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Viscosimetric Determination of Cellulase Activity in the Intestine of the Sea Urchin: Reaction Mechanism and Equilibrium Constant for Cellulase Stabilization with Calcium

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Intestines of the sea urchin *Paracentrotus lividus* contain a cellulase which is stabilized by calcium. The apparent rate constant k for the inactivation of the enzyme was determined at various calcium ion activities in a buffer solution in which the buffering substances did not react with calcium ions. The rate constant k_u for the unstabilized enzyme and the rate constant k_s for the stabilized enzyme were calculated. The logarithm of $(k-k_s)/(k_u-k)$ was plotted *versus* the logarithm of the calcium ion activity, and a straight line was fitted to the points. The inclination of such a line gives the number n of calcium ions reacting with each of those groups of the enzyme which have an influence upon its stability, and the intersection of the line with the axis of abscissas gives the value of the logarithm of the equilibrium constant K for the stabilization of the enzyme, divided by n.

In the stabilization of the cellulase studied, each of its chemical groups which have an influence on its stability react with one calcium ion. The equilibrium constant for this stabilizing reaction is 0.0009 mole/liter. The optimum pH value for the stability of this cellulase is about 6.8 and is not significantly affected by the calcium ion activity. The rate constant for the inactivation of the calcium-free cellulase is about 4 times as large as that of its calcium complex. The optimum pH value for activity is about 6.1.

Metal ions combine with some enzymes to form complexes which may have a different enzyme activity or a different enzyme stability than the metal-free enzyme. A classical example of the first-mentioned effect is the reaction between silver and β-fructofuranosidase, the mechanism of which, including the influence of pH, was investigated by Myrbäck.[1] The stabilization of malt

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α-amylase with calcium may be mentioned as an example of how a metal ion can have an effect on the stability of an enzyme.[2,3]

The influence of various metal ions and various organic substances on the activity of cellulases has been studied by several scientists and has been recently reviewed by Halliwell [4] and by Mandels and Reese.[5] However, no reports have been found in the literature indicating an influence of a metal ion on the *stability* of a cellulase.

We found a new type of cellulase, one which is *stabilized* by calcium ions, in the Mediterranean sea urchin *Paracentrotus lividus* Lk. The cellulase occurs predominantly in the intestines, but relatively small amounts of this enzyme were also found in the gonads. The *activity* of this cellulase is not affected by calcium ions. In this respect this new cellulase resembles malt α-amylase, the stability but not the activity of which depends on the activity of calcium ions.

The experimental procedure for this investigation was the following. In a few introductory experiments the optimum pH value for activity and the optimum pH value for stability were determined. Reaction mixtures were then made up, containing the cellulase, calcium ions of various concentrations, and buffer salts giving the optimum pH value for stability. These reaction mixtures were immersed into a thermostatically controlled water bath of 42°C, and samples were withdrawn at suitable intervals. The cellulase activity of these samples was measured, and the rate constants for the denaturation of the cellulase were calculated.

An investigation of the stabilization of a cellulase with calcium ions comprises several problems: the determination of the calcium activity with sufficient accuracy, the determination of the enzyme activity with sufficient accuracy, and the treatment of the data obtained for the interpretation of the results.

The determination of calcium ion activity in the presence of proteins and buffer substances which may react with calcium ions is not very easy.[6] In the beginning of this investigation we used acetate buffers in the reaction mixture, but we soon found that the reaction between acetate ions and calcium ions could not be neglected; it would either introduce serious errors or would make the calculations of the results inconveniently complicated. Thus, it erroneously appeared from the preliminary experiments, when acetate buffers were used, that two calcium ions might be involved in the stabilization reaction. In order to be able to calculate the calcium ion activity reliably from the total calcium concentration in the mixture, we avoided buffer substances which form complexes easily with calcium ions. Hence, imidazole buffers were used in the main part of this investigation. The concentration of the purified enzyme preparation used could be kept low because a viscosimetric method was used for the activity determination, and so the amount of calcium in the complex was small compared to the amount of calcium added to the reaction mixture. Consequently, the calcium ion activity could be calculated from the calcium concentration and the ionic strength of the mixture.[7] The following form of Debye and Hückel's equation was used in the calculation of activity coefficients:

$$-\log f = \frac{0\,51 \cdot z^2 \cdot \sqrt{\mu}}{1 + \sqrt{\mu}} \qquad (1)$$

where f is the activity coefficient, z is the electric charge and μ is the ionic strength.

The cellulase activity was measured viscosimetrically. The following expressions give a value which is proportional to the number of linkages broken between the basic units of the substrate [8,9]

$$A = c^2 \frac{\mathrm{d}(1/\eta_{sp})}{\mathrm{d}t} \tag{2}$$

$$A = c^2 \frac{\mathrm{d}(1/\ln \eta_r)}{\mathrm{d}t} \tag{3}$$

Here A is the enzymic activity in the reaction mixture, c is the concentration of the substrate in the reaction mixture, η_{sp} is the specific viscosity, and η_r is the relative viscosity. The relative viscosity is the quotient between the viscosity of the mixture and the viscosity of the solvent; in the determination of enzymic activity one usually gets a value for the relative viscosity with sufficient accuracy if the flow time for the reaction mixture is divided by the flow time for water. The specific viscosity equals the relative viscosity minus 1. These expressions were deduced from Staudinger and Heuer's [10] equation for the relationship between viscosity, concentration, and molecular weight of solutions of polymeric homologous substances. A more accurate equation for this relationship has been given by Mark,[11] but it would not give an expression for enzymic activity as simple as eqn. (2) or (3).

Eqn. (2) can be used for most measurements, but if the viscosity of the solution is many times higher than the viscosity of the solvent, eqn. (3) should be used.[9] If the viscosity of the solution is considerably less than twice that of water, the influence of buffer salts on the viscosity of the solvent should be considered. If low molecular weight constituents are present in the preparation of the substrate used, or if the enzymic depolymerization is allowed to proceed so far that a noteworthy amount of low molecular weight substances are formed, considerable deviations from the linearity may be noticed, some of which may be caused by an inhibitory effect on the enzyme.

In the determination of enzymic activity, the inverse value of the specific viscosity (or the inverse value of the natural logarithm of the relative viscosity) is plotted *versus* the time for which it is valid. This time is usually obtained as follows. A stopwatch is started when the enzyme and the buffered substrate are mixed. A reading is taken when the flow reading begins, and half the flow reading is added (the flow reading is taken with another stopwatch).[12] A straight line is fitted to the points, and the inclination of this line is multiplied by the square of the concentration of the substrate in the mixture.

A convenient unit for the expression of the enzymic activity (H.U./ml) was suggested by Lundblad:[13,14] the activity, obtained when the substrate concentration is measured in g/ml and the time is measured in seconds, is multiplied by 10^9.

Eqns. (2) and (3) imply that the same expression for enzymic activity, proportional to the number of bonds hydrolyzed, is obtained irrespective of the original degree of polymerization of the substrate solution. However, it is implied in the deduction of the equations that the molecular weight distribu-

tion of the substrate is the same as that obtained at the breakdown of a single giant molecule; if this is not approximately true, some deviations from the expected viscosity change may occur in the early part of the enzymic degradation.[8,15] The substrate concentration will, of course, have an influence on the apparent enzymic activity,[16,17] and running viscosimetric activity determinations at various substrate concentrations, using eqn. (2) or (3) for the calculation of the activity, one can subsequently calculate the substrate constant (Michaelis' constant) for an enzyme.[18]

Eqns. (2) and (3) were deduced under the assumption that the enzyme has the capacity to hydrolyze any linkage between the basic units with equal ease; if only bonds at one end of a molecule can be broken enzymically, another expression is obtained.[8] Thus, if a straight line fits to the plottings according to eqn. (2) or (3) from an enzymic degradation, the conclusion can be drawn that the enzyme attacks all or almost all linkages between the basic units with equal ease.

