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Viscosimetric Determination of the "Michaelis Constant" for Proteolytic Enzymes and its Use for the Determination of Reaction Mechanisms

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A method is given for the viscosimetrical determination of the "Michaelis constant" for proteolytic enzymes with gelatine as substrate. The procedure is demonstrated with trypsin but primarily intended for very weak enzyme preparations. Using gelatine with a high intrinsic viscosity — which can easily be obtained by fractionating precipitation of solutions of commercial gelatine with ethyl alcohol — it is possible to determine "Michaelis constants" with values as low as about 0.1 per cent. The use of "Michaelis constants" at various pH values for finding out the mechanism by which an enzyme and its substrate combine, is discussed.

The viscosimetric method is more sensitive than other methods for the determination of the activity of proteolytic enzymes. Unfortunately, gelatine is almost the only substance that can be used as substrate, since a high viscosity is needed. This is a draw-back in investigations of reaction mechanisms, as substrates with only one type of linkages would be preferable, but such high polymeric, water soluble substances are not easily available [1].

The viscosimetric method is of particular interest for investigations of those proteolytic enzymes which are so weak that other methods cannot be applied. Several such enzymes with great physiological interest are known, *e. g.*, the sequence of proteolytic enzymes appearing and disappearing in the early development of a fertilized sea urchin egg, recently studied by one of us [2,3], and the proteolytic enzymes of many bacteria.

The investigations hitherto carried out on the weak proteolytic enzymes have almost exclusively concerned their occurrence and activity-pH-curve. However, it would also be of great interest to study their reaction mechanisms. Two different problems then arise: the specificity of the enzymes as to the amino acids whose linkages can be split, and the protolytic form (acid or base form) of the enzymes (and possibly also of the substrate) which can enter into the enzyme-substrate compound. The first problem will not be considered here, as gelatine was the only available substrate.

THEORETICAL AND EXPERIMENTAL

The method for finding out the mechanism by which an enzyme combines with its substrate was originally worked out for saccharase. The first interpretation of a chemical reaction with the enzyme molecule was given by Henri [4] as early as 1902: the enzyme and the substrate combine according to the law of mass action; the enzyme and an inhibitor combine also according to the law of mass action and the enzyme-substrate compound is the instable compound which is split *. Hudson [5], and Michaelis and Davidsohn [6] introduced the theory that the enzymes are amphoteric electrolytes, and that the branches of the activity-pH-curve correspond to two dissociation curves for the enzyme; shortly thereafter Michaelis and Menten [7] took up ** Henri's theory about the enzyme-substrate compound and its dissociation constant. This constant is nowadays understood as a combination of rate constants [8]. The two theories, one about the influence of pH and the other about the influence of the substrate concentration, were combined by Myrbäck [9,15], who gave the following equation in 1926

$$\frac{v_{max}}{v} = \frac{1}{(1 + K_a/[H^+] \cdot [1 + K_m/[S] \cdot (1 + K_b/[OH^-])]} \qquad (1)$$

* Henri [4]: "Je suppose qu'une partie z de ce ferment se combine avec une partie du corps à dédoubler; qu'une autre partie y du ferment se combine avec une partie des produits de l'hydrolyse; et enfin qu'il reste une portion X du ferment qui reste libre. Je suppose, en plus, que ces combinations se produisent suivant la loi de l'action des masses. On obtient ainsi les trois équations suivantes:

$$(a-x)X = \frac{1}{m} z \qquad Xx = \frac{1}{n} y \qquad \Phi = X + y + z$$

On peut supposer .... que la combinaison z entre les corps à dédoubler et le ferment est une combinaison intermediaire instable, qui se décompose en régénérant une partie du ferment. Dans ce cas la vitesse de la reaction sera proportionelle à la quantité de cette combinaison z; donc on aura

$$\frac{dx}{dt} = \frac{K\Phi\ (a-x)}{1 + m\ (a-x) + nx}."$$

This is the correct equation for the competitive inhibition. If the denotations now in use are substituted for Henri's notations: $a-x = [S]$, $x = [I]$, $\Phi = [E_{total}]$, $X = [E]$, $y = [EI]$, $z = [ES]$, $1/m = K_m$, $1/n = K_I$ and $K\Phi = V/K_m$, his equations are written

