

DESIGN OF A CAPILLARY VISCOMETER FOR ASSAY OF COLLAGENASE ACTIVITY BY 'CONTINUOUS' OBSERVATION OF THE HYDROLYSIS OF A SOLUBLE COLLAGEN WITH A TIME-OF-FLIGHT FLOWMETER*

ABSTRACT

The design of a capillary viscometer for semi-automated assay of collagenase activity is described. The rate of flow of a solution of a soluble derivative of collagen undergoing hydrolysis by the collagenase is measured "continuously" by an electronic flow meter. Since the reciprocal of the flow-rate is proportional to the viscosity and the latter is a (double exponential decay) function of the collagenase concentration (as well as of time), collagenase concentration can be determined from the flowrate at a given incubation time. The data are collected automatically, stored in a computer, and analyzed at the analyst's convenience. The viscometer is suitable for use in research laboratories investigating damage to hides (and therefore to leather) caused by bacterial digestion of collagen.

INTRODUCTION

In earlier work¹ we demonstrated that pepsin-treated porcine skin collagen is a suitable substrate for viscometric assay of collagenase activity. However, that work also indicated the unsuitability, for kinetic work, of measuring the viscosity with an Ostwald-type (Cannon-Fenske) viscometer. The procedure is very labor intensive, and data for early incubation times cannot be obtained. We have therefore undertaken the development of a relatively simple apparatus providing automatic acquisition of viscosity data at a high frequency.

There are, of course, sophisticated viscometers on the market which could be used for collagenase assay. But these cost \$5,000 to \$100,000. Our work required a device made with components already available in our laboratory which could evaluate the collagenase activity of a sample in 10-20 min.

MATERIALS AND METHODS

Flowrates were measured with a Thermalpulse II flowmeter (Figure 1, items #2 & 3) manufactured by M-Tek, Inc., Pittsburgh, PA. The flow cell (Figure 1, #2) is made of 25% glass-filled Teflon, and has a nominal flowrate range of 0.3 to 5 ml/min. The flowmeter is provided with circuitry and a connector for RS-232 interfacing with a computer (serial ASCII) and has the capability of providing flowrate readings as frequently as once every 15 seconds. A flowmeter with these capabilities can be purchased for ca. \$3500. The instrument is useful for applications such as HPLC and FIA (flow injection analysis), in addition to the application described here.

The reservoir (Figure 1, #1), which contained the solution whose viscosity was being measured, was a model R9 packing reservoir manufactured by Pharmacia Biotech, Inc., Piscataway, NJ. The conical adaptor was not used. The screw cap assembly, including the nipple, was attached to the bottom of the acrylic cylinder (instead of to the top).

The lower of the two capillaries was Teflon, 0.037 in i.d., 0.065 in o.d. It was connected to the bottom of the reservoir with the nipple (see above), and to the bottom of the

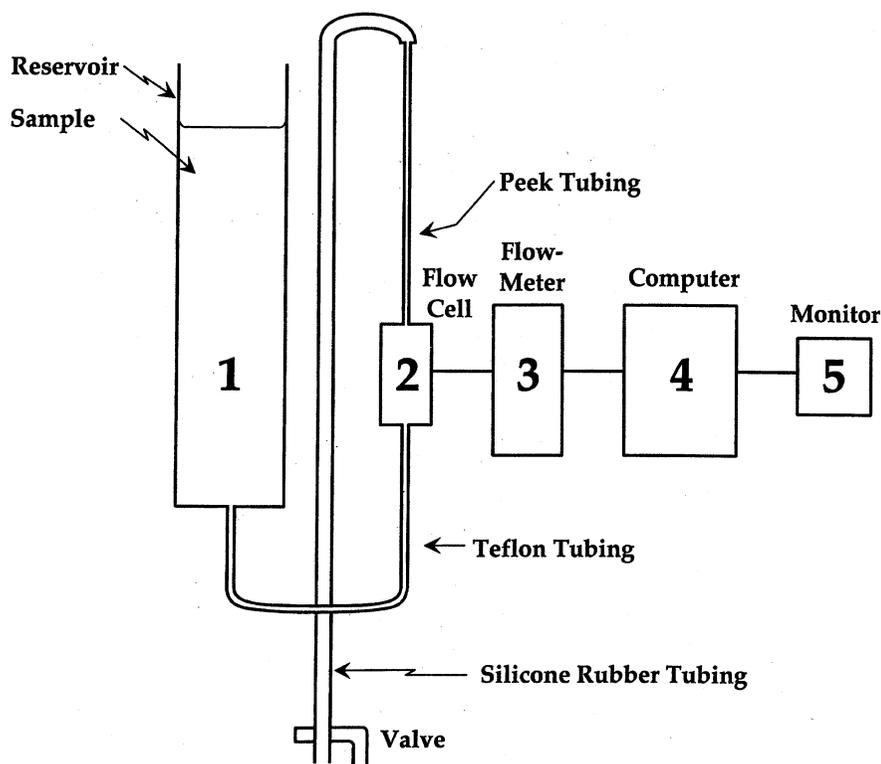


FIGURE 1. — Schematic of the viscometer. The parameters selected are as follows (see text): for the lower (Teflon) capillary, 0.037 in (i.d.) x 25.5 cm (1); the upper (Peek) capillary, 0.030 in (i.d.) x 20 cm (1); the exit (Silicone rubber) tubing, 1/16 in (i.d.) x 68. cm; the initial hydrostatic pressure, i.e., the initial level of the meniscus in the reservoir relative to the outlet (bottom of the valve), 35. cm. For other details, see Materials and Methods section.

flow cell with a type P-206X flangeless Delrin nut (with a type P-200X flangeless ferrule) obtained from Upchurch Scientific, Oak Harbor, WA. The upper capillary, of Peek tubing, 0.030 in i.d., 1/16 in o.d., also obtained from Upchurch Scientific, was connected to the top of the flow cell with a nut (and ferrule) of the same kind. The upper end of the second capillary was connected to silicone rubber tubing, 1/16 in i.d., 1/8 in o.d. The lower end of the tubing was connected to a miniature inert valve (Figure 1) with a 180° flow path manufactured by the Hamilton Co., Reno, Nevada. The inlet and outlet of the valve were male Luer connectors.

The vertical positions of the meniscus in the reservoir and the point of exit of the liquid from the valve were measured with a cathetometer.

Once operating parameters for the viscometer were determined (see Results and Discussion), the following conditions were used, unless otherwise indicated: sample volