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The determination of cellulase activity by viscometry

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The enzymatic activity of cellulase has been estimated by the decrease of viscosity of a hydroxyethylcellulose solution as a function of incubation time. This decrease is a function of the applied shear stress. It is proposed that the viscosity be measured in an ordinary Ostwald viscometer under conditions where the viscosity is Newtonian, that is, the decrease of viscosity by enzymatic activity is independent on the shear stress.

Methods for determining the activity of cellulase preparations have been recently discussed by Courtois & Bui Khac Diep (1965; 1967) who recommended the use of hydroxyethylcellulose as the most suitable substrate. It is accepted that the cellulose derivatives are attacked only if the degree of substitution is small, and these authors recommended an appropriate preparation procedure. The starting material is Whatman Cellulose Powder (CF II for chromatography), and this is hydroxylated at room temperature by ethylene oxide in alkaline solution.

Viscometry has been recommended for the assay of hydrolases and is most useful for the assay of enzymatically catalysed reactions consisting of simultaneously and successive breakdown of a polymeric molecule, which cannot be defined by a simple molecular scheme.

The enzyme response could be assayed by the rate of release of the reducing groups, as has been shown by Courtois & Bui Khac Diep (1965; 1967) the determination of the reducing saccharides liberated by hydrolysis of the β-1,4-linkages, and the quantitative determination of the glucose in the hydrolysate, lacked accuracy and reproducibility. The viscosities of the solutions were measured in a Haage Rotovisco apparatus. The decrease in viscosity of the solutions, being in direct relation to the amount of cellulase incubated, allowed for an easy assay in the initial period of breakdown of the proposed substrate.

Our aim has been to effect closer control of some physical factors involved and of the precision. For a control method a capillary viscometer of simple construction was thought to fit the laboratory needs and to give more dependable results.

Cellulose ethers can be considered as random coiled macromolecules (Ferry, 1961). The position of each segment (subunit) of the macromolecule relative to its neighbours gives rise to a configurational entropy, S, according to the Boltzmann formula:

$$S = k \ln P$$

where S = entropy, k = Boltzmann constant, P = number of configurations of the random coiled macromolecule.

Each linkage of such a subunit acts as a so called "entropy spring". By the action of viscous flow, the position of a segment relative to its neighbours changes, causing a change in configurational entropy. On deformation there will be some energy

storage. Further calculations indicate that such a solution behaves as a non-Newtonian fluid (Ferry, 1961).

Of the different devices for measuring viscosity, we considered the movement of concentric cylinders (Couette type) and the rate of flow through a capillary type viscometer (Phillipoff, 1942; Van Wazer, 1966).

The Haaze Rotovisco viscometer was recommended by Courtois & Bui Khac Diep (1967), and satisfactory results were obtained with collaborative tests made under the auspices of the F.I.P. Commission for standardization of pharmaceutical enzymes. But owing to the cost it seemed desirable also to develop a method using an ordinary capillary viscometer.

Methods of expressing the enzymatic activity

The enzymatic activity may be calculated by a formula like that of Courtois & Bui Khac Diep (1967).

$$\text{Enzymatic activity} = \frac{-1}{P} \cdot \frac{100 \times \left(\frac{d\eta}{dt}\right)}{\eta_0 - \eta_w}$$

where P = the amount of cellulase in the test solution (in mg), $d\eta/dt$ the decrease of viscosity η as a function of the incubation time t over the linear part of the $\eta - t$ curve, η_0 = the viscosity of the test solution at zero incubation time (blank value) and η_w = the viscosity of water.

Although using this formula no absolute calibration of the viscometer is necessary, the value of η_w is low relative to η_0 and it is difficult to determine accurately. Also this formula requires that $(d\eta/dt)/(\eta_0 - \eta_w)$ is constant, but it is also a function of the initial viscosity η_0, and experiment shows that if η_0 undergoes a small variation $\delta\eta_0$, due to the preparation or pipetting of the initial solution, $d\eta/dt$ is not affected, so the value of enzyme activity depends on the value of η_0, even though the value of $d\eta/dt$, which measures the enzymatic activity of the cellulase solution, remains reasonably constant. As this method is liable to errors which have nothing to do with the action of the enzyme itself, it seems more realistic to define enzyme activity as:

$$-\frac{1}{P}\frac{d\eta}{dt}$$

which gives more reliable results (η being expressed in $mNsm^{-2}$ and t in s). The hydroxycellulose sample must show a linear decrease of the viscosity due to the action of the enzyme.

