structures[7,8,9] II or III, depending on the nature of the substituent X. When X is a good leaving group like acetyl or pyridinium product **III** is formed with a second equivalent of acid (CH_3CO_2H, $C_5H_5NH^+$) being generated by the expulsion of the leaving group in the process[6,9]. However, the exact stoichiometry of these reactions has not yet been established with a precision adequate for analytical applications. In fact, the alkalimetric titres of cephalosporin C

Fig. 1. Some cephalosporins (**I**) and proposed structures (**II, III**) of the products formed by their β-lactamase-catalyzed hydrolysis. Cephalosporin C: $R=(CO_2H)CH(NH_2)(CH_2)_3-$, $X=CH_3CO_2-$; cephacetril: $R=CNCH_2-$, $X=CH_3CO_2-$; deacetyl cephalosporins: X=OH; deacetoxy cephalosporin C: $R=(CO_2H)CH(NH_2)(CH_2)_3-$, X=H; cephaloridine: $R=C_4H_4SCH_2-$, $X=C_5H_5N\oplus-$; deacetyl cephalosporin C lactone is formed from deacetyl cephalosporin C by lactonization of the neighbouring $-CH_2OH$ and $-CO_2H$ groups on the 6-membered ring.



fall short 10~20% of stoichiometry[6] when the material is assumed to be pure and dry.

In the present work the stoichiometry of acid formation in the lactamase-catalyzed reactions was established by using the esterase to assay highly purified samples of cephalosporin C and cephacetril, and also to prepare analytically defined solutions of the deacetyl derivatives. The proper choice of conditions for such titrations is discussed as well as factors which may lead to real or apparent deviations from stoichiometry in β-lactamase-catalyzed hydrolysis.

Experimental

Apparatus

Radiometer titration assembly (pH Meter PHM 64, Titrator TTT 60, Autoburette ABU 13) Metrohm electrode EA 147 and a thermostated titration vessel furnished with a magnetic stirrer and an inlet and outlet for nitrogen; a 2.5 ml burette containing 0.100 N NaOH for the assay of cephalosporins, a 0.25 ml burette for determining enzyme activities; Zeiss spectrophotometer RPQ 20 A.

Assay of cephalosporins

The pH-stat titrations of cephalosporins were carried out at 25°C under nitrogen. The sample was dissolved in 5 ml buffer (0.1 M KCl/10 mM K-phosphate) in the titration vessel and the pH was adjusted immediately to the set point by automatic titration. Reaction was then started by adding enzyme. Stock solutions of the nearly pure enzymes, which were stored at 4°C with little or no loss of activity in 1 month, contained 1,400 units/ml of lactamase P-99 or 600 units/ml esterase; the enzyme storage medium contained 0.1 M KCl/2 mM phosphate pH 7.0 (lactamase) or 0.1 M KCl/20 mM phosphate pH 8.0 (esterase). Specific activities of the two enzymes were 107 and 395 units/mg protein respectively, protein assays being based on optical densities at 280 and 260 nm. Addition of these enzyme solutions to the titration buffers produced no change in pH.

Materials

Cephaloridine was purchased from Glaxo, the other cephalosporins were obtained from the CIBA-GEIGY research laboratories. Deacetyl cephacetril was prepared by deacetylating the parent compound with the immobilized esterase[6] and lyophilizing the solution. The sample therefore contained all the sodium acetate produced by the reaction and a little sodium phosphate. Impurities in the analyzed samples were determined quantitatively by high pressure liquid chromatography using defined mixtures of the main component and the other cephalosporins for calibration. Nucleosil 10 C 18 was the stationary phase and 5 mM $NaClO_4$ in 0.1% isopropanol/99.9% water the mobile phase; for

Table 1. Chromatographic purity of the employed cephalosporins.

