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KINETIC STUDIES OF A β-LACTAMASE BY A COMPUTERIZED MICROACIDIMETRIC METHOD

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Summary

On-line computerized treatment of enzyme kinetic data allows the precise measurement of Michaelis—Menten constants (K_m and V) from a single progress curve. This method has been used to determine the kinetic constants of a β-lactamase extracted from an *Escherichia coli* strain. In the profile of enzymatic activity there obtained, K_m and V are a function of the pH. From these results some information is derived about the mechanism of the enzyme—substrate binding.

Introduction

As the number of bacteria resistant to antibiotics is increasing, the effectiveness of these substances is decreasing. Very often such bacteria produce enzymes which chemically modify these antibiotics. β-Lactamases (penicillin amido-β-lactam-hydrolase EC 3.5.2.6) are the enzymes which are able to cleave an amide bond in penicillins and cephalosporins and make the penicilloic thus obtained completely inactive.

Due to the importance of the problem a few attempts have been made to obtain a classification of β-lactamases [1—4]. These classifications are essentially based on the profile of activity of these enzymes on various substrates and on the reaction of the enzyme with various inhibitors or antiserums [5].

Recently Richmond and Sykes [4] reported a classification in which the β-lactamases are grouped in five major classes which are further divided into subclasses.

Very often the inactivation of β-lactam antibiotics is followed by the microiodometric method [6—11] but the mechanism of the action of iodine with breakdown products of penicillins and cephalosporins is uncertain [12]. Iodine can react either with the antibiotic, even unaltered, or with the enzyme and various other components.

The reaction velocities are determined by measuring the quantity of substrate hydrolyzed per unit of time and generally corresponds to the maximum velocity V. The maximum velocity of penicillin G is taken as the standard $V = 100$. The Michaelis-Menten constant, K_m, is frequently overlooked.

We have recently developed a computerized microacidimetric method for the determination of Michaelis—Menten constants [13,14]. Now the computer works on a statistic program in which the various data are recurrently weighted in order to obtain a higher accuracy. This computation also gives a correlation constant which indicates if the experimental data fits the proposed Michaelis—Menten equation.

This paper deals with a β-lactamase from *Escherichia coli*. It gives a profile of the enzymatic action where K_m and V are a function of the pH, the curves varying with the chemical structure of the substrates. We have also studied the influence of some inorganic ions and temperature.

Material and Methods

Bacterial strain. We used an *E. coli* K 12 which is resistant to ampicillin and carbenicillin, and produces a β-lactamase.

Enzymatic extract. The *E. coli* is grown and treated as described previously. The bacterial extract has been purified by affinity chromatography [15]. We found that in the two preparations there is no significant difference in the Michaelis—Menten constants at least for ampicillin and penicillin G as is shown in Table I. So we always used the crude cell-free bacterial extract in the following experiments.

Kinetics. The reaction kinetics are monitored by the computerized microacidimetric method [13,14], using a Mettler pH stat and a Wang 600 "minicomputer". The pH stat and the computer are connected on line by means of a home-made interface. A crystal clock monitors the whole system. This new modification allows more precise determinations.

Results and Discussion

Methodology

The computerized microacidimetric method is very convenient for the study of the kinetic constants of β-lactamases. A single experiment shows, at a given temperature and pH: (a) That the β-lactamase follows the Michaelis—Menten equation, so we generally obtain a very high correlation. A bad correla-

TABLE I

Kinetic constants (K_m, V at 37° C, pH 7) of penicillin G and ampicillin for (1) crude cell free enzymatic extract and (2) purified enzyme.

Substrate		K_m (μM)	V (rel.)
Penicillin G	1	21	100
	2	20.5	100
Ampicillin	1	29.6	92
	2	29.5	90

tion is indicative of high experimental errors, such as a bad pH regulation. (b) The computation also gives the K_m and V values, even when the experiment is conducted with an insufficient amount of substrate in order to obtain an initial maximum velocity. The determination of K_m and V is obtained with an initial substrate concentration between 0.1 K_m and 20 K_m.

The dilution of the enzyme has to be chosen in order to obtain a reaction time varying from 10 to 20 min. If the reaction time is longer than 30 min there is a loss in the accurancy of the determination due to the higher influence of some experimental errors. A too rapid reaction does not allow the pH stat to correctly regulate the pH.

