Determination of Michaelis Constants for β- Lactamase Reaction by Spectrophotometric Methods

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Abstract

Direct spectrophotometric assay of β-lactamase activity toward cephalosporins was found readily applicable to penicillins too. The differential UV absorption spectra of various penicillins and their corresponding penicilloic acids were determined. The appropriate experimental conditions have been examined and the spectrophotometric assay seems adequate for the study of several substrates in a mixture. Moreover this method was found suitable for the computerized analysis of the kinetic data for the determination of Michaelis constants of the various penicillins. The use of the integrated form of the rate equation for the evaluation of the best estimates of Michaelis constants was found to be advantageous.

Keywords: Michaelis Constants, β- Lactamase reaction

Introduction

Most of the chemical methods used for the determination of enzymatic hydrolysis of penicillins and cephalosporins utilize the formation of a new carboxyl group which follows the cleavage of the β-lactam ring. The appearance of this free acidic group in the molecule makes it possible to use pH-stat alkalimetric titration (1), to follow the colour shift of an acid-base indicator (2), and to measure manometrically (3) the CO_2 evolved. The measurement of the generated penicilloic acid is performed also by use of several iodometric methods, based on the formation of a colourless iodine-penicilloic acid complex (4,5). Many improvements and refinements of the above-mentioned techniques have been devised in recent years, yet in some cases experimental difficulties or shortcomings are still involved in the use of these methods.

The determination of the liberated penicilloic acid, in the course of the reaction, actually measures the overall effect including the spontaneous nonenzymatic hydrolysis of the substrate. Moreover, the where the substrate is accompanied by another derivative susceptible to the enzyme activity, all methods relying only on penicilloic acid formation, fail to distinguish between the different contributions to the reaction rate. Consequently, the measurement of the simultaneous hydrolysis of two or more substrates is impossible. The assay of the hydrolysis of type-A penicillins, such as methicillin or oxacillin, by titration with NaOH was found to yield nonlinear reaction curves (6) presumably due to a progressive inactivation of the enzyme. (Type-A penicillins are derivatives carrying side-chains which

interfere with the catalytic activity of penicillanase and labilize the enzyme.) In general the alkalimetric titration technique or the direct iodometric assay are not readily applicable to the study of enzymatic hydrolysis of analogs or substrates which either alter the susceptibility of the enzyme to inactivation by iodine and high pH (7), or react with iodine (8). Where the enzyme activity is highly pH dependant, the acidimetric method (acid-base indicator) should be limited to measurements of the initial reaction rates only, as the activity is altered with the pH change in the course of the reaction. Finally, when the free carboxyl group undergoes further reaction (as in 6-APA) (9), the reaction course cannot be followed by the usual assay procedures (10).

Direct spectrophotometric determination has been used for the assay of cephalosporins (11) and has been suggested for measuring the rate of hydrolysis of ampicillin (12), but was not adopted as a routine assay for penicillinase activity. On the contrary, in a comparative study of assay methods for β-lactamase, direct spectrophotometry was recommended for cephalosporins, but declared inapplicable for penicillins, as substrates (13).

The aim of the present work is to find experimental conditions, appropriate for the direct spectrophotometric assay of the activity of β-lactamase on natural and semisynthetic penicillins. It is also demonstrated that the continuous curves of single assays are especially suitable for computerized treatment of the kinetic data for the determination of Michaelis constants.

MATERIALS AND METHODS

Penicillins. Benzylpenicillin, phenoxymethylpenicillin, methicillin, ampicillin, carbenicillin, oxacillin, 6-amino-penicillinanic acid (6-APA).

Solutions. Buffer 0.1 M phosphate, pH = 7.0; all solutions were prepared with double distilled water.

β-Lactamase, (P 0389, Sigma,from *Baccillus cereus* strain 569/H).

Spectra and kinetic measurements. All spectra and kinetic measurements were carried out with a Varian-Techtron model 635 and a Cary model 14 recording spectro- photometers, equipped with a thermostatted cell holder, which maintained a temperature of $30 \pm 0.2\ ^0C$.

The absorbancies of the solutions of substrate and product were compared and the values of $\Delta\varepsilon\ (\lambda)$ were determined using Eq. (1).

$$\Delta\varepsilon\ (\lambda) = \varepsilon_s(\lambda) - \varepsilon_p(\lambda) = (OD_s(\lambda) - OD_p(\lambda))/\ l * [S] \qquad (1)$$

for each of the substrates studied, where [S] is the substrates concentration and l is the optical path. The reaction rates and the enzymatic activity were calculated for the various substrates using the corresponding $\Delta\varepsilon\ (\lambda)$, where the unit of activity was taken as the amount of enzyme required to hydrolyse 1 μmole of substrate in 60 min at 30 ^0C, pH=7.

