Biochimica et Biophysica Acta, 481 (1977) 759—762

BBA Report

BBA 61318

AN IMPROVED pH-STAT METHOD FOR DETERMINING MICHAELIS-MENTEN CONSTANTS

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(Received September 1st, 1976)

Summary

In pH-stat measurements of enzyme activities at low substrate levels, changes of substrate concentration may be eliminated by the controlled feeding of substrate. Linear response curves are obtained, and errors associated with the visual estimation of initial rates and with other factors are eliminated.

The determination of Michaelis-Menten constants by pH-stat titrations, and also other methods, frequently leads to a family of reaction curves of the type shown in Fig. 1. The most commonly used procedure to calculate K_m from such data is based on initial rates, inferred from the estimated initial slopes. The uncertainty in such estimates increases with increasing curvature

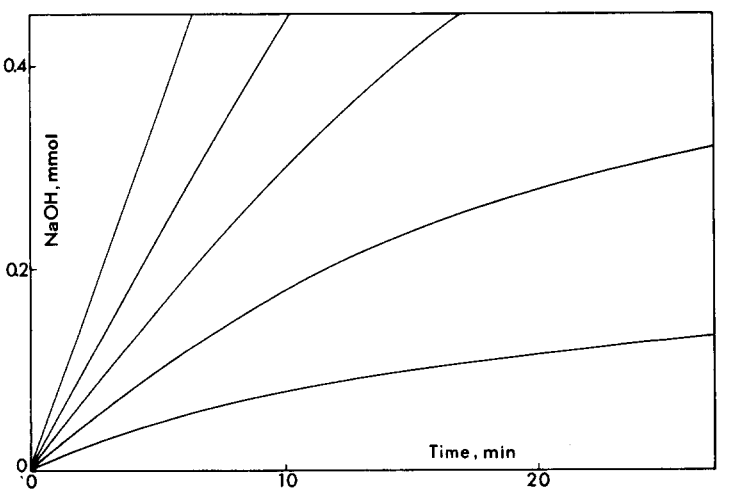

Fig. 1. Recordings of pH-stat titrations at a fixed esterase concentration and the following initial concentration levels of substrate: 0.8, 2, 4, 8, 82 mM. For details, see text.

and may lead to appreciable errors in K_m, especially when Lineweaver-Burke plots are used for the final calculation [1]. Further problems arise in work with immobilized enzymes. When diffusion limitations are very severe on account of the large size of the particles or other factors, appreciable time may be required to establish a steady state concentration of substrate within the catalyst [2, 3]. In such situations the initial rates may not correspond to the steady state values and lead to erroneous values of K_m (app.).

Subject to certain conditions, these difficulties may be resolved by coupling the automatic titration burette to a second one, containing an equimolar solution of substrate. The substrate concentration in the reaction solution then remains practically constant during the run because each mol of substrate transformed by the enzyme, and titrated by the acid or the base, is replaced by an equivalent quantity, delivered by the second burette.

The titration curves shown in Fig. 1 represent the rates of hydrolysis of (2-methoxy ethyl) acetate by an esterase from *Bacillus subtilis* [4] at the given substrate concentrations and a fixed concentration of the enzyme. The experiments were done with 200 ml of a solution containing 100 mM NaCl and 5 mM sodium phosphate, pH 8.0, at 25°C under nitrogen. NaOH (0.1 M) was delivered from a 5-ml Metrohm autoburette "Multi-Dosimat" 415 connected to the Metrohm titrator E 526. The curves shown in Fig. 2, representing the same range of substrate concentrations, were obtained when the first burette was linked to a second one containing 0.1 M substrate.