The method of plotting the inverse value of the specific viscosity *versus* time as part of the calculation of enzymic activity from viscosimetric measurements — first suggested by one of us [8] — has been used for amylase,[19–22] cellulase,[21,23–29] chitinase,[21] dextranase,[30–32] hyaluronidase,[33–36] lysozyme,[37] polymetaphosphatase,[32,38,39] and proteolytic enzymes.[13,14,18,40–43]

It has been recently pointed out by Whitaker [44] that units of activity have often been based on the expression $d(1/\eta_{sp})/dt$, and that this expression does not give a direct measure of the bonds split. For example, Thomas [28] misunderstood eqn. (2) and stated erroneously that this equation predicts a linear relation between the enzyme concentration and $(1/\eta_{sp})/dt$ "in the presence of excess substrate", irrespective of the substrate concentration. In order to test this misconception he determined the value of $d(1/\eta_{sp})/dt$ at five selected cellulase concentrations, using three carboxymethylcellulose solutions of differing initial specific viscosity which he published (the concentrations,

Table 1. Thomas' viscosimetric assay of cellulase at various enzyme concentrations [E] and at various substrate concentrations;[28] he published the initial specific viscosity but not the sodium carboxymethylcellulose concentration of reaction mixtures with different substrate concentrations. It is assumed [45] that $\ln \eta_r$ is proportional to the substrate concentration c_s; *i.e.*, $\ln \eta_r = C c_s$. Activity values are listed for various enzyme concentrations; they are Thomas' values for $d(1/\eta_{sp})/dt$ multiplied by the square of the concentration times its proportionality factor. For such high viscosities, $1/\ln \eta_r$ instead of $1/\eta_{sp}$ should have been plotted *versus* time. The values for the lowest enzyme concentrations may have been affected by instability of the substrate in absence of ferricyanide.

η_{sp}	2.60	3.60	4.33
η_r	3.60	4.60	5.33
$\ln \eta_r$	1.28	1.53	1.67
$C^2 c_s^2$	1.64	2.34	2.79
16 [E]	395	384	332
8 [E]	202	202	184
4 [E]	103	107	106
2 [E]	56	61	53
[E]	31	30	31

however, of these substrate solutions are not given). Of course, he obtained differing results for what he erroneously calls "enzyme activity" but what actually is $d(1/\eta_{sp})/dt$. We can conclude from Thomas' article that he obtained his three substrate solutions by diluting the same stock solution. If we assume that the natural logarithm of the initial relative viscosity of the substrates is proportional to their concentrations,[45] and if we multiply Thomas' values for $d(1/\eta_{sp})/dt$ with the square of these logarithms, we get the values for the enzymic activity at various substrate concentrations given in Table 1. The relative viscosities of Thomas' substrates are so high that eqn. (3) should have been used, and it must be expected that if instead eqn. (2) is used, somewhat too small values will be obtained at the higher substrate concentrations;[9] furthermore, a spontaneous decrease in viscosity (which can be prevented by ferricyanide) may have given too high values at the lowest enzyme concentration; otherwise the agreement between the results is in some instances even surprisingly good. Additionally, we can conclude that the affinity between enzyme and substrate was high.[16-18]

Enebo *et al.*,[26] however, using ethyl-hydroxyethylcellulose solutions, did not obtain an entirely linear relationship between the activity and the concentration of a preparation consisting of at least two enzymes, one being a typical hydrolytic β-polyglycosidase, and the other a nonhydrolytic enzyme of "transglucosidase" type.

