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Kinetics of the Hydrolysis of Methyl-valerate Catalyzed by Horse Liver ali-Esterase

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The hydrolysis of methyl-valerate catalyzed by horse liver ali-esterase was investigated by continuous automatic titration of the liberated acid. The reaction was studied with different initial concentrations of enzyme and substrate and followed to a degree of reaction of 99 %. The following chronometric integral can describe the reaction

$$t = A \cdot \frac{a}{E} \cdot a + B \cdot \sqrt{\frac{a}{E}} \left[\ln \frac{1 + \sqrt{a}}{1 - \sqrt{a}} - 2\sqrt{a} \right]$$

where t is time, a initial substrate concentration, E enzyme concentration, a degree of reaction and A and B are constants. It is pointed out that the appearance of square roots in the chronometric integral indicates a reaction between two enzyme-containing molecules. A mechanism of this type is suggested.

Several attempts have been made to elucidate the kinetics of the ali-esterases. It is very often described as a zero order reaction [1–3]. Schønheyder [6] has described it in a particular case as a combination of a zero and a first order reaction, while others [4–5] have described similar cases by a Michaelis-expression, which actually amounts to the same. Many of the investigators have only studied the beginning of the reaction (*i. e.* less than 50 % degree of reaction) and it should be well known that this often leads to misinterpretations of the experimental data. It is the purpose of this investigation to study the kinetics of the enzymic ester hydrolysis including also high degrees of reaction. The substrate was methyl-valerate. This ester was chosen because of its rapid reaction in the presence of esterases [7–10].

The enzyme was obtained from horse liver which has a high content of esterase [11] and is easy to obtain in large quantities.

EXPERIMENTAL

Enzyme. Fresh horse liver was obtained directly from the slaughter house. It was disintegrated in a Waring blendor and washed four times with acetone in order to remove water and fat. After each washing the powder was drained on a Büchner funnel with moderate suction and after the last one it was dried overnight at room temperature. The dry powder was stored in the refrigerator until use. Solutions for the experiments were made by stirring the powder with ten times its weight distilled water for a few hours at room temperature. After centrifugation the clear dark red-brown supernatant was used for the experiments.

Substrate. Methyl-valerate was prepared from butanol (1) which was converted into valeronitril as described by Adams and Marvel [12]. From this nitrile the ester was prepared by interaction with methanol and sulfuric acid. The product was distilled in a Stedman column [13,14], b. p. 127.8/128.3 (uncorr.) at 767.4/768.6 mm Hg.

Apparatus. The hydrolysis of the ester was followed by continuous automatic titration in a pH-stat [14a]. All experiments were made at pH = 8.0, which is the optimum pH [2] and 20.0° C. The reaction chamber was a 100 ml beaker. The experiments were performed in 50 ml 0.1 M KCl, carefully adjusted to pH = 8 with KOH. A suitable amount of ester was added and the experiment was started by adding the desired amount of enzyme solution. Corresponding values of time and amount of KOH added from a syringe necessary to keep pH constant were read. The concentration of ester was found as the titration value when the reaction had finished.

The stability of the enzyme was checked in the following way: An enzyme solution was kept in a thermostat at 20° C. A sample of 3 ml was removed and an experiment performed in the manner described above and continued for about 10 min. This was repeated at suitable intervals for the next 20 hours. As will be seen from Fig. 1 there was no measurable alteration of enzyme activity during this time. The last enzyme sample was heated to about 95° C for 30 min. As also shown by Fig. 1 there was no measurable activity left after this treatment.

The positive interception on the ordinate axis is due to the fact that the enzyme solution has a pH lower than the experimental pH and the final adjustment of this pH is made by the pH-stat. It is seen from the figure that the amount of KOH necessary to make this adjustment is well-defined and it is of course subtracted from all measurements used for the kinetical determinations.