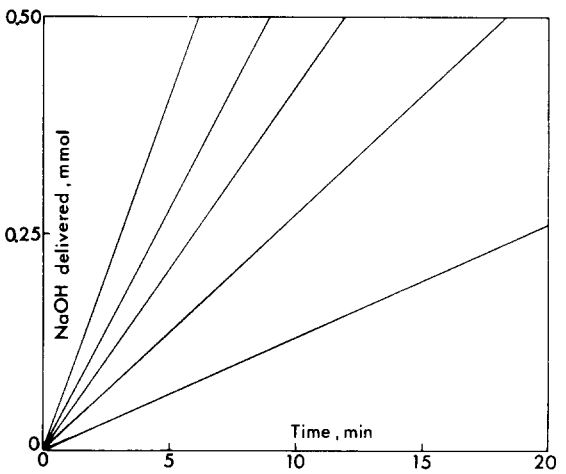

Fig. 2. Recordings at the same substrate concentration levels obtained by the substrate-compensation method.

Before the actual runs were carried out, a suitable speed setting was selected for the first burette to make sure that the speed (rate of piston displacement during the 'on'-phase of the control cycle) was just sufficient to keep up with the highest reaction rate. The electrical outlet from the controler was then connected also to the second burette, which was subsequently 'synchronized' with the first one in trial runs at different settings of its speed dial. Minor adjustments of this setting were made, when necessary, during the actual runs. The differences between the readings of the two burettes never exceeded 0.004 ml.

As shown by the results, the rate curves were perfectly linear in the experimental range. The plot of the rates v (per enzyme unit) against v/s (where s is the substrate concentration) is shown in Fig. 3. The Michaelis-Menten constant calculated from this plot, K_m = 4.5 mM, is identical with the value reported earlier [4].

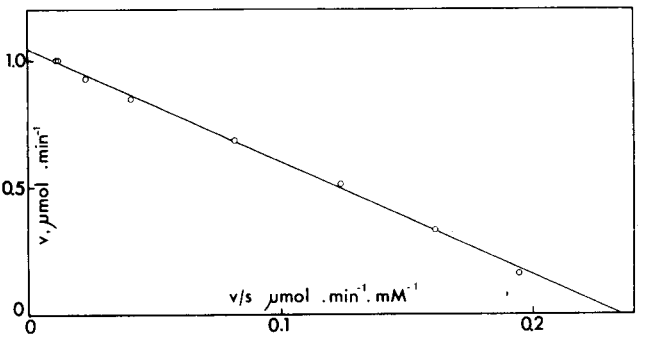

Fig. 3. Plot of the rates (per enzyme unit) versus the ratio of rate/substrate concentration.

Linear titration curves were also obtained with a controlled-pore glass conjugate of the esterase (550 Å pores) having an activity of the order of 60 units/ml carrier under standard test conditions (Konecny, J., unpublished).

There are three restrictions on the use of this method. In the first place the hydrolysis of substrate must be free from side reactions and must have well defined stoichiometry. Otherwise the rate of substrate delivery will not correspond to the rate of substrate hydrolysis. Such a situation may arise if the reaction products undergo slow follow reactions generating acid. In the second place, non-enzymatic hydrolysis must be sufficiently slow so that spontaneous decomposition of the substrate in the burette is negligible in the course of the measurements. This is true of (2-methoxy ethyl) acetate [4]. Finally, the quantity of substrate in the titration vessel at the lowest concentration selected (which depends on the K_m of the reactions) should preferably not be less than about one third of the total quantity delivered by the burette. Otherwise significant errors in substrate compensation may arise from small differences in substrate and base concentrations in the two burettes, from impurities in the substrate and other factors.

The small increase in the volume of the reaction solution in the course of the run (from 200 to 210 ml in the present case) leads to a corresponding decrease of substrate level. It can be eliminated by using a slightly higher concentration of the fed substrate than of the base. However, the incremental value required for compensating the dilution varies with the initial concentration of substrate in the titration vessel, and the work involved is hardly worthwhile except in most accurate work.

Finally, in working with free enzymes with a K_m of the magnitude of the *B. subtilis* esterase, the curvature of the plots shown in Fig. 1 can be reduced also by using 0.01 M in place of 0.1 M base. This, however, calls for a corresponding reduction of the buffer concentration in order to maintain the same sensitivity (pH change per µmol acid produced or base added). Such

changes are, however, not admissible in work with immobilized enzymes, the activities of which change with buffer concentration when the reaction involves the generation of acid or base [4] or when the support carries an electrical charge [5].

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