$$[S] \cdot [E] = K_m\ [ES], \quad [I] \cdot [E] = K_I\ [EI]', \quad [E_{total}] = [E] + [EI] + [ES]$$

$$v_i = \frac{V\ [S]/K_m}{1 + [S]/K_m + [I]/K_I}$$

For graphical computations the last equation is nowadays preferably written in the following form [7]

$$\frac{1}{v_i} = \frac{1}{V} + \frac{K_m}{V} \cdot \left((1 + \frac{[I]}{K_I}\right) \cdot \frac{1}{[S]}$$

** Michaelis and Menten [7]: "Die untersuchungen von Henri sind deshalb bedeutungsvoll, weil es ihm gelang, von rationellen Vorstellungen über das Wesen der Fermentwirkung ausgehend zu einer mathematischen Formulierung des Ganges der Fermentwirkung zu gelangen, die sich den Tatsachen in vielen Punkten ganz gut anschloss. Von diesen Vorstellungen Henris werden wir auch in dieser Arbeit ausgehen."

Myrbäck also drew attention to the following facts. The enzymes are ampholytes; they can take up and give off hydrogen ions, and this exchange of protons (protolysis) can — in the case of saccharase — be written in the following way [13] (N stands for a basic group containing nitrogen).

$$\left.\begin{matrix} \text{⁻EN:} \\ \text{HEN:} \end{matrix}\right\} + 2\text{H}^+ \rightleftharpoons \left.\begin{matrix} \text{⁻ENH}^+ \\ \text{HEN:} \end{matrix}\right\} + \text{H}^+ \rightleftharpoons \text{HENH}^+ \qquad (2)$$

Only those protolytic forms of this enzyme which have a lone electron pair at the nitrogen can combine with the substrate: hydrogen ions will thus cause a competitive inhibition of the enzyme in sufficiently acid solutions. One consequence of this competitive inhibition in the case of saccharase is that the acid branch of the pH curve depends on the substrate concentration whereas the apparent pH value for optimum activity also depends on the substrate concentration.

In some enzyme systems, the substrate may be an acid or a base or even an ampholyte. It is not unusual then that only one of the protolytic forms of the substrate can be acted upon by the enzyme.

It is evident that the "Michaelis constant", *i. e.*, the substrate concentration at which half of the enzyme is bound to the substrate, is influenced by pH, if only a restricted number of protolytic forms of an enzyme and its substrate can combine. It is therefore possible to find out the mechanism by which the enzyme combines with the substrate, *i. e.*, the protolytic (acid or base) forms which can combine, by measuring the enzymatic activity at various pH values and various substrate concentrations [9–16]. In a few cases the enzyme combines not with one but with, *e. g.*, two molecules of the substrate, and this reaction mechanism can also be found out by such measurements [17].

It has been emphasized by Myrbäck that it is very important to distinguish between the apparent and the true "Michaelis constant". The apparent value of "Michaelis constant" is the value obtained in the usual way without consideration of the protolytic (acid or base) form of the enzyme and substrate; it is often valid only for the particular pH at which it was measured; the true value of the "Michaelis constant" is calculated with regard to the protolytic forms which can actually combine and is hence independent of pH. It can be defined [14] by *e. g.*, the following equation:

$$K_m = \frac{[\text{S}]\,[:\text{NEH}]}{[\text{S:NEH}]} \qquad (3)$$

It is evident that the rather common habit of determining the "Michaelis constant" at just the pH optimum for activity is of hardly any interest other than that it gives us some information about how high the substrate concentration shall be in an ordinary determination of enzymic activity if almost the whole quantity of enzyme shall be utilized and if small variations in the substrate concentrations shall be insignificant.

The viscosimetric method seems not to have been used previously for the determination of the "Michaelis constant" for any enzyme and this investiga-

tion was made in order to find out the conditions for determining the apparent value of "Michaelis constant" for proteolytic enzymes, using a viscosimetric method. If such a method can be applied, it will be possible to investigate the mechanism for proteolytic enzymes which are too weak to be assayed by other methods.

