

Kinetics of the Enzymatic Splitting of Hyaluronic Acid

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The enzymatic splitting of hyaluronic acid is studied by means of viscosimetry. The reaction is followed to more than 90 % reduction in specific viscosity. It is shown that the experiments conform in that interval with either of the following two expressions:

$$E t = A (1/y - 1) + B (1/y^2 - 1) \quad (1)$$

$$E t = C (e^{Dy} - e^D) \quad (2)$$

where y is a measure of the remaining viscosity and E is the total enzyme concentration. A possible reaction mechanism corresponding to the first of these expressions is discussed and some other possible mechanisms are mentioned.

Since 1934, when hyaluronic acid was first isolated by K. Meyer¹, many investigations have provided evidence of the extensive occurrence of this substance, and essential contributions have been made towards elucidation of its constitution². Hyaluronidase, too, has received the attention of a great many investigators, but apart from clinical uses, the enzyme has been employed mostly as a convenient means of examining whether or not a particular liquid of high viscosity contained hyaluronic acid³. Much fewer investigators have been engaged in studies of the kinetic problems connected with the enzymatic decomposition of hyaluronic acid, and most of these have dealt only with splitting to the extent of 20—30 %, as a rule with the purpose of finding a convenient, fairly unique, method of standardizing a particular preparation of enzyme.

Most of the available studies of the kinetics of the reaction describe the inception of the reaction as a first-order reaction⁴. In a more recent publication, Dorfman⁵ describes the reaction as a specific instance of the classical expression of Michaelis and Menten⁶.

The investigations herein recorded lead to a mathematical expression by which it is possible, at the given experimental conditions, to describe more than 90 % of the reaction. This expression, as will be seen, is different from a first-order expression.

The concentration of hyaluronic acid was determined viscometrically in a modified Ostwald viscosimeter as described by Dalgaard-Mikkelsen, Kvorning and Rasbech⁷. The capacity of the apparatus was 0.6 ml; the content between the two marks was 0.2 ml. All experiments were carried out at 20° C. The flow time for distilled water was 45.0 seconds.

The substrate employed was potassium hyaluronate, produced from umbilical cords as described by Jensen⁸. The substrate as well as the enzyme were dissolved in a McIlvaine⁹ phosphate-citrate buffer (0.09 M Na_2HPO_4 + 0.0105 M citric acid + 0.06 M NaCl; pH = 7.0.) The hyaluronidase used was a commercial preparation "Invasin-Lundbeck". The buffer was sterilized by heating to 100° C before use, and saturated with toluene. The viscosity of the substrate solution remained constant for several days.

Measurements were performed on a solution consisting of 0.80 % potassium hyaluronate solution and a solution of enzyme, in equal volumes. The two solutions were carefully mixed by stirring. Time was calculated to equal zero at the moment stirring was initiated. The cleansed viscosimeter was washed through with a small amount of the experimental solution and subsequently filled with 0.5 ml of it. At suitable intervals determination was made of the flow time of the solution. The time of measurement was taken as the time the solution began to run through plus half the time it occupied in running through. The viscosity at time zero was determined on a 0.40 % solution of substrate to which no enzyme was added.

The stability of the enzyme at the experimental temperature was determined in the following manner:

A solution of enzyme was placed in a thermostat at 20° C. A sample of 0.5 ml was removed and subsequently mixed with 0.5 ml of the substrate; the viscosity of the solution was then determined 3–4 times during the next fifteen minutes; the viscosimeter was cleansed and the experiment repeated at suitable intervals during the following 48 hours. As it will be seen from Fig. 1 there was no measurable alteration in the activity of the enzyme during the first 48 hours.