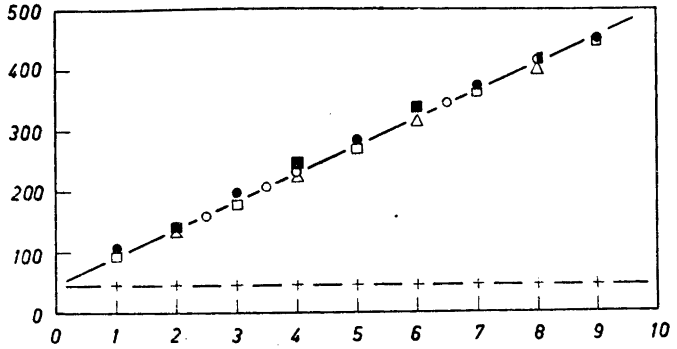

Fig. 1. Experiment with enzyme at different ages. Abscissa: Time in minutes; ordinate: Scale readings on the syringe. One scale reading equals 4.98 10^{-7} eqv. Age of enzyme: O — O — O*: 0 min.* □ — □ — □*: 70 min.* △ — △ — △ *158 min.* ● — ● — ●*: 1 210 min.* ■ — ■ — ■*: 1 283 min.* + — + — +*: Boiled enzyme.*

Tables 1—4. The tables indicate the measured values of time (t_{exp}) and degree of reaction (a), together with times calculated (t_{calc}) from the suggested chronometric integral. If the enzyme concentration used in these experiments equals 1 M the constants A and B are 3 310 and 17.42 minutes, respectively.

Table 1. $a = 13.15 \times 10^{-3}$ M.

t_{exp} min	a	t_{calc} min
0	0	0
3	0.0707	3.10
6	0.1400	6.17
9	0.2028	8.96
12	0.2764	12.26
16.5	0.3742	16.68
20.5	0.4578	20.50
29	0.6357	28.83
33	0.7170	32.78
37	0.7920	36.58
41	0.8710	40.89
46	0.9550	46.56
48	0.9700	48.02
50	0.9870	50.42

Table 2. $a = 6.70 \times 10^{-3}$ M.

t_{exp} min	a	t_{calc} min
0	0	0
2	0.0983	2.21
4	0.1840	4.17
6	0.2612	5.95
8	0.3511	8.05
10	0.4410	10.18
12	0.5183	12.04
14	0.5965	13.97
16	0.6713	15.86
18	0.7584	18.17
20	0.8272	20.12
22	0.8919	22.18
24	0.9480	24.44
28	0.9902	27.73

Table 3. $a = 3.04 \times 10^{-3}$ M.

t_{exp} min	a	t_{calc} min
0	0	0
2	0.2167	2.26
4	0.4056	4.30
6	0.5387	5.81
8	0.7090	7.87
10	0.8483	9.83
12	0.9412	11.63
14	0.9845	13.32

Table 4. $a = 1.08 \times 10^{-3}$ M.

t_{exp} min	a	t_{calc} min
0	0	0
1	0.2435	0.93
2	0.4435	1.75
3	0.6609	2.75
4	0.8000	3.50
5	0.9565	4.89

RESULTS

Experiments were performed at four different substrate concentrations, with the same enzyme concentration. It was found that under these circumstances the reaction could be described by the expression:

$$t = A \cdot a\alpha + B \cdot \sqrt{a}\left[\ln \frac{1+\sqrt{\alpha}}{1-\sqrt{\alpha}} - 2\sqrt{\alpha}\right]$$

where t is time, α degree of reaction, a initial substrate concentration and A and B are constants. The results of these experiments are given in Tables 1—4.

Tables 5—8. The tables indicate the measured values of time (t_{exp}) and degree of reaction (α), together with time calculated (t_{calc}) from the suggested chronometric integral. With the arbitrary values for enzyme concentration used here the constants A and B are 5 699 and 17.39 minutes respectively.

Table 5. $a = 1.314 \times 10^{-2}$ M. $E = 2.005$.

t_{exp} min	α	t_{calc} min
0	0	0
3	0.0865	3.26
6	0.1673	6.32
9	0.2489	9.43
11	0.3018	11.46
15	0.4105	15.67
18	0.4856	18.60
21	0.5608	21.57
24	0.6351	24.55
27	0.7131	27.74
30	0.7818	30.64
33	0.8519	33.75
36	0.9142	36.80
39	0.9614	39.66
42	0.9871	42.14

Table 6. $a = 1.259 \times 10^{-2}$ M. $E = 2.978$.

t_{exp} min	α	t_{calc} min
0	0	0
2	0.0776	1.89
4	0.1626	3.97
6	0.2425	5.95
8	0.3246	8.00
10	0.4022	9.95
12	0.4791	11.90
14	0.5589	13.96
16	0.6305	15.84
18	0.7067	17.89
20	0.7791	19.91
22	0.8455	21.88
24	0.9067	23.88
26	0.9552	25.88
28	0.9873	28.03
30	0.9963	29.61