A prerequisite for the application of a viscosimetric method is that the influence of the substrate concentration on the result of the viscosimetrical measurements can be eliminated. This is possible according to the theory for the viscosimetrical determination of enzymic activity, published by one of us [18].

One general difficulty in the viscosimetrical determination of a "Michaelis constant" is that it is necessary to get a substrate with a high intrinsic viscosity [19-21] which implies a high degree of polymerization, as the solutions actually used must have a sufficiently high viscosity to give results of acceptable accuracy, even if the concentration of the substrate is lowered so much that it approaches the value of the "Michaelis constant". This difficulty is noticeable in the case of proteolytic enzymes.

Enzyme

It would have been tempting to start immediately with, *e. g.*, one of the proteolytic enzymes in newly fertilized eggs, which have been detected by one of us [2,3]. However, the way in which these enzymes appear and disappear makes it plausible to assume the presence of some inhibitor, and it is evident that the crude preparations previously used must be purified before the properties of these enzymes are studied further. As already pointed out, the aim of this investigation is more general: to find the conditions for determining the "Michaelis constant" for proteolytic enzymes viscosimetrically. We therefore considered it appropriate to use a pure, crystalline enzyme, trypsin, for this first investigation. As substrate we used a high polymer gelatine preparation.

High polymer gelatine

It has already been mentioned that it is important to get a gelatine with a high intrinsic viscosity, and there are two reasons for this. The first is the following. If two gelatine preparations of different intrinsic viscosity are used for making up solutions of the same viscosity, the solution containing the gelatine with the higher intrinsic viscosity is more dilute than the other solution. If, in determining the proteolytic activity of enzyme solutions, we shall have the same drop in viscosity in unit time with both solutions, we must have a more dilute enzyme solution when we use the substrate solution with the more high molecular gelatine and the lower concentration. This can be concluded from the formula derived by one of us [18]

$$A = c_s^2 \frac{\mathrm{d}\ 1/\eta_{sp}}{\mathrm{d}t} \tag{4}$$

where A is the enzyme activity, c_s is the substrate concentration in the reaction mixture, and η_{sp} is the specific viscosity of the reaction mixture at the time t.

Hence, by choosing a gelatine preparation with a higher intrinsic viscosity than usual we can work with more dilute substrate solutions and thereby increase the sensitivity of the method so that we can either measure the activity of enzyme solutions which otherwise are too weak to be assayed or we can carry out the measurement in much shorter time than otherwise or we can get a higher accuracy by having a greater drop in viscosity during the measurements.

The other reason for procuring a gelatine preparation with a high intrinsic viscosity is the following. As already mentioned, it is possible to use a more dilute substrate solution if the preparation has a high intrinsic viscosity than otherwise, and in determining the "Michaelis constant" we should also be able to use substrate solutions with low concentrations, at least concentrations which approach the apparent value of the "Michaelis constant".

As a result of these considerations, we decided as a first goal to find a gelatine with so much higher intrinsic viscosity than the preparations previously used extensively by one of us, that the sensitivity of the viscosimetric method for the determination of proteolytic activity thereby would be expected to increase ten times.

The intrinsic viscosity of a solution of gelatine changes very little with alterations in pH and alterations in salt concentration if pH is 4.7 (the isoelectric point) and if the sodium chloride concentration of the solution is 4.7 % (0.8 M); at this salt concentration there is a viscosity maximum [19]. Viscosimetrical determinations with gelatine are usually carried out at 35.5 °C[20]. We considered it appropriate to carry out the experiments for the assay of the intrinsic viscosities of our gelatine preparations under these conditions. It may be mentioned that other conditions have also been used, *e. g.*, by Pouradier and Venet [19]. The determinations were made as follows. Three solutions with different concentrations (for commercial preparations 4, 3, 2, and 1 %, for preparations with higher intrinsic viscosity 2.0, 1.5, 1.0, and 0.5 %) were made up, and the viscosity was measured. The calculations were carried out according to Mead and Fuoss' formula [21]

$$\frac{\ln \eta_r}{c} = [\eta] - \beta[\eta]^2 c \qquad (5)$$

where η_r is the relative viscosity, $[\eta]$ the intrinsic viscosity, c the concentration of the high polymer substance in grams per 100 ml, and β a constant which is characteristic for the high polymer substance.