Eriksson and Lindvall [29] in the determination of cellulase activity with a carboxymethylcellulose of "medium viscosity" type, degree of polymerization 150, found that plottings of $1/\eta_{sp}$ *versus* time do not lie about a straight line when the enzymic breakdown is allowed to go so far that the inverse value of the specific viscosity of the mixture is increased up to sevenfold and discuss this observation. It appears from that work and is evident from the deduction of eqn. (2) that this equation is not strictly valid when the depolymerization has gone very far and a considerable amount of low-molecular fractions of the substrate is present, fractions which may even act as inhibitors of the enzyme.[26] Thus, it is important in viscosimetric assay of enzymic activity to use substrate preparations with a high degree of polymerization.[18]

Furthermore, the molecular weight distribution may have some influence [8,15] and, for electrically charged polymeric substances, the expression must be multiplied by an ionic factor if values of enzymic activity at various compositions of reaction mixtures are to be compared.[33,38,41] One can obtain relative values for this ionic factor by measuring the viscosity of solutions of the same concentration of the same batch of substrate at various pH values, various salt concentrations *etc.*

Finally, as already mentioned, the concentration of the substrate has an influence on the apparent activity of the enzyme,[16-18] and, accordingly, increased activities at increased substrate concentrations have been noticed in some investigations.[18,24,30,35,38]

THEORETICAL

A method for the calculation of the equilibrium constant of complexes between enzymes and their stabilizing metal ions has been described earlier by one of us.[46] That method concerned complexes in which one enzyme

molecule reacts with one metal ion. In this work both the number of metal ions in the complex and the dissociation constant are investigated.

The following symbols are used:

E_s the more stable form of the enzyme (the enzyme-calcium complex),
E_u the less stable form of the enzyme (the calcium-free enzyme),
f the activity coefficient for the stabilizing metal ion,
K the dissociation constant for the complex between the enzyme and the metal ion,
k the rate constant measured for the inactivation of the enzyme,
k_s the rate constant for the inactivation of the more stable form of the enzyme,
k_u the rate constant for the inactivation of the less stable form of the enzyme,
M the stabilizing metal ion (calcium),
n the number of metal ions reacting with a group affecting the stability of the enzyme.

Concentrations are expressed by brackets.

The dissociation of the stabilized enzyme can be written as follows:

$$E_s \rightleftharpoons E_u + nM \tag{4}$$

The activity factor can be estimated only for the metal ion, and so the equilibrium equation is written

$$K = \frac{[E_u] \cdot [M]^n \cdot f^n}{[E_s]} \tag{5}$$

If the total concentration of the enzyme is [E],

$$[E] = [E_s] + [E_u] \tag{6}$$

The differential equation for the inactivation of the enzyme at constant temperature can be written as follows:

$$d[E] = -[E] \cdot k \cdot dt = -[E_u] \cdot k_u \cdot dt - [E_s] \cdot k_s \cdot dt \tag{7}$$

Thus,

$$k = \frac{[E_u] \cdot k_u + [E_s] \cdot k_s}{[E]} \tag{8}$$

Eqn. (6) is used for the elimination of [E]; and both the numerator and the denominator are divided by $[E_u]$:

$$k = \frac{k_u + ([E_s]/[E_u])k_s}{1 + ([E_s]/[E_u])} \tag{9}$$

Eqn. (5) is used for the elimination of $[E_s]/[E_u]$:

$$k = \frac{k_u + ([M]^n \cdot f^n \cdot k_s/K)}{1 + ([M]^n \cdot f^n/K)} \tag{10}$$

This equation is solved for K, and we obtain

$$K = [M]^n \cdot f^n \cdot \frac{k - k_s}{k_u - k} \tag{11}$$

As will be shown later, this equation can be transformed further to become more suited for the experimental determination of the value of n.