Table 7. $a = 1.274 \times 10^{-2}$ M. $E = 3.990$.

t_{exp} min	a	t_{calc} min
0	0	0
2	0.1128	2.08
4	0.2197	4.08
6	0.3230	6.03
8	0.4262	8.01
10	0.5265	9.96
12	0.6209	11.85
14	0.7168	13.83
16	0.8097	15.86
18	0.8894	17.80
20	0.9521	19.73
22	0.9830	21.30
24	0.9919	22.17

Table 8. $a = 1.330 \times 10^{-2}$ M. $E = 4.970$.

t_{exp} min	a	t_{calc} min
0	0	0
2	0.1392	2.15
3	0.2042	3.18
4	0.2699	4.22
5	0.3300	5.18
6	0.3943	6.21
7	0.4537	7.18
9	0.5717	9.13
10	0.6339	10.19
12	0.7491	12.23
14	0.8516	14.21
16	0.9420	16.40
18	0.9880	18.50

Four experiments with different enzyme concentrations, but with almost the same substrate concentration were performed. Tables 5—8 show the measured and the calculated times. The following expression was used for the calculations:

$$t = \mathrm{A} \cdot \frac{a}{E} a + \mathrm{B} \cdot \sqrt{\frac{a}{E}} \left[\ln \frac{1 + \sqrt{a}}{1 - \sqrt{a}} - 2 \sqrt{a} \right]$$

The constants A and B used for the calculations were found in the following manner: The chronometric integral was written as:

$$t = \mathrm{A}' \cdot a + \mathrm{B}' \cdot \left[\ln \frac{1 + \sqrt{a}}{1 - \sqrt{a}} - 2\sqrt{a} \right]$$

and the constants A′ and B′ were determined for every experiment. It turned out that empirically A′ could be represented by Aa/E and B′ by $B\sqrt{a/E}$ where A and B are common to all experiments in which the relative values of E are known. The deviation between measured and calculated times seen in some of the experiments are supposed to be due to difficulties in obtaining exactly the

desired enzyme concentration. It may be, however, that the deviations are due to the approximation introduced between formula (12) and (12 a) in the following discussion of the reaction mechanism.

REACTION MECHANISM

To explain the occurrence of the square root of the initial substrate concentration we have assumed that two molecules of enzyme in the same or in different forms combine with one molecule of one of the reactants. As the situation is similar to the state of affairs at certain photochemical reactions, *cf. e. g.* the classical work of Bodenstein *et al.*[15] and Jost [16], some such assumption seems very natural or even necessary.

As it is known that enzymic ester hydrolyses are often inhibited by the acid and not by the alcohol [6] we assume that in our case it is the valerate ion which combines with the enzyme, and suggest the following reaction scheme:

$$X_1 \rightleftharpoons X_2 \qquad (\pm 1)$$
$$X_2 + OH^- \rightleftharpoons X_3 \qquad (\pm 2)$$
$$X_3 + MV \rightarrow X_1 + MOH + V^- \qquad (+3)$$

with the additional equilibrium:

$$2X_3 + V^- \rightleftharpoons X_4 \qquad (\pm 3a)$$

where X_{1-4} are different enzyme forms, MV methyl-valerate, MOH methyl alcohol and V^- valerate ion. From this scheme we can theoretically deduce a chronometric integral identical with the empirical one: Using the steady state method as described by Christiansen [17,18] and with the symbols used by Christiansen we have:

$$s = x_1 w_1 - x_2 w_{-1} \qquad (4)$$
$$s = x_2 w_2 - x_3 w_{-2} \qquad (5)$$
$$s = x_3 w_3 \qquad (6)$$

where s is the steady state rate and the w_i's are reaction probabilities for the steps indicated. The other small letters indicate the concentration of the substance symbolized by the same capital letter.

Solutions of the equations yield:

$$x_1 = s\ (1 + w_{-1}/w_2 + w_{-1}w_{-2}/w_2 w_3)/w_1 \qquad (7)$$
$$x_2 = s\ (1 + w_{-2}/w_3)/w_2 \qquad (8)$$
$$x_3 = s/w_3 \qquad (9)$$

As it is known that the reaction has an energy of activation [19] we assume that X_2 must be on a higher energy level than X_1.