With a view to examining whether the chronometric integral of the reaction could be represented as $Et = f(y)$ (E = the concentration of enzyme, t = time and $1-y$ = the degree of reaction) four experiments were carried out in which the ratios of the concentrations of enzymes were 12/6/4/3. The experiments were followed until 91–94 % transformation. Fig. 2 shows viscosity as a function of time in all four experiments. In Table 1 the ratios of the periods of time consumed in obtaining a given degree of reaction are recorded for a number of different degrees of reaction. These ratios, it will be seen, were constant within about 1 %, although they had not quite the expected values. The variations can probably be accounted for by difficulty in

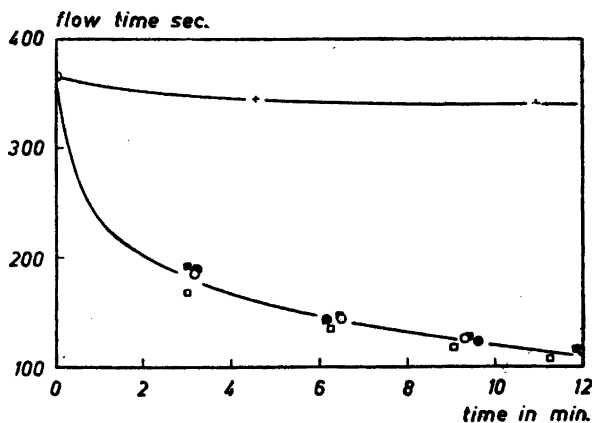


Fig. 1. Experiment with enzyme at different ages. —○—○— 6.0 min; —□—□— 83.5 min; —●—●— 483.0 and —■—■— 2872 min. —+—+— enzyme heated to abt. 100° C for 4 min.

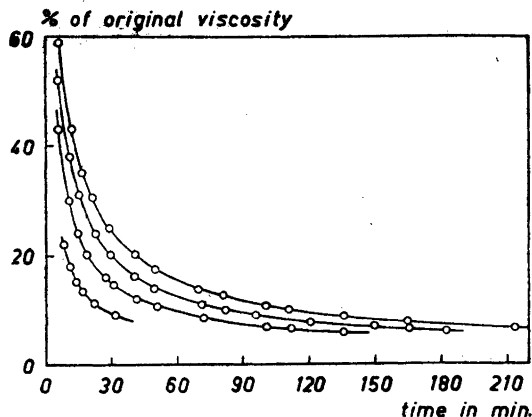


Fig. 2. Four experiments with different enzyme concentrations.

producing solutions of enzyme exactly of the desired concentration. The constancy of the values was taken as a guarantee that the chronometric integral of the reaction could be expressed in the aforementioned general manner. The results of measurements in the experiment with enzyme concentration "4" are recorded in Table 2.

On a purely empirical basis it was found that throughout the range here examined, the reaction could be described by one of the following two expressions:

$$Et = A (1/y - 1) + B (1/y^2 - 1) \quad (1)$$

$$Et = C (e^{Dy} - e^D) \quad (2)$$

If $E = 1$ the experiment recorded in Table 2 yields the following results: $A = 5.424$; $B = 0.339$; $C = 76.4$ and $D = 1/13$.

Further, in Table 2, the differences Δ_1 and Δ_2 were found between the t -values calculated on the basis of (1) and (2), respectively, and the t -values measured.

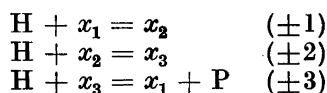
Table 1. The table indicates the ratio between the times necessary to reach a given degree of reaction at different enzyme concentrations.

Degree of reaction	Ratio between enzyme concentrations		
	12 : 3	6 : 3	4 : 3
0.48	—	—	1.38
0.70	—	2.06	1.36
0.80	4.36	2.09	1.37
0.83	4.39	2.09	1.37
0.86	4.37	2.09	1.37
0.89	4.37	2.08	1.40
0.93	—	2.03	1.34
Average	4.37	2.07	1.37

Table 2. The table indicates (some of) the measured values for the experiment with enzyme-concentration 4. y is $\eta_{\text{spc}}/\eta^{\circ}_{\text{spc}}$, where η_{spc} is the specific viscosity of time t and $\eta^{\circ}_{\text{spc}}$ is the specific viscosity at zero time, and Δ_1 and Δ_2 is the difference between calculated and measured times for the mathematical expressions 1 and 2 respectively.