Compound/sample designation	Relative weight of the components					
	P[a]	DA[b]	DAO[b]	DAL[b]	S[b]	U[c]
Cephalosporin C/A	100	0.2	<0.1	0.2	0.1	0.1
" /B	100	0.4	<0.1	0.3	<0.1	
" /C	100	0.2	<0.1		0.1	0.8
" /D	100	2.8	0.8	0.9	0.9	0.6
Deacetyl cephalosporin C		100	0.5	0.4	0.2	0.2
Deacetoxy cephalosporin C	0.8	<0.1	100			
Cephacetril	100	0.5				
Deacetyl cephacetril		100				1

a) P stands for the parent compound, *i.e.* for cephalosporin C or, in the case of the last two samples, for cephacetril; b) the symbols represent the following derivatives of the parent compounds: DA deacetyl, DAO deacetoxy, DAL deacetyl lactone, S sulfone; c) unknown components; their values are based on areas under the peaks relative to the area under the main component taken as 100.

analyses of cephacetril and deacetyl cephacetril the mobile phase was 25 mM $NaClO_4$ in 1.5% acetonitrile/98.5% water. The values given in Table 1 are the relative weights of the main component and of the impurities in the samples. Composition of the samples in terms of weight percent may be calculated from these values and the alkalimetric titres in Table 2 (Concerning the limitations of such a calculation when the impurity levels are high see "Discussion").

Table 2. Lactam titres of five cephalosporins at pH 8 and pH 7.

Compound[a]	Lactamase, units	Lactam titre, % of theory	
		pH 8	pH 7
Cephalosporin C[b]	15	90.2	90.4[c]
Cephacetril	109	98.3	97.9
Cephaloridine	20	96.9	93.8
Deacetyl cephalosporin C	15	88.4	88.2[c]
Deacetyl cephacetril	58	71.5	71.6
Deacetoxy cephalosporin C	33	89.6	88.2

a) All materials except cephaloridine, which is a zwitterion, were Na salts; b) sample A; c) with 3 mM in place of 10 mM phosphate in the titration medium the following titres were obtained: cephalosporin C 90.6%, deacetyl cephalosporin C 88.3%

Enzymes

Isolation of the esterase has been described elsewhere[4]. A new procedure for lactamase P-99 was developed which takes advantage of the high IpH of the protein and permits processing large quantities of enzyme in smaller columns. Ten g of salt-free lyophilized crude cell extract (14 units/mg) were suspended in 200 ml 5 mM K-phosphate pH 6.5, centrifuged at 100,000 × *g* for 2 hours, and the clear supernatant (150 ml, 100,000 units) was applied to a 1,400 ml column of DEAE cellulose (diam. 5 cm) previously equilibrated with the same buffer. On elution with 5 mM phosphate pH 6.5 the lactamase with a specific activity of 350 units/mg protein emerged in 85% yield as the first peak. Adsorption of the eluent on 200 ml CM cellulose preequilibrated with the same buffer and subsequent elution with ten column volumes of phosphate gradient (5 mM to 100 mM) pH 6.5 gave a 90% yield of nearly pure enzyme with a specific activity of 395 units/mg. The solution, adjusted to pH 7 with phosphate, was lyophilized without loss of activity. Solutions having a low buffer content were prepared by gel filtration through Sephadex G-25.

Assay of enzymes

Both enzymes were assayed by pH-stat titration at 25°C in 6 ml reaction medium: Esterase in 0.1 M NaCl/10 mM pH 8.0 phosphate containing 1% v/v 2-methoxyethyl acetate, the lactamase in 0.5 M KCl/5 mM phosphate pH 7.0 containing 0.42% w/v cephaloridine. Enzyme units are defined in terms of μmole substrate/minute hydrolyzed.

Results

In preliminary studies the stoichiometric nature of the esterase-catalyzed hydrolysis was established with 2-methoxyethyl acetate, a stable[10] and defined substrate. With such a material the concentration of enzyme, which determines the titration time, does not affect the titre. Cephalosporins, however, are subject to non-enzymatic decomposition and excessively long reaction times may therefore lead to errors.