The rate constants k_s and k_u can be determined by experiments at particularly high and at particularly low metal ion concentration. It is also possible to calculate k_s and k_u by a series of alternating approximations, using measurements at several metal ion activities. For that purpose, eqn. (11) is transformed as follows for the determination of k_s:

$$K(k_u - k) = k \cdot [M]^n \cdot f^n - k_s \cdot [M]^n \cdot f^n \tag{12}$$

$$k = k_s + K \cdot \frac{k_u - k}{[M]^n \cdot f^n} \tag{13}$$

Eqn. (13) implies that if the rate constant k experimentally determined is plotted *versus* the function $(k_u - k)/[M]^n \cdot f^n$, and a straight line is fitted to the points, the intersection with the axis of ordinates gives the value k_s, and the inclination of the line equals the dissociation constant for the enzyme-metal complex.

Similarly for k_u:

$$k_u - k = \frac{[M]^n \cdot f^n}{K} (k - k_s) \tag{14}$$

$$k = k_u - \frac{1}{K} (k - k_s) \cdot [M]^n \cdot f^n \tag{15}$$

Eqn. (15) suggests that the experimentally determined rate constant k is plotted *versus* $(k - k_s) \cdot [M]^n \cdot f^n$, and that a straight line is fitted to the points; the intersection of this line with the axis of ordinates gives the value of k_u, and the inclination of the line gives the negative inverse value of the dissociation constant.

Finally, the number of metal ions reacting with each group of the enzyme having an effect on the stability of the enzyme, is obtained after the following transformation of eqn. (11):

$$\log K = n \log [M] \cdot f + \log \frac{k - k_s}{k_u - k} \tag{16}$$

$$\log \frac{k_u - k}{k - k_s} = - \log K + n \log [M] \cdot f \tag{17}$$

Eqn. (17) implies that if $\log (k_u - k)/(k - k_s)$ is plotted *versus* the logarithm of the metal ion activity, a straight line may be fitted to the points. The inclination of the line equals the number n of metal ions reacting with each group of the enzyme affecting its stability. It is obvious that this number must be a cardinal number. Hence, the way to find the correct number is to carry out a statistical test in the regular way to find out whether or not the inclination of the straight line, fitted according to the method of least squares, differs significantly from 0, 1, 2, *etc.*[47] Eqn. (17) further implies that one can read the value of $(\log K)/n$ at the point of intersection between the straight line fitted to the points and the axis of abscissas.

EXPERIMENTAL

The cellulase. The cellulase preparation used in these experiments was obtained from the intestines of the sea urchin *Paracentrotus lividus*, collected in the Bay of Naples in the month of April. It was purified according to the method of Weidenhagen.[48] A 5 % solution of tannic acid was added to a filtered extract of the intestines as long as a precipitate formed. This was centrifuged off, and the tannic acid extracted with acetone at as low temperature as possible without ice formation. The remainder was dried in vacuum.

The viscosimetric assay. The activity of the enzyme was measured viscosimetrically with a commercial carboxymethylcellulose preparation as substrate. The reaction mixture usually consisted of 1 ml of enzyme solution, 1 ml of phosphate buffer and 2 ml of a 0.6 % substrate solution. Reaction mixtures with low enzyme activity also contained potassium ferricyanide. This substance was included in the buffer solution. The flow time was measured at 30°C in viscosimeters whose flow time for water was about 20 sec. We found that the flow time decreased continuously for mixtures which contained no active enzyme but contained reducing substances, *e.g.*, hydrogen sulfide, glutathione, cysteine, or ascorbic acid, and that ferricyanide stabilized the flow reading. When relatively large enzyme concentrations were used in the reaction mixtures, the same activity was found both in the presence and in the absence of ferricyanide. The buffer was made up to give a pH value of 6.1 (except in experiments for measuring the influence of pH on the activity of the cellulase). Typical plottings for the calculation of the enzymic activity are shown in Fig. 1. The influence of pH on the activity is shown in Fig. 2. The optimum pH value for activity was 6.1, a value which was obtained from several experimental series.