EXPERIMENTAL

Apparatus

For the Rotovisko viscometer used, the cup was of the DMK type. The viscometer was calibrated with a heavy oil with a viscosity $\eta = 1{\cdot}62$ Nsm^{-2}, showing Newtonian behaviour.

The capillary viscometer used was of the Ostwald type and so constructed that the efflux time was about 30 s for the hydroxyethylcellulose solution. This viscometer was calibrated with an aqueous 50% glycerol solution, the viscosity of which was determined using another capillary viscometer with an efflux time for water at 25° of about 60 s ($\eta = 893{\cdot}7\,\mu$ Nsm^{-2}). In this way the short efflux time for the water in the measuring viscometer can be deduced. A fast efflux time for the measuring viscometer (30 s) has the advantage of minimizing the error on incubation time of the enzyme solution.

Preparation of hydroxyethylcellulose and its solutions

The hydroxyethylcellulose ether was prepared according to Courtois & Bui Khac Diep (1967). As enzymatic assay results may depend on the sample of hydroxyethylcellulose used (degree of polymerization, degree of substitution and homogeneity), samples showing a linear decrease in viscosity as a function of the incubation time were sought. Solutions were prepared by swelling a known amount of a sample in water over 12 to 20 h. After diluting with water to the mark, the whole solution was ultrasonicated or treated in a Servall mixer for 10 min. This solution was allowed to stand for 24 h to reach equilibrium.

Rheological behaviour of hydroxyethylcellulose solutions

To test the rheological behaviour of hydroxyethylcellulose solutions in the capillary viscometer, the pressure difference, Δp, was changed by an electromagnetic manostat.

Cellulase activity was assayed as follows: 2 ml of a suitable diluted cellulase solution, previously centrifuged, was added to a solution containing 16 ml of 2% hydroxyethylcellulose and 2 ml of buffer (0·45 ml 0:2 M Na_2HPO_4 and 0·55 ml 0·1 M citric acid). This test solution was kept at 25° in the viscometer and the viscosity measured every 2 min. Viscosity measurements were continued for 20 to 30 min and the curve of the efflux time as a function of the incubation time constructed. The incubation times were read exactly at the midpoint of the efflux time.

Blank value

It has been claimed that a blank test can be made after destroying the cellulase activity by boiling the solution for 30 min. We found that a residual activity remained. However by our different definition of cellulase activity the necessity for a blank value was eliminated.

The blank value could be obtained by taking 2 ml of water instead of the destroyed cellulase, or more conveniently by extrapolating the incubation time to zero. Interaction between the denatured cellulase and substrate did not alter viscosity values. The extrapolation of the efflux times to the initial incubation time avoids a supplementary pipetting of the hydroxyethylcellulose solution, which is best avoided, in view of the high viscosity of the solution.

RESULTS AND DISCUSSION

In Fig. 1A the viscosity (measured in the Rotovisko apparatus) as a function of the incubation time is shown for a given cellulase preparation. It appears that there is a linear relation, at least up to 25 min, between the apparent viscosity and the time of enzyme action. Since the viscosity of a hydroxyethylcellulose solution is a function of the rate of shear (or shear stress), it may be expected that the decrease of viscosity $-d\eta/dt$ to will also be dependent on the shear stress. This is indeed so as seen in Fig. 1B. It is also found that for higher values of shear stress that $d\eta/dt$ remains roughly constant.

The activities determined by the Rotovisko viscometer (at high shear stress) were markedly lower than those obtained by the capillary viscometer method.

The conditions for the assay of the enzymatic activity of a cellulase were also investigated with a modified Ostwald viscometer (Fig. 2).