The determination of K_m in this way is independent of the initial substrate concentration and of the enzyme concentration. The determination of V is also independent of the initial substrate concentration but directly proportional to the enzyme concentration.

The enzyme we are dealing with is a better penicillinase than a cephalosporinase, so we have dealt mainly with penicillins. In terms of "physiological efficiency" [5] the ratio of V/K_m (relative units) for penicillins lies between 100 and 50, for carbenicillin it is 16 and for cephalosporins it lies between 5 and 1.

Influence of pH

Although both K_m and V vary as a function of pH, these variations may

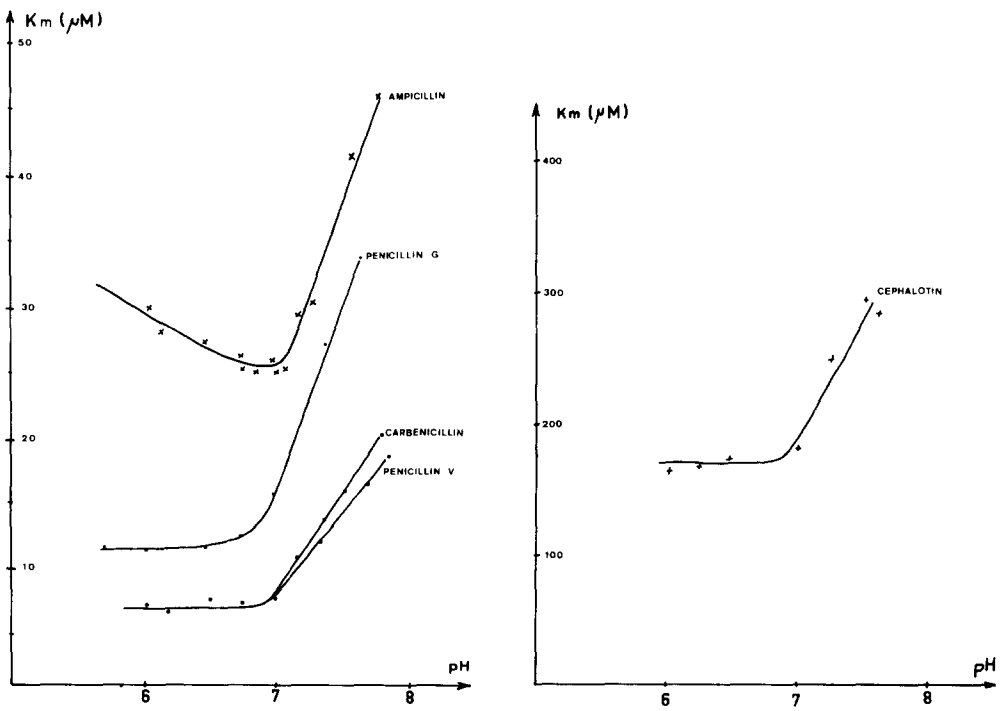

Fig. 1. Variation of K_m for penicillins as a function of pH. Temperature 37°C.

Fig. 2. Variation of K_m for cephalothin as a function of pH. Temperature 37°C.

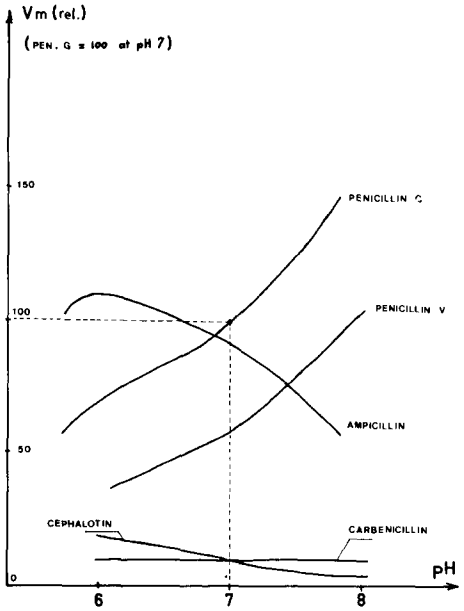

Fig. 3. Variation of V for penicillins and cephalothin as a function of pH. Temperature 37°C.

be independent of one another. The pH zone from 6 to 8 gives the most pertinent information concerning the enzyme–substrate reaction.