The rate constant for the inactivation. Reaction mixtures of 6 ml were made up with imidazole buffer of pH 6.9 (except in experiments for measuring the influence of pH on the stability of the cellulase), calcium chloride, sodium chloride, and enzyme. The amount of sodium chloride was usually chosen so that the ionic strength of the mixture was 0.02. For example, one mixture consisted of 3.98 ml of enzyme solution, 0.25 ml of imidazole buffer of the ionic strength 0.06, 0.5 ml of a 0.019 M $CaCl_2$ solution and 1.27

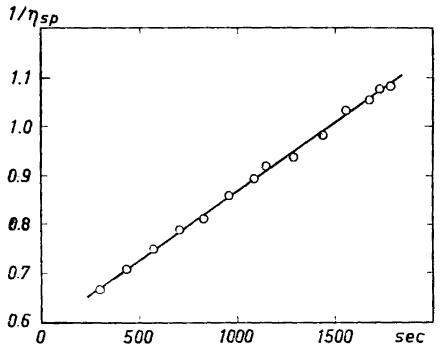

Fig. 1. The viscosimetric assay of cellulase activity. Plot of $1/\eta_T$ *versus* time (sec). The flow time at 30°C for the Ostwald viscosimeter used was 20.0 sec. 2 ml of a 0.61 % carboxymethylcellulose solution were mixed with 1 ml of phosphate buffer solution pH 5.9, containing ferricyanide, and 1 ml of enzyme solution. The inclination of the line is 0.274×10^{-3} sec. Hence, the activity of the enzyme solution was $4 \times (0.305)^2 \times 0.01^2 \times 0.274 \times 10^{-3} \times 10^9 = 10.1$ H.U./ml.

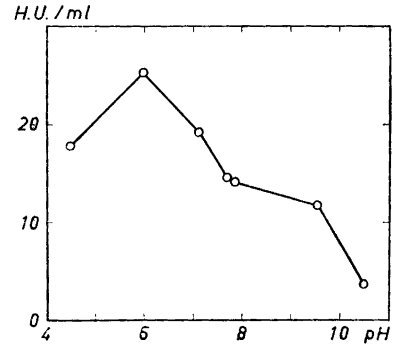

Fig. 2. The influence of pH on the activity of the cellulase.

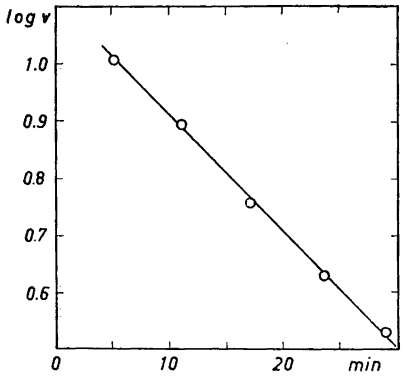

Fig. 3. The rate constant for the inactivation of the enzyme. Plot of the logarithm of the enzyme activity *v versus* time (min). The inclination of the line is 0.0146 min^{-1}, and so the rate constant is $2.303 \times 0.0146 = 0.036$ min^{-1}.

ml of a 0.06 M NaCl solution. The mixture, in a thin-walled aluminum test tube, was immersed in a thermostatically controlled water bath at 42°C. Samples were withdrawn after 5, 11, 17, 23, and 29 min. The viscosimetric measurement of the sample taken after 5 min is shown in Fig. 1. The logarithm of the activities were plotted *versus* time for the calculation of the rate constant for the inactivation as shown in Fig. 3. Graphs for the calculation of the dissociation constant of the enzyme-metal complex and for the calculation of the rate constants for the inactivation of the enzyme-metal complex and the metal-free enzyme from measurements at several metal ion activities are shown in Figs. 4 and 5.