Thus the natural logarithm of the relative viscosity was divided by the concentration in per cent, and the quotient was plotted in a diagram *versus* the concentration. A straight line was fitted to the points. Its intersection with the axis of ordinates gave the intrinsic viscosity.

Previously, in the viscosimetrical determination of proteolytic activity, one of us [2,3] has used a commercial preparation of granular gelatine (Fisher Sc.). This gelatine has an intrinsic viscosity of 0.21. In order to get a sample with higher intrinsic viscosity, we procured eight other commercial gelatines. The following values were obtained for their intrinsic viscosities: 0.43, 0.38, 0.38, 0.37, 0.38, 0.38, 0.36, and 0.38. We also considered these preparations to have too low intrinsic viscosity. Hence, we decided upon preparing a gela-

tine with a higher degree of polymerization by fractionated precipitation of a solution of one of the commercial gelatine preparations by ethanol according to Pouradier and Venet [19,22].

The high polymer gelatine, used in the following experiments, was prepared in the following way. 200 g of a commercial gelatine preparation with the intrinsic viscosity 0.38 (measured at 35.5 °C and pH 4.7 in a 0.8 M sodium chloride solution) were dissolved in 4 500 ml of water. At a temperature of 42 °C 5 200 ml of abs. alcohol were added, whereby a strong precipitate was formed. The mixture was heated so that the precipitate dissolved, whereupon it was left in a thermostat room at 42 °C till the following morning. Then a layer containing 51 % of the gelatine had separated out on the bottom of the container. The layer was taken out and dissolved in 1 650 ml of water. Part of this solution was precipitated at −5 °C with acetone, and the precipitate was dried *in vacuo*. The intrinsic viscosity of the preparation was 0.71. The preparations we obtained in other fractionation experiments usually showed no significant difference from this preparation. However, as a result of some successive fractionation of 20 g of gelatine we obtained 0.78 g of a preparation with the intrinsic viscosity 1.0.

Those properties of the various gelatine preparations which are of special interest in this investigation are listed in Table 1.

Table 1. The properties of some gelatine preparations.

$[\eta]$	Molecular weight	Concentration in per cent		Relative enzyme concentration for the same decline in viscosity
		$\eta_r = 2$	$\eta_r = 3$	
0.21	40 000	3.4	5.6	1
0.38	100 000	1.9	3.2	0.3
0.71	300 000	1.0	1.7	0.09
1.0	500 000	0.7	1.2	0.05

The relative molecular weights of the preparations (relative to the Fisher gelatine previously used by one of us) was calculated according to the formula of Mark [23]

$$[\eta] = K \cdot M^a \tag{6}$$

where M is the molecular weight, and K and a are constants. For gelatine, a has the value 0.885 according to Pouradier and Venet [22]. Another figure is given by Williams, Saunders, and Cicirelli [34], who — from measurements carried out under iso-electric conditions and in the presence of salt, using preparations with intrinsic viscosities between 0.137 and 0.35 — found the values $K = 2.9 \cdot 10^{-4}$ and $a = 0.62$. Using their values, we have calculated the weight average molecular weights of our preparations as given in Table 1. As it is not quite certain that their viscosity determinations were done under the same conditions as ours, and as two of our preparations have much higher intrinsic viscosities than their highest sample, the molecular weights computed for our preparations are not very accurate but give at least the right order of magnitude.

For viscosimetric determinations of enzymic activity solutions with the relative viscosity $\eta_r = 3$ are suitable. The concentration of solutions with this viscosity were read from the graphs for the calculation of the intrinsic viscosity for the various preparations. The relative enzyme concentrations, which give the same decline in viscosity for equally viscous ($\eta_r = 3$) solutions of the various gelatine preparations was computed according to equations (4) and (6); the squares of the concentrations at which solutions of the various preparations have the relative viscosity 3 were divided by the square of the concentration, at which a solution of the previously used gelatine with the intrinsic viscosity 0.21 has the relative viscosity 3.