RESULTS AND DISCUSSION

Hydrolysis of 6-APA. The relative rate of hydrolysis (benzylpenicilline =100) of 6-aminopenicillanic acid (6-APA) has not been well established, and the values recorded in the literature differ widely from one report to another [from 0% (10) to 40% (15)].

The hydrolysis product may undergo further changes after the cleavage of the β-lactam ring; consequently, the acidimetric, manometric and alkalimetric methods cannot be used reliably for assaying the hydrolysis of 6-APA. Moreover, this substrate as well as its decomposition product react with iodine thus complicating the measurement of the hydrolysis reaction by the iodometric method. It seemed advantageous to check the reaction of this penicillin by using direct spectrophotometric technique. To this effect the uv absorption spectrum of 6-APA in 0.1 M phosphate buffer, pH=7, was recorded at 30 ^0C and compared

with the spectrum of the enzymatic decomposition product of the substrate. The results are shown in (**Figure1**) along with the differential spectrum for this system. Considering figure 1 it is obvious that the reaction is accompanied by a marked decrease in the optical density; this change in OD with time may easily serve as a measure for the progress of the reaction.

In general it is desirable to follow the hydrolysis of the penicillin around the peak of its differential spectrum. However, when higher substrate concentrations are required one is forced to study the reaction at the "off-peak" wave lenghts to avoid high absolute absorption. This might happen when the enzyme-substrate affinity is low [as in the case of 6-APA and exopenicillinase from *B.cereus* 569/H (16)] and substrate concentration ought to be high in order to keep saturating conditions. As a consequence the sensitivity of the measurement is reduced. When the enzyme-substrate affinity is high, the choice of the wavelenght is not limited as above. Yet, on studying such systems one is encountered with a different difficulty in the measurement of K_M values, as the introduction of lower initial concentrations of the substrate results with smaller OD changes.

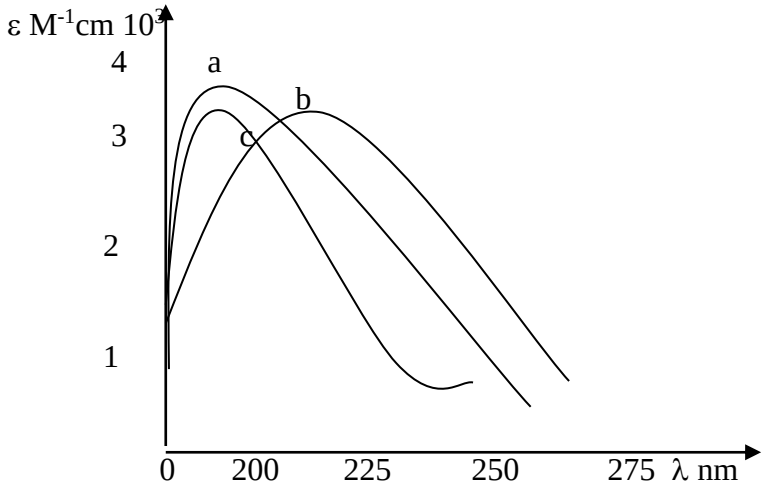

Figure 1. Ultraviolet absorption spectra of 6-APA solution in 0.1 M phosphate, pH=7, before (a line) and after (b line) enzymatic hydrolysis. The third curve c which refers to the right-hand side ordinate is the differential absorption spectrum of 6-APA and its hydrolysis product.

To overcome this it is possible to use either the extended sensitivity range of the spectrometer (0-0.1 OD), or to increase *l* using longer optical path, or to switch to wavelenghts
closer to the peak of the differential spectrum, thus increasing Δε.

As can be seen in Fig.1 the catalytic hydrolysis of 6-APA can be conveniently studied by measuring Δ OD (t) = $l*\Delta\varepsilon\ (\lambda)*[S]\ (t)$ at any wavelenght between 210 and 265 nm. From the change of the absorption with time and knowing Δε (λ) the absolute reaction rate is readily calculable.

The change in OD with time at 265 nm during the enzymatic hydrolysis of 6-APA in 1M phosphate, pH =7, at 30 ^{0}C was followed and a typical reaction curve is presented in (**Figure 2**).