The first-order rate constants *k* for the non-enzymatic decomposition of cephalosporin C[1] and cephacetril at 25°C as a function of pH are shown in Fig. 2. At pH 8 these rates were 0.5 and 2% per hour, the deacetyl derivatives being much more stable. Curves 1 and 2 represent the normalized rate versus pH curves for the lactamase P-99 catalyzed hydrolysis of 10 mM cephaloridine (in 0.5 M KCl/3 mM phosphate) and the esterase-catalyzed hydrolysis of various cephalosporins and other substrates[4]. Initial rates of lactamase-catalyzed hydrolysis of various cephalosporins (concentration about 0.03 equivalent/l) in 0.5 M NaCl/10 mM phosphate pH 7.0, expressed as μmole substrate/min./lactamase unit were approximately as follows: Deacetyl cephalosporin C 3.2, deacetoxy cephalosporin C 2.0,

cephalosporin C 1.9, deacetyl cephacetril 1.4, cephaloridine 1.0, cephacetril 0.3. For the esterase values of V_{max} are all in the neighbourhood 1 μmole substrate/min./enzyme unit[4] at pH 8.

Typical titration curves of the substrates are reproduced in Fig. 3, with the first part of curve 5 showing the automatic adjustment of the solution at pH 8.00 prior to the addition of enzyme. The average deviation between duplicate titrations was 0.5%. As shown in Table 2, a change of the titration pH from 8 to 7 had no effect on the results obtained with cephalosporin C, cephacetril and their deacetyl derivatives; these results were also unaffected by reducing and increasing the lactamase concentration by a factor of 2. The term "lactam titre" represents the nominal content of the anhydrous compound in the samples, on the assumption that hydrolysis of the first three compounds generates 2 equivalents, that of the others 1 equivalent of acid per mole.

Fig. 2. Normalized rate *vs.* pH curves for 1) lactamase-catalyzed hydrolysis of cephaloridine, 2) esterase catalyzed hydrolysis of various substrates. Curves 3 and 4 represent the first-order rate constants k for the non-enzymatic decomposition of cephalosporin C and cephacetril.

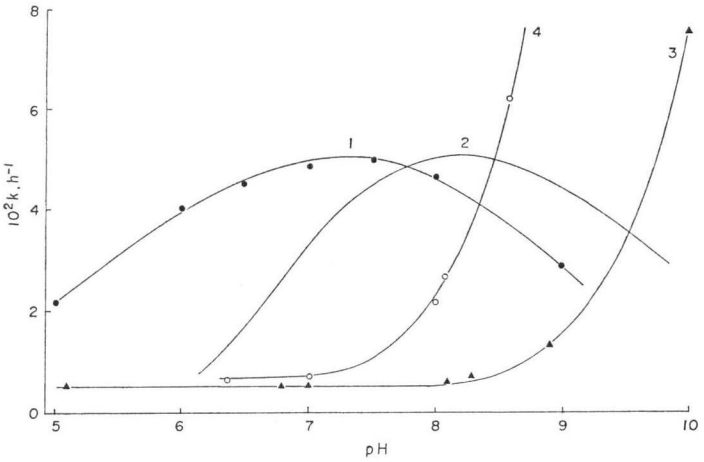
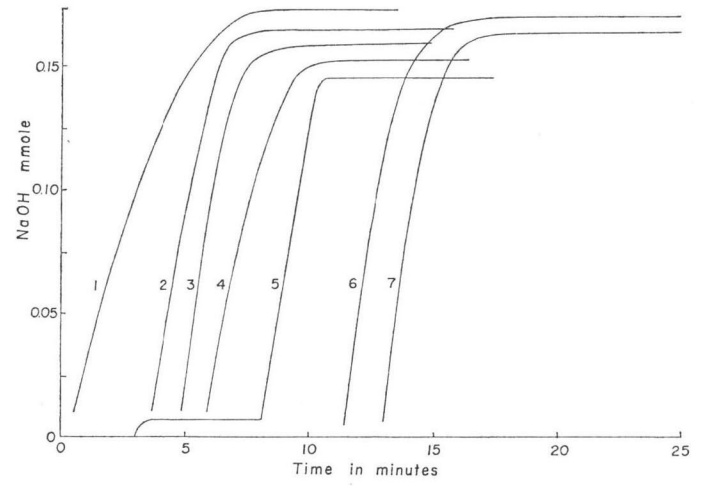


Fig. 3. Titration curves at pH 8 for the following substrates in the presence of the specified quantities (units) of lactamase 1) cephacetril, 72, 2) cephalosporin C, 15, 3) deacetyl cephalosporin C, 29, 4) deacetyl cephacetril, 43, 5) cephaloridine, 29. Curves 6 and 7 represent titrations of cephalosporin C and cephacetril with the esterase (113 units) as catalyst.