The calcium ion activity. In the example mentioned, the ionic strength was 0.02. The activity factor was calculated as follows, using eqn. (1). $-\log f = 0.51 \times 4\sqrt{0.02}$

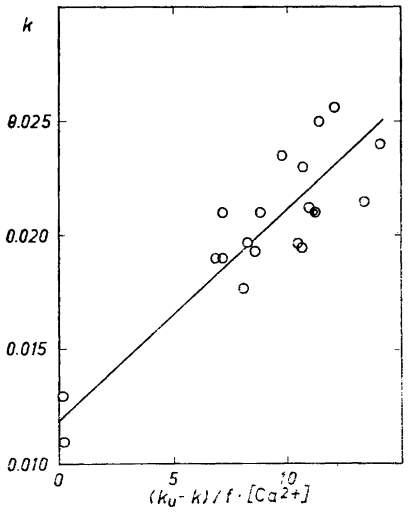

Fig. 4. Graph for the calculation of the rate constant for the inactivation of the cellulase-calcium complex and for the calculation of the dissociation constant (eqn. 13).

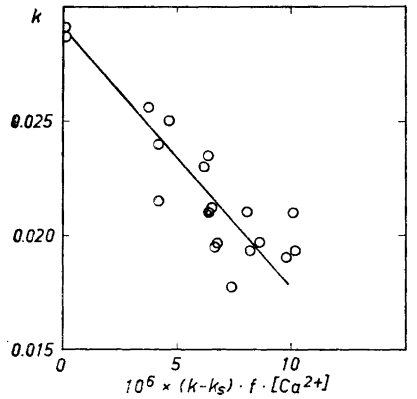

Fig. 5. Graph for the calculation of the rate constant for the inactivation of the calcium-free cellulase and for the calculation of the dissociation constant (eqn. 15).

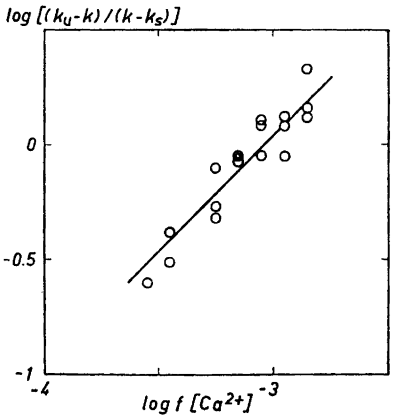

Fig. 6. Graph for the determination of the number of calcium ions reacting with a group affecting the stability of the cellulase, and for the calculation of the dissociation constant of the complex. A line with the inclination 1 is statistically satisfactory.

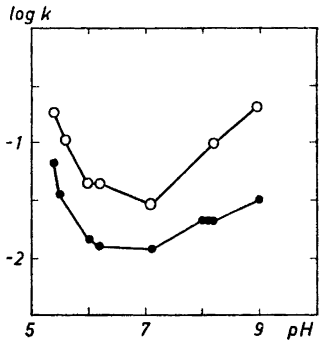

Fig. 7. The effect of pH on the stability of the cellulase. Dots represent the more stable form (the cellulase-calcium complex), and rings represent the less stable form (the calcium-free cellulase).

$(1 + \sqrt{0.02}) = 0.252$; $f = 0.56$. The calcium concentration in the mixture was 0.0016 (a 0.019 M solution diluted 12 times), and so the estimated calcium activity was 0.00089.

The stabilization mechanism. The rate constant k for inactivation at 42°C was determined for various calcium ion activities, including high activities (for k_s) and negligibly low activities (for k_u). The values were plotted according to eqn. (17), and the result is shown in Fig. 6. It can be seen from this graph that the value of n, the number of calcium ions reacting, is 1, because only a line with the inclination 1 fits in. The value of log K is read as the intersection between the line and the axis of abscissas as 0.95—4. Hence, $k = 0.0009$. The rate constant was also measured at various pH values for the stabilized and the calcium-free cellulase, and the result is shown in Fig. 7. The optimum pH value for stability is about 6.8 and seems not to be significantly affected by the calcium ion activity.

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