The efflux times of the hydroxyethylcellulose were measured as a function of the applied external pressure (held rigorously constant). According to Poiseuille's law,

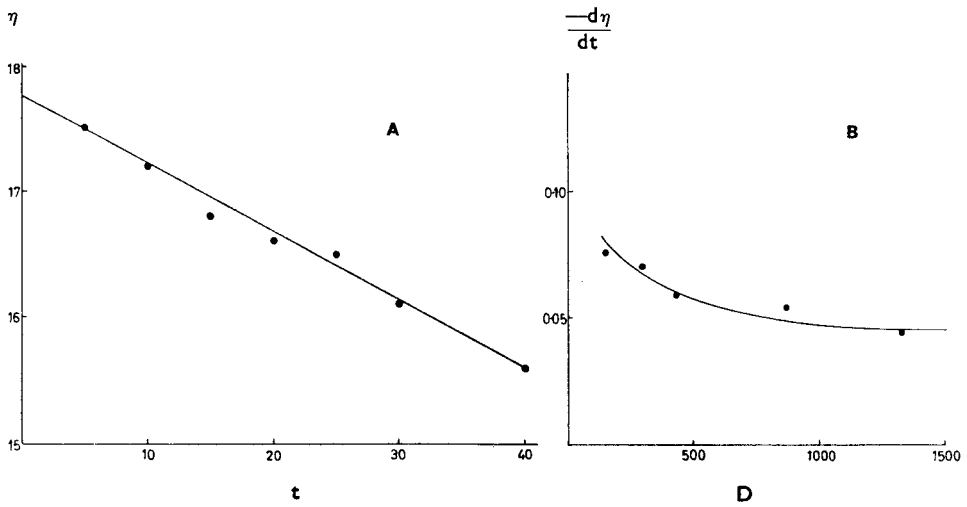

FIG. 1A. Decrease of viscosity (η, $mNsm^{-2}$) for a hydroxyethylcellulose solution, by cellulase as a function of incubation time (t in minutes) (D = 870, Rotovisco) ($-d\eta/dt$ = 0·9 pNm^{-2}).
B. Decrease of the viscosity ($-d\eta/dt$) for a given cellulase solution as a function of the rate of strain D (Rotovisco).

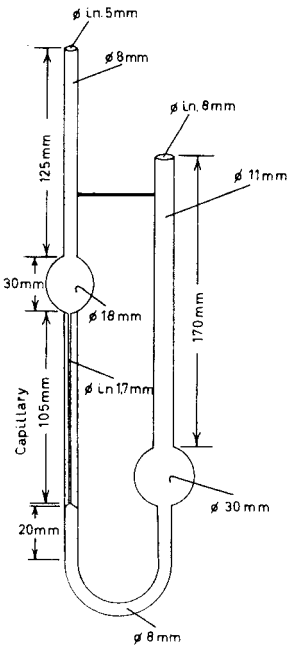

FIG. 2. Capillary viscosimeter for the assay of the cellulase activity. Efflux time for a 3% hydroxyethylcellulose solution ≈30 s.

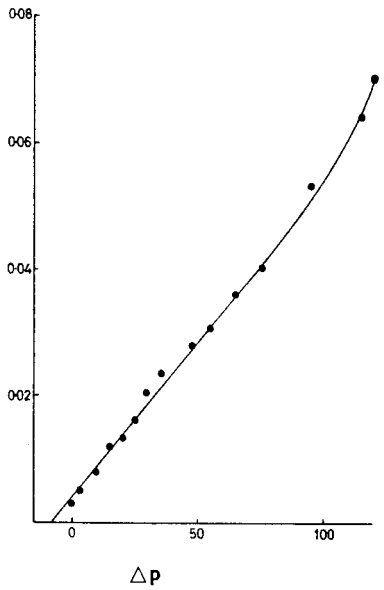

FIG. 3. Inverse of the elution time ($t^{-1}s$, ordinate) as a function of the applied pressure difference (Δp in mm Hg) for a 3% hydroxyethylcellulose solution.

for a Newtonian fluid a linear relation exists between the pressure difference Δp and t^{-1} (t = efflux time). For the results in Fig. 3 it appears that this linear relation holds for hydroxyethylcellulose solutions up to a pressure difference of about 50 mm Hg. Thus in this region the solution shows a Newtonian behaviour.

The pressure difference Δp, causing flow consists of two components: one the hydrodynamic pressure difference p_o, the other the applied pressure difference p, thus

$$\Delta p = p_o + p$$

From Fig. 3 it appears that the mean value of p_o is about 8 mm Hg.