The stoichiometry for four of these substrates was determined by measuring the quantity of acid generated at pH 8 per unit weight of cephalosporin C and cephacetril in the following reactions: 1) Direct hydrolysis with lactamase (L_1), 2) direct hydrolysis with esterase (E), and 3) lactamase-catalyzed hydrolysis of the deacetylated solutions (L_2) from the preceding reaction (2). As is apparent from the results in Table 3 the stoichiometric relation $2E=2L_2=L_1$ holds within 1% for the purified materials.

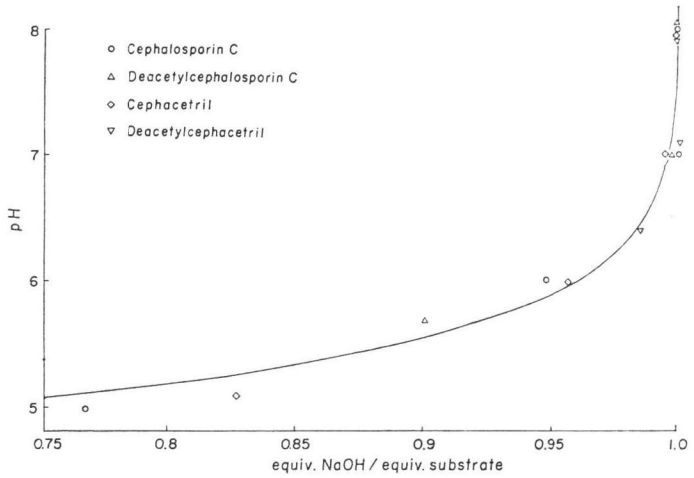
Table 3. Relation between the acetoxy titre E and the lactam titres L_1 and L_2[a]

	Lactam titre %	Impurities[b]		$(E+L_2)/L_1$	L_2/E	L_1/E
		identified	unknown			
Cephacetril	98.3	0.5	—	1.002	1.000	1.996
Highly purified cephalosporin C	90.2[c]	0.5	0.1	1.003	0.988	1.982
	88.3[c]	0.8	—	1.000	0.998	1.998
	86.7[c]	0.3	0.8	1.006	0.992	1.980
	87.4	1.3	—	1.003	1.010	2.004
Other samples of cephalosporin C	69.1[d]	5.4	4.2	0.998	1.034	2.038
	82.2[c]	5.0	0.6	1.001	1.040	2.038

a) The symbols represent the quantity of base consumed per gram cephalosporin C or cephacetril in their hydrolysis by esterase (E), by lactamase directly (L_1) or by lactamase after preceding hydrolysis with esterase (L_2); b) parts by weight, relative to main component, taken as 100; c) samples A, B, C and D, in the given order; d) originally highly purified sample, which decomposed on prolonged storage.

For the impure samples of cephalosporin C, which had a higher content of β-lactam rings than of hydrolyzable acetoxy groups, $L_2 > E$. However, $L_1 = E + L_2$ for all materials; this shows that the total quantity of acid produced by direct hydrolysis with lactamase was equal to that produced by the esterase and lactamase, applied sequentially. No acid was generated when the non-specific acetyl esterase was added to solutions previously hydrolyzed with lactamase.

Fig. 4. Relation between the quantity of base consumed and the titration pH for cephalosporin C, deacetyl cephalosporin C, cephacetril and deacetyl cephacetril. Line represents the theoretical titration curve of an acid with pK 4.6.



The products formed by lactamase hydrolysis are weak acids. The quantity of base consumed in the titrations, expressed relative to the value at pH 8, is therefore a function of titration pH, as shown in Fig. 4*. The curve drawn through the data is the theoretical titration curve of an acid with pK 4.6.