Fig. 2 will account for the energy levels of the enzyme during the reaction. From this it is seen that $w_{-2}/w_3 \ll 1$ and eqns. 7, 8 and 9 can be rewritten as follows:

$$x_1 = s\ (1 + w_{-1}/w_2)/w_1 = q_1 s \qquad (7a)$$
$$x_2 = s/w_2 = q_2 s \qquad (8a)$$
$$x_3 = s/w_3 = q_3 s \qquad (9a)$$

From (3a) we get

$$x_4/x_3^2 v = K \qquad (10)$$

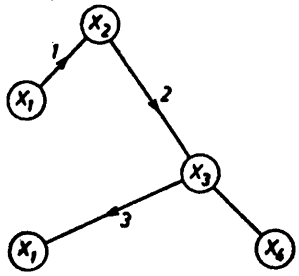

Fig. 2.

where K is an equilibrium constant and v means the concentration of valerate ions. For the total enzyme concentration we have

$$E = x_1 + x_2 + x_3 + x_4$$

Regarding the energy levels it is obvious that x_2 can be neglected and we then have

$$x_4 = E - (x_1 + x_3) \tag{11}$$

Introducing (7a), (9a) and (11) in (10) and solving the equation we find

$$\frac{2}{s} = \frac{q_1 + q_3}{E} + \sqrt{\left(\frac{q_1 + q_3}{E}\right)^2 + \frac{4K \cdot v}{E} \cdot q_3^2} \tag{12}$$

If we require this expression to conform with that derived from the chronometric integral by differentiation we must assume that:

$$\left(\frac{q_1 + q_3}{E}\right)^2 \ll \frac{4K \cdot v}{E} \cdot q_3^2$$

Regarding the energy levels we get:

$$q_3 \ll q_1$$

and eqn. 12 is then reduced to

$$2/s = q_1/E + 2q_3\sqrt{K \cdot v/E} \tag{12a}$$

From the reaction scheme it is seen that

$$\begin{aligned} w_1 &= k_1 \\ w_{-1} &= k_{-1} \\ w_2 &= k_2' \cdot c_{OH^-} \quad \text{or, at constant pH,} \\ w_2 &= k_2 \\ w_3 &= k_3\ (a - v) \end{aligned}$$

where the k's are constants and a is the initial substrate concentration. Introducing this in (12a) we find

$$2/s = 2\mathrm{d}t/\mathrm{d}v = 1/E\ (1/k_1 + k_{-1}/k_1k_2) + 2\sqrt{1/E} \cdot \sqrt{K} \cdot \sqrt{v}/k_3\ (a - v)$$

or

$$\mathrm{d}t/\mathrm{d}v = \mathrm{A}/E + \mathrm{B} \cdot \sqrt{1/E} \cdot \sqrt{v}\,/(a - v) \tag{12b}$$

where A and B are new constants. Integrating this equation and introducing the degree of reaction $\alpha = v/a$ we get

$$t = \mathrm{A} \cdot \frac{a}{E} \cdot \alpha + \mathrm{B} \cdot \sqrt{\frac{a}{E}} \cdot \left[\ln \frac{1+\sqrt{\alpha}}{1-\sqrt{\alpha}} - 2\sqrt{\alpha} \right]$$

which is identical with the empirically found chronometric integral.

It should be noted that (12b) only differs from an ordinary combination of a zero and a first order expression by the factor $\sqrt{E \cdot v}$ in the last term.

DISCUSSION

The reaction scheme suggested here is different from any one earlier found in the literature. It should be noticed that we have followed the reaction nearly to completion, while many of the earlier investigators only studied the first 50 % or less of the range. An exception is Schønheyder [6] who has published an experiment in which he follows the hydrolysis of 1-caprylyl-glycerol by rabbit liver esterase until 98.5 % degree of reaction. However, even his experiments are often followed only to 45—90 % degree of reaction.

It should be mentioned that investigations carried out in order to study the ali-esterase have been performed with enzymes isolated from many different sources and with the most different substrates. We have no reason to be sure that different enzymes will act on different substrates in the same way.

It should be mentioned too, that the chronometric integral found empirically by us could possibly be explained by other reaction schemes. The scheme mentioned must be considered as a suggestion which explains our experimental results, but we cannot postulate that it is the only possible explanation, observe, however, the remark p. 1263 and in the synopsis.

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