The enzyme activity was measured for a cellulase sample with a 3% hydroxyethylcellulose solution. Since the elution time for this solution was about 35 s, it was easy to make measurements every 2 min. From Fig. 4 it appears that there is a linear decrease of the viscosity as a function of the incubation time. Deviations occur before 5 min and these points were not considered. Since pipetting the viscous solution is not very accurate, the initial viscosity of the test solutions may differ, however the decrease ($d\eta/dt$) remains constant. The results for three different runs were ($-d\eta/dt$) 472, 460, 488 mNm^{-2}. The accuracy of a determination is within 5%

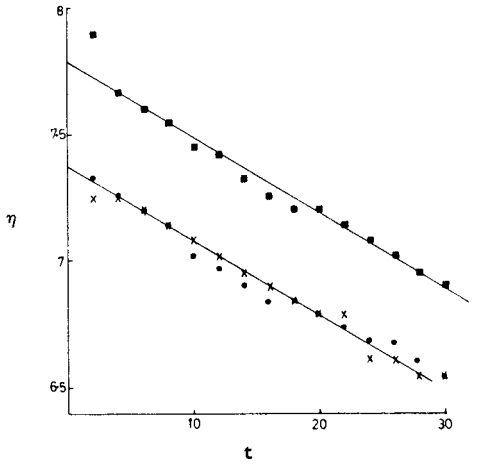

FIG. 4. Decrease of the viscosity (η, $mNsm^{-2}$) of a hydroxyethylcellulose solution by cellulase as a function of the incubation time (t min). Results of three separate runs.

Table 1. *Determination of the enzyme activity of cellulase. Effect of the amount cellulase added*

P (μg cellulase per 20 ml reaction solution)	$-d\eta/dt$ nNm^{-2}	Enzyme activity
100	800	8·00
100	800	8·00
100	892	8·92
100	733	7·33
100	783	7·83
100	967	9·67
200	1717	8·57
200	1667	8·33
200	1650	8·25
200	1700	8·50
200	1500	7·50
200	1717	8·57
300	2450	8·17
300	2550	8·50
400	3033	7·58
400	3017	7·54

We also varied the enzyme concentration. The enzyme activities were calculated by $-(1/P)(d\eta/dt)$, P being expressed in μg cellulase in the incubation solution of 20 cm^3 (Table 1). Units of enzyme activity are expressed as decrease of viscosity per μg enzyme in 20 ml solution.

Values for 5, 10, 15 and 20 μg cellulase/cm^3 of reaction solution were obtained using different substrates. They were most regular for a cellulase activity region of 8·20 to 8·80 units contained in 200 μg of enzyme preparation in the reaction mixture. For one substrate, 30 replicate assays gave a mean value of 8·36 with a standard deviation of $\pm$0·10.

Samples of one hydroxyethylcellulose solution were measured in 3 viscometers for an enzyme concentration of 10 μg/ml reaction mixture. The values are in Table 2.

Table 2. *Enzymatic cellulase determination for one hydroxyethylcellulose solution in 3 different Ostwald viscometers*

P (μg cellulase in 20 ml reaction solution)	Viscometer	$-d\eta/dt$ nNm^{-2}	Enzyme activity
200	1	1800	9·00
200	1	1650	8·25
200	1	1783	8·91
200	1	1717	8·58
200	2	1817	9·08
200	3	1800	9·00
200	3	1733	8·67

The optimal substrate concentrations were 2 to 3%. Enzymatic activity was not independent of the hydroxyethylcellulose concentration. In view of the complexity of the viscosity factors involved in such a system, this result is not surprising.

The method may be considered as a relative one and the unit definition has no absolute significance as the cellulase preparations are mostly mixtures of enzymes.

As a control procedure, the use of a simple capillary method is recommended and could easily be adapted for Pharmacopoeia purposes.

A difficulty remains in that the results for enzymatic activity are to some extent dependent on the sample of hydroxyethylcellulose used, even if it is prepared exactly according to recommendations. Some commercial samples examined did not show a linear relation between the decrease of viscosity with incubation time.

A further objective would be to relate the decrease of viscosity quantitatively with the breakdown of the macromolecule. But the calculation of the number of subunits split up per unit of time, or the reducing groups released, is still difficult to determine with accuracy.

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