It is evident from Table 1 that the sensitivity of the viscosimetrical method for the determination of enzymic activity is increased about ten times, if the fractionated gelatine with the intrinsic viscosity 0.71, which could easily be prepared, is substituted for the Fisher gelatine previously used. This improvement was, however, somewhat counteracted by the fact that the slow decrease in viscosity in the absence of enzyme was greater in the case of the more high molecular gelatine.

Activity determinations

The enzymic activity at various substrate concentrations was determined as follows [22-26]. Using a fractionated gelatine with the intrinsic viscosity 0.71, we made up six solutions of the concentrations 2.00, 1.75, 1.50, 1.25, 1.00, and 0.75 %, respectively, in phosphate buffer of pH 6.50 and the ionic strength 4/30 (in order to get the ionic strength 0.1 in the final reaction mixture). Merthiolate (0.01 %) was added as a preservative. The solutions were kept in a thermostat bath at 35.5 °C.

Two experimental series were carried out, using solutions of crystalline trypsin. The reaction mixtures were made up of 1 ml of enzyme solution and 3 ml of substrate solution. They were transferred to an Oswald viscosimeter which had been calibrated carefully with sugar solutions [27], and the viscosity measured at various times as usually. In calculating the specific viscosities, due allowance was made for the viscosity and density of a corresponding water solution of the buffer salts. The measurements at different substrate concentrations were made in a randomized order.

CALCULATION OF THE RESULTS

The computation of the activity from viscosimetrical measurements is usually done graphically, whereby the inverted values of the relative viscosities $1/\eta_{sp}$ are plotted *versus* time, whereupon a straight line is fitted by the eye to the points and the inclination read [18,24,27]. However, in this case we preferred to calculate the inclinations of the lines by the method of least squares. The inclinations were multiplied by the squares of the substrate concentrations in the reaction mixtures according to eqn. (4) [18].

The activity v was expressed [18,28] in $m\mu A$ ($A \cdot 10^{-9}$). We also calculated $[S]/v$, where [S] is the substrate concentration in the reaction mixture, expressed in per cent. In a diagram, $[S]/v$ was plotted *versus* [S] [17] and a straight line was fitted to the points (Figs. 1—2). The "Michaelis constant" can — analogously to a similar suggestion by Dixon [29] — be read as the point of

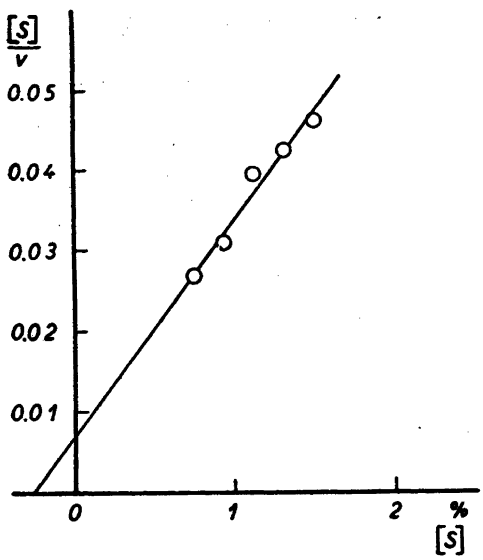

Fig. 1.

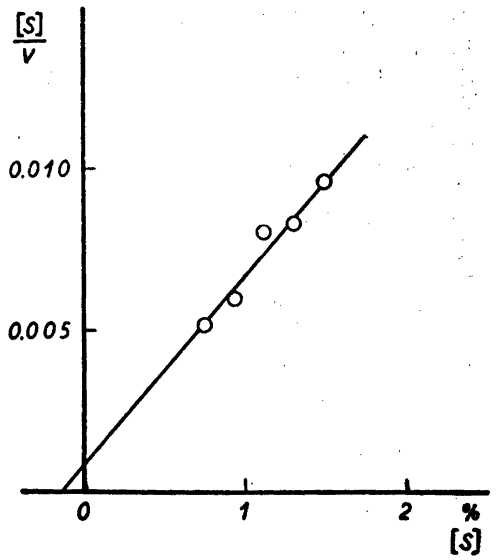

Fig. 2.

Graphs for the calculation of the "Michaelis constant" for trypsin. The value of the constant — however, with negative sign — is read at the intersection between the abscissa and the fitted line.

intersection — however, with positive sign — between the fitted line and the abscissa *.