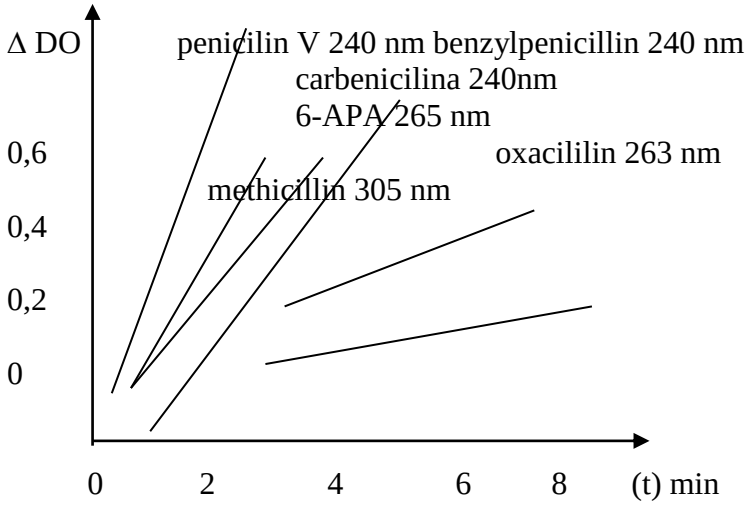

Figure 2. The dependence of Δ OD on time during the hydrolysis of several penicillins by β-lactamase at pH=7,30 ^{0}C, 1 cm optical path. Benzylpenicillin 0.5mg/ml; β-lactamase 12 units/ml; carbenicillin 1 mg/ml, 61 units/ml;penicillin V 1 mg/ml,24 units/ml; 6-APA 10 mg/ml, 3000 units/ml; methycillin 3 mg/ml, 610 units/ml;oxacillin 10 mg/ml, 1400 units/ml, 0.2 cm optical path.

Evidently the spectra of both the substrates and of their products exhibit similarity regarding the location of the main absorption band (near 210 nm). Nevertheless spectral differences between each penicillin and its penicilloic acid, even if small, are significant. Furthermore, the differential spectra of certain different substrates do not coincide. For instance, the hydrolysis of benzylpenicilline or phenoxymethylpenicilline (penicillin V) do not cause any OD change at 265 nm, where the hydrolysis of 6-APA was followed. In such cases it is possible to follow the decomposition of each substrate independently.

The enzymatic hydrolysis of benzylpenicillin, penicillin V, and carbenicillin were followed at 240 nm, and the hydrolysis of methicillin and oxacillin at 305, and 263 nm, respectively. The corresponding reaction plots are displayed in (**Figure 2**).

Table 1. Spectrophotometric Determination of Relative Rates of Hydrolysis [a] and Michaelis Constants [b] of Various Penicillins

Substrate	λ(nm)	Δε ($M^{-1}cm^{-1}$	Relative rate [a] of hydrolysis(%)	μM)
Benzylpenicillin	240	500	100	52
Penicillin V	240	405	148	64
Carbenicillin	240	400	35	120
6-APA	265	14	133	1400
Methicillin	305	41	3.5 (5)[c]	490
Oxacillin	263	-240	3.0 (5)[c]	240

a V values are presented as relative rates of hydrolysis, where that of benzylpenicillin is taken at 100.
[b] β-Lactamase is the exoenzyme of *Bacillus cereus* 569/H.
[c] The value in the parentheses refers to the initial rapid phase of the reaction.

It should be noticed that the rates of hydrolysis of type-A penicillins (methicillin and oxacillin) achieved a constant value only several minutes after the start of the reaction. In the present work relative rate of hydrolysis of these penicillins is based on the second, linear phase of the reaction. In contrast to these results, when the hydrolysis of either methicillin or oxacillin was followed in a pH-stat by titration with 0.01M NaOH, the reaction curves obtained were not linear throughout. On the contrary, the plots exhibited a time dependent decrease in the reaction rate. Indeed, when the reaction came to an end, regeneration of the reaction could be achieved by the addition of a new batch of the enzyme rather than of the substrate, indicating a progressive inactivation of the enzyme.

Similar kinetics was observed when the iodometric assay of benzylpenicillin by penicillinase was followed (at any arbitrary wavelenght between 300 and 600 nm) in a spectrophotometer. The decay of the absorbance due to the iodine was slightly nonlinear, indicating again inactivation of the enzyme which becomes appreciable with time. Thus the linear reaction plots displayed in Fig.2, manifest an evident advantage of the direct spectrophotometric assay.

Based on the respective values of $\Delta\varepsilon$ (λ) for various penicillins, the relative rates were calculated. The results summarised in Table 1 are in a satisfactory agreement with the literature (17). The different values of V for oxacillin reported previously, presumably reflect the average rate of different stages of the same reaction.

Determination of Michaelis constant. The direct spectrophotometric assay of the enzymatic hydrolysis provides the user with a continuous curve of the OD vs time. Consequently
the concentration of the substrate [S] and the instantaneous reaction rate $d[S]/dt$ are accurately recorded at any moment of the reaction. An appropriate choice of the wavelenght enables one to determine the reaction kinetics at various substrate concentrations without losing accuracy and sensitivity. One may also achieve unsaturating the reaction conditions by reducing [S] along with increasing the optical path,*l*.