t min	flow time sec	y	Δ_1	Δ_2
0	555.4	1	0	0
5.89	310.9	0.5210	+0.01	+0.15
11.10	237.4	0.3770	-0.09	+0.05
15.25	204.0	0.3115	-0.11	+0.03
18.99	184.9	0.2741	-0.46	-0.35
22.74	167.2	0.2394	+0.06	+0.10
26.20	156.4	0.2183	-0.01	-0.07
29.45	148.0	0.2018	-0.02	-0.11
34.75	137.4	0.1810	-0.21	-0.45
40.36	127.6	0.1618	+0.35	+0.06
44.98	121.3	0.1495	+0.69	+0.33
49.42	116.9	0.1408	+0.44	+0.01
55.26	111.6	0.1305	+0.43	-0.02
60.49	108.6	0.1246	-0.90	-0.59
63.01	106.2	0.1199	+0.04	-0.44
70.86	101.2	0.1101	+0.60	+0.27
82.07	96.2	0.1003	-0.06	-0.09
98.08	90.5	0.0891	-0.31	+0.56
120.46	85.3	0.0789	-3.09	-0.44
133.92	82.3	0.0731	-2.15	+2.61
149.87	80.0	0.0686	-4.62	+2.09
165.5	77.5	0.0637	-2.7	+7.5
180.5	76.2	0.0611	-6.8	+6.1

Expression (1) could possibly cover a three step reaction:



where H is a molecule of hyaluronic acid, P is the reaction products and x_1 , x_2 and x_3 are different combinations of enzyme and substrate. Treating this reaction as described by Christiansen^{10, 11}, we have, with the symbols used by Christiansen:

$$\begin{aligned} s_1 &= x_1 w_1 - x_2 w_{-1} \\ s_2 &= x_2 w_2 - x_3 w_{-2} \\ s_3 &= x_3 w_3 - x_1 w_{-3} \end{aligned}$$

and furthermore:

$$E = x_1 + x_2 + x_3$$

and in the case of stationarity:

$$s = s_1 = s_2 = s_3$$

Assuming now that w_{-3} equals zero we get by solving the equations:

$$x_1/s = 1/w_1 + w_{-1}/w_1 w_2 + w_{-1} w_{-2}/w_1 w_2 w_3 \quad (3)$$

$$x_2/s = 1/w_2 + w_{-2}/w_2 w_3 \quad (4)$$

$$x_3/s = 1/w_3 \quad (5)$$

putting: $w_1 = k_1 \cdot y \cdot a$ and $w_2 = k_2 \cdot y \cdot a$ and $w_3 = k_3 \cdot y \cdot a$ where a is the initial substrate concentration and further:

$$w_{-1} = k_{-1} \text{ and } w_{-2} = k_{-2}$$

and $1/s = dt/dy$

we get by addition of (3), (4) and (5)

$$\begin{aligned} -E \, dt/dy &= K_0 \, 1/y + K_1 \, 1/y^2 + K_2 \, 1/y^3 \\ \text{where} \quad K_0 &= 1/k_1 \, a + 1/k_2 \, a + 1/k_3 \, a \\ K_1 &= k_{-1}/k_1 k_2 \, a^2 + k_{-2}/k_2 k_3 \, a^2 \\ K_2 &= k_{-1} k_{-2}/k_1 k_2 k_3 \, a^3 \end{aligned}$$

By integration we get:

$$E \, t = K_0 \ln 1/y + K_1 (1/y - 1) + 1/2 K_2 (1/y^2 - 1)$$

Assuming that K_0 is small as compared to K_1 and K_2 we see that this expression is identical with the one found empirically when $K_1 = A$ and $K_2 = 2B$.

This result would be imaginable if k_{-1} is large in proportion to k_1 , which would mean that the first reaction is near equilibrium.