K_m. The plots of K_m against pH present the same pattern (Figs 1 and 2) with a small variation, if any, between pH 6 and 6.8. When the pH increases up to 7, the affinity of the enzyme for all the substrates decreases rapidly.

Cephalothin is not a good substrate because the K_m is high and the V low. For cephaloridin we came to the same conclusion. At pH 7 (37°C) its V is good but its K_m is very high.

Following the interpretation of these curves by Cleveland [26], it seems that the active site of the enzyme involves a basic moiety of a pK 7.5.

Only the protonated state of this moiety allows the enzyme–substrate bindings.

V. The schemes of the curves of V as a function of pH are much more complex than those obtained for K_m (Fig. 3). The ampicillin curve presents a maximum at pH 6, whereas the carbenicillin velocity is independent of the pH.

For penicillin G and V the maximum velocities increase with pH but we have been unable to detect an optimum pH because while V increases K_m increases dramatically and at a pH higher than 8 the correlation decreases too much for accurate measurement.

Hou and Poole [21,22] have shown recently by means of classical pH stat titration that K_m and V are capable of significant variation with pH and the nature of substrate.

Fig. 3 shows the variation of V for various substrates as a function of pH (temperature is 37°C). Penicillin G at pH 7 is the standard V = 100. A consequence of this figure is that the "classical" profile of activity of an enzyme at a given pH may have important variations as a function of pH.

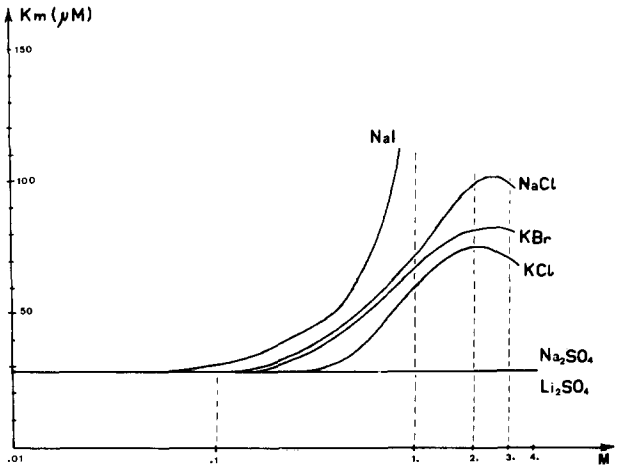

Fig. 4. Variation of K_m for ampicillin at 37°C and pH 7 as a function of the concentration of salts.

Influence of ions

We have studied the influence of monovalent cations (Li^+, Na^+, K^+) and some mono- and divalent anions (Cl^-, Br^-, I^-, SO_4^{2-}).

These ions have little influence on the enzyme activity. Generally it is necessary to reach a concentration of 0.5 M before any influence on K_m or V is detected (Figs 4 and 5).

The influence of a high concentration of NaCl had been used in the affinity chromatography of β-lactamase [15], the enzyme being released from the column with 1 M aqueous NaCl.

NaI gives results which differ from the other salts. At 1 M the enzymatic activity is rapidly destroyed. This may be due to the oxidation of I^- to I_2 which is a strong inhibitor at low concentrations.

NaF and KCN have no influence up to 0.1 M in concentration.

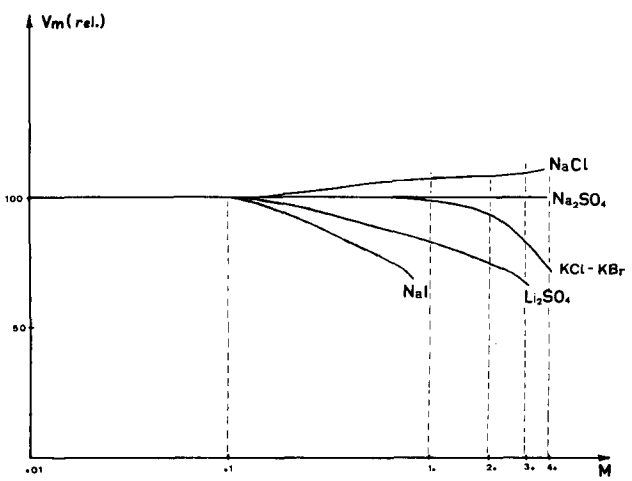

Fig. 5. Variation of V for ampicillin at 37°C and pH 7 as a function of the concentration of salts.