DISCUSSION

We can see from Figs. 1—2 that all reaction mixtures used for the activity determinations, had a higher substrate concentration than corresponds to the "Michaelis constant". It turned out that the accuracy of the activity determinations at the actual substrate concentration 0.56 % (using the 0.75 % stock solution) was so low that the determinations could not be taken into consideration. The relative viscosity of the actual reaction mixtures was only 1.4, and even an error of only 0.2 seconds in the reading of a flow time had a marked influence on the result. Hence, the measurements with the 0.75 % stock solution of gelatine were not considered in the calculations. The values of the Michaelis constant as read from Figs. 1 and 2 are 0.03 and 0.01 % respectively.

* A linear relationship for the calculation of "Michaelis constant" can also be obtained in two other ways, but this way not only makes it possible to read the value of the constant directly in a graph but also has the advantage of being more suitable than the other for numerical calculation of Michaelis constant by the method of least squares. This depends on the fact that for such a calculation one variable should be actually independent, in this case [S], and that the accuracy of the other variable, in this case [S]/v, should be independent of the value of the first variable. The first condition is met in this case, and also if we plot $1/v$ *versus* 1/[S], but not if we plot v *versus* v/[S]; however, the second condition is not met with if we plot $1/v$ *versus* 1/[S] but is approximately met with if we plot [S]/v *versus* [S].

It is evident that the accuracy in the determination of the constant is rather low as a consequence of the considerable extrapolation. It would have been desirable to have measurements at lower substrate concentrations; however, for such measurements it would have been necessary to use a gelatine with higher intrinsic viscosity than 0.7.

It is of interest to compare the value of "Michaelis constant" obtained by this viscosimetrical method to values obtained by other methods. Northrop [30] measured the activity of trypsin on gelatine solutions at pH 6.0 using a conductometric method. For one experimental series (*loc. cit.*[30] Table V) he gives the value 0.5 % for the "Michaelis constant". From another experimental series (*loc. cit.*[30] Table VI) we have calculated the value 0.3 %.

Northrop [31] also investigated the influence of trypsin on gelatine solutions of different concentrations by measuring the viscosity at various times. As the change of viscosity at the breakdown of gelatine proceeds differently at the beginning than later on — probably [24] as a consequence of the various types of amino acid linkages which are not split with equal ease — and as Northrop made only a few measurements, however, over a wide interval, it is not possible to calculate the "Michaelis constant" from his experiments.

We can conclude from the experiments that it is possible to determine the "Michaelis constant" of proteolytic enzymes viscosimetrically using gelatine as substrate. However, with a gelatine with the intrinsic viscosity 0.7 (as measured at 35.5 °C and pH 4.7 in 0.8 M sodium chloride solution) the sensitivity of the method permits only measuring of constants with higher value than about 0.1 %. The sensitivity can, however, be increased somewhat by using a gelatine with higher intrinsic viscosity than 0.7. As already mentioned in this paper a gelatine with the intrinsic viscosity 1.0 can be prepared from commercial gelatine by a few fractionated precipitations with ethyl alcohol at 42 °C according to Pouradier [19]. It is evident that it would be highly desirable to use such a gelatine preparation for the reaction mixtures with the lowest substrate concentrations, *e. g.*, about 0.5 %, in order to increase the accuracy The accuracy of the determinations can, of course, be increased also by increasing the number of experiments in each series.

It should finally be mentioned that in reaction mechanism determinations when viscosimetric activity measurements are carried out at different pH values, it is necessary to correct the values for the influence of pH on the viscosity of the substrate [32], and that it may be desirable in calculating the activities to use the more accurate formula [32,33] $A = J\, c_s^2\, (\mathrm{d}\ 1/\ln\ \eta_r)/\mathrm{d}t$ instead of the formula given here as eqn. (4). However, the accuracy of the determination of the "Michaelis constant" must not necessarily be very high, as the value is usually independent of the pH in some region (the true value of the constant) and changes very much with the pH in some other region (apparent values of the "Michaelis constant"); the logarithm of the factor by which the constant changes if pH is changed by one unit gives the number of hydrogen ions which take part in the proton exchange necessary for the combination of the enzyme and substrate.

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