Discussion

As is apparent from the results, lactamase-catalyzed hydrolysis of cephalosporin C, cephacetril

* Side reactions take place at pH 5 and manifest themselves by a) a rather rapid increase in pH when the burette ceases to deliver base and b) an increase of the titres to the given limiting values when the titration time is reduced by increasing the concentration of catalyst. These follow reactions are much faster than the γ-lactonization of deacetyl cephalosporin C.

and their deacetyl derivatives generates stoichiometric quantities of acid and may therefore be used for determining the quantity of the anhydrous compounds in chromatographically pure samples. Mixtures of the cephalosporins and deacetyl cephalosporins may also be assayed, hydrolysis with esterase giving the total acetoxy groups, and subsequent hydrolysis with lactamase the total β-lactam rings. However, before using this two-step procedure to determine the β-lactam content of arbitrary mixtures it is necessary to ascertain that the lactamase (or mixture of lactamases) hydrolyzes all the compounds efficiently and that all compounds generate stoichiometric quantities of acid. (The lactamase-catalyzed reactions described here also proceed satisfactorily in biological fluids like urine).

Titrations involving hydrolysis of the acetoxy side chain with the esterase are applicable to other cephalosporins. The quantity of acid produced by lactamase may, however, deviate from stoichiometry if the unstable primary products partake in side reactions involving pH change, or if cleavage of the β-lactam ring changes the pK of acid and basic substituents which are not directly involved in the reaction. The quantity of base consumed in the titration also depends on the pK of the expelled species X. The increase of the titre of cephaloridine with titration pH (Table 2) is at least partly due to the relatively high pK of the generated pyridinium ion (pK 5.2), which is incompletely neutralized at pH 7.

With regard to the stability of the products, 5×10^{-5} M cephalosporin C, deacetyl cephalosporin C, deacetoxy cephalosporin C and cephaloridine in 10 mM phosphate pH 7 were hydrolyzed completely by 4 units lactamase/ml within 30 minutes, the optical densities of the products at 227 nm subsequently declining at a rate of 8, 8, 4 and 2% per hour. Spectra of the first two products, unlike the others, were nearly identical with adsorption maxima at 227 nm. Thus both substrates probably give rise to the same principal product.

When deacetyl cephalosporins cannot be assayed *via* lactamase-catalyzed hydrolysis on account of their low reactivity,* analytically defined solutions for calibrating working standards by liquid chromatography may be obtained by enzymatic deacetylation of defined solutions of their parent compounds. With 0.1% substrate in 50 mM phosphate pH 7 and 4~10 units/ml esterase deacetylation at 25°C is usually complete within 30 minutes. This procedure may also be used for determining the molar extinction coefficient of the deacetyl derivatives and for the UV-spectrophotometric assay of the working standards.**

Lactamase P-99 immobilized by the same method as the esterase[4,13] on controlled-pore glass is a very stable catalyst. However, substrates which are less reactive than cephalosporin C require awkward or impractical catalyst volumes to complete the titration in a short time.

Acknowledgements

The purified samples of the cephalosporins were kindly supplied by Dr. W. VOSER and Mr. K. WEISS. The chromatographic methods were developed and the samples analyzed by Dr. H. MAJER and Mr. E. FELBER.

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* In our experience, based on about a dozen cephalosporins, lactamase P-99 is rather insensitive to the nature of the substituent X but sensitive to R (see Fig. 1). The converse seems to apply to lactamase II from *B. cereus*[11,12]. The rates of hydrolysis of deacetyl cephacetril and deacetyl cephalosporin C under pH-stat conditions were found to be an order of magnitude slower than those of the parent compounds, while deacetoxy cephalosporin C and certain deacetyl cephalosporin γ-lactones reacted very slowly, if at all. Neither lactamase hydrolyzed 7-ACA and 7-DACA.

** Molar extinction coefficients of a number of cephalosporins and of the products of their lactamase-catalyzed hydrolysis will be reported together with NMR data later.

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