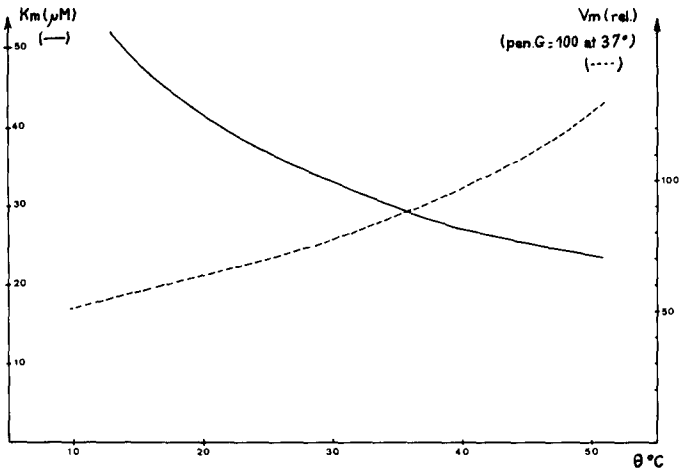

Fig. 6. Variation of K_m and V for ampicillin at pH 7 as a function of temperature

It would appear that this enzyme has a low sensitivity towards the ionic strength of the medium.

Influence of temperature

The experiments were conducted with ampicillin at pH 7. We can see in Fig. 6 that the enzymatic activity increases with temperature: K_m decreases while V increases. These results are in agreement with the Arrhenius equation: $V = 39\,413\ e^{-1882/T}$ (relative $V = 91$ when $t = 37°C$ or $T = 310°K$); $K_m = 0.10065\ e^{1762/T}$ M.

Above 50°C the thermal inactivation of the enzyme begins and the results deteriorate. At 65°C this inactivation is rapid and after 15 min all enzymatic activity had disappeared.

In 1962 Novick [6—11] found an optimal temperature of 55°C for a staphylococcal β-lactamase.

Discussion

If one tries to measure a small amount of penicillin present in a growth medium for example, the microacidimetric method proves inadequate. For kinetic studies, however, the problem is essentially different, as the quantity of antibiotic is not critical and the accuracy and reproducibility of the experiment are more important aspects. It is in this latter case that a pH stat technique comes into its own [12—14, 21—25]. When the treatment of kinetic data is computerized on line the errors introduced by the operator are less important.

The experimental results reported here show that the examination of K_m constants is as important as that of V. Conclusions drawn from V only could be false because some substrates have a high V and low affinity for the enzyme, or vice versa. For example cephaloridin has a V higher than the V of penicillin G. The affinity of the enzyme for cephaloridin is very poor; so, at the biological concentrations, its hydrolysis is very much lower than penicillin G, so that an *E. coli* producing this enzyme is sensitive to cephaloridin.

The active site of our β-lactamase may involve a protonated moiety of pK ~ 7.5 which plays a key role for the formation of the enzyme—substrate complex. The presence of an imidazole group seems to be very probable.

An imidazole group was proposed by Banfield [16] as the active site of a β-lactamase from *Bacillus subtilis* and he obtained the same variations as us for K_m as a function of pH for penicillin G: slow variations between 5, 9 and 7, increasing rapidly between 7 and 8. De Pue et al. [17] have made the same observations and also recently Scott [18] with the β-lactamase R-TEM. The sequence of some β-lactamases have been determined by Ambler and Meadway [19] and in each case they found the presence of at least one histidine group. These various results are in good agreement with our observations.

Conclusion

Until now the characterization of β-lactamases has not often involved the kinetic constants of the enzyme. In antibiotherapy the kinetic aspect is fundamental as the efficiency of an antibiotic is a compromise between its activity on the bacteria, and its sensitivity towards β-lactamase.

The kinetic constants of our enzyme are in good agreement with those in Richmond's class III [4]. But as he measured only the maximum velocities he described these enzymes as "Enzymes with approximately equal activity against penicillins and cephalosporins". The introduction of the K_m may lead to different conclusions.

On-line computerized treatment of enzyme kinetic data allows the precise measurement of K_m and V from a single curve. A single reaction treated in this way gives more precise results than a large number of experiments conducted with the other techniques.

The precise determination of the kinetics parameters of the β-lactamases is an important factor that we must use in order to obtain a better classification and a better understanding of these enzymes.

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