The 3-step reaction here suggested should be taken with no slight reservation, however. Notwithstanding the fact that such a reaction may offer an explanation of the expression found, it seems rather unlikely that the reaction should, in fact, require that 3 molecules of hyaluronic acid combine with 1 molecule of enzyme before the decomposition takes place.

We have made no attempt to find a mechanism of reaction to be covered by formula (2), but we wish to draw attention to the mathematical relationship between the two expressions, as a development in series of (2) would give

$$(1-1) + (1/y-1) + K' (1/y^2-1) + K'' (1/y^3-1) + \dots$$

Neither expression (1) nor expression (2) is of the first-order form and the rectilinearity obtained by Lundquist⁴ and others by plotting the logarithm of the concentration of hyaluronic acid against time cannot be claimed to support the view that we have to do with a first-order reaction. In those experiments the splitting did not exceed 30 %, and at degrees of reaction that low it is often possible to make any expression applicable to the experiment if only the right constants are chosen. In certain instances it may, of course, be convenient to use a first-order constant for the purpose of standardizing a preparation or the like, but from such experiments we cannot conclude that the reaction actually follows the pattern of a first-order reaction.

The view advanced by Dorfman⁵ appears to us to be somewhat artificial, *inter alia* because it is based upon the assumption that all enzymatic processes take place in two steps, and in two steps only, which is an unpermissible abstraction from established facts. The aforementioned state of equilibrium of the first step of the reaction may be responsible for the fact that such calculations may yield satisfactory results.

One of the crucial points of all viscometric investigations of hyaluronidase is the assumption that the viscosity may readily be used instead of the concentration in the ordinary kinetic expressions. Dorfman⁵, among others, has conducted experiments to show that the specific viscosity ($\eta_{rel} - 1$) is directly

proportional to the concentration of hyaluronic acid. It is hardly correct, however, to assume that the viscosity measured has any relation to the actual concentration of a well defined substance. It should be taken only as a unique value which can be used to describe the reaction.

If we consider Staudinger's¹² equation $\eta_{\text{spc}} = K \cdot M \cdot c$ where M is the number of units of the high molecular substance and c the concentration in weight %, assuming then that the molecules are split near the centre^{13, 14} we find that η is inversely proportional to the number of molecules, that is $\eta = K_1 \cdot 1/n$ where n is the number of molecules and c , which is a constant for a given solution irrespective of the size of the molecules, is integrated in the constant K_1 .

In the following, mention is made of a mechanism of reaction which appears to us to be more likely than the 3-step reaction, although we failed to obtain the same good agreement with the results found.

Assuming that the enzyme splits an equal number of bonds per time unit, the following expression would apply:

$$dn/dt = A \quad (A \text{ constant})$$

This gives $t = 1/A (n - n_0)$

where n_0 is the number of molecules at time zero, and n the number at time t . If n is introduced as inversely proportional to η and hence to y we get $t = B (1/y - 1)$. It will be seen that this is the first and most important term in our expression (1). The second term of the expression, the effect of which may be said to be that more time is consumed in obtaining a given degree of reaction than would be the case if the first term were the only one, could be accounted for qualitatively in the following manner:

If the molecules of the substrate are not split at the very centre, the reduction of the viscosity, according to Staudinger's formula, would not be so great as it would be if the fragments were of equal size. The result could also be explained by imagining that the enzyme acted less on the fragments than on the larger molecules. Further, it has been established that the enzyme may also act as transglycosidase¹⁵, and so it may also be assumed that the activity of the enzyme as such will increase with a rise in the number of fragments. One or several of the factors here mentioned might explain the occurrence of the second term of the expression.

The aspects of these possibilities are so complex, however, that we have not been able to subject them to a theoretical analysis. A promising way of attacking the problem might be to use the method described by W. Kuhn¹⁶. Studies of the reaction with other initial concentrations of the substrate may throw light on some of these problems.

The authors thanks are due to professor, dr.phil. J. A. Christiansen and to mag.scient. C. E. Jensen for active interest in this investigation. Financial support from the "Carlsberg Foundation" is gratefully acknowledged.

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Received July 5, 1955.