Providing the reaction obeys a regular Michaelis-Menten kinetics,

$$E + S \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} ES \xrightarrow{k_2} E + P \qquad (2)$$

and in the absence of any interference such as product inhibition, the reaction rate is given by:

$$-\frac{d[S]}{dt} = v = k_2[E]_0 \frac{[S]}{[S] + K_M} \qquad (3)$$

where $k_2\ [E]_0 = V$.

The calculation of the instantaneous reaction rate $d[S]/dt$ naturally requires choosing very small increments of *t*. Such a procedure results in large experimental fluctuations of the measured rates, while the thus calculated values of K_M are highly dependent on the increments. It has been previously noted that on choosing larger time quanta for the calculation of *v*, a better correlation of the results to the estimated constants was obtained. However, this was accompanied by a marked increase in the values obtained for K_M. An arbitrary choice of Δt according to a desired level of confidence, resulted in an arbitrariness of the estimates for the constants.

By adopting the integrated form of Eq. (3) rather than the differential one the user can avoid the necessity of differentiating [*S*] with respect to *t*. Hence, following Stein and Kremer (22), integrating Eq. (3) over *t*, and dividing by $\Delta[S]$, one gets after rearrangements:

$$\frac{-\Delta t}{\Delta[S]} = \frac{1}{V} + \frac{K_M}{V}\,\frac{\Delta ln[S]}{\Delta[S]} \qquad (4)$$

Thus an analysis of the kinetic curve of a single assay will yield reliable values of V and K_M.

The values of K_M and V for several penicillins were evaluated using other procedures using Eq. (4) as before and the results are presented in Table 1. On comparing the values of Michaelis constants thus obtained with those previously reported in the literature (17), a satisfactory agreement is seen.

The above results confirm the applicability of the direct spectrophotometric method, which is quick and reliable to the study of the β-lactamase activity on penicillin derivatives also.

References

1. (a) MURTAUGH, J.J. AND LEVY, G.B. *J. Amer. Chem. Soc*. **67**, 1042; 1984 (b) HOU,J.P. AND POOLE, J.W. (1972) *J. Pharm. Sci*. **61**, 1994.
2. SAZ, A.K, LOWERY, D.L., AND JACKSON, L.J. *J. Bacteriol*. **82**, 298,1989.
3. (a) HENRY, R.J. AND HOUSEWRIGHT, R.D. *J. Biol. Chem*. **167**, 559 1987 (b) POLLOCK, M.R. *Brit. J. Exp. Pathol*. **31**, 739.1990.
4. PERRET, C.J. *Nature (London)* **174**, 1012, 1993.
5. (a) NOVICK, R.P. *Biochem. J.* **83**, 236 1992; (b) CITRI, N.1984 *Meth. Med. Res*. **10**, 221; (c) SARGENT, M.G. *J. Bacteriol*. **95**, 1493 1984; (d) ZYK, N. *Anti. Microb. Ag. Chemother*. **2**, 356.
6. SAMUNI,A. AND CITRI, N. *J. Biol. Chem*, 40, 1901, 1988.
7. CITRI, N. AND GARBER , N. *J. Pharm. Pharmacol*. **14**, 784, 1990.
8. ALICINIO, J. F *Anal. Chem*. **33**, 648.1987.
9. BATCHELOR, F. R. *Nature (London)* **191**, 910, 1992.
10. BEAR, T.A. AND MERTES, M.P, *J. Med. Chem*. **16**, 85, 1987.
11. O'CALLAGHAN,C.H., MUGGLESTON, P.W., AND ROSS, G.W. *Anti. Microb. Ag. Chemother*. p **57**, 1986.
12. JANSSON, J.A. *Biochim. Biophys. Acta* **99**, 171, 1987.
13. ROSS, G.W., CHARTER, K.V., HARRIS, A.M., KIRBY, S.M., MARSHALL, M.J., AND O'CALLAGHAN, C.H, *Anal. Biochem*. **54**, 9, 1990.
14. CITRI, N., GARBER, N., AND SELA, M, *J. Biol. Chem*. **235**, 3454, 1987.
15. CITRI, N.,AND ZYK, N. *Biochim. Biophys. Acta* **99**, 427, 1990.
16. CITRI, N., GARBER, N., AND KALKSTEIN, A. *Biochim. Biophys. Acta* **92**, 562,1989.
17. CITRI, N. (1973) *Adv. Enzymol*. **37**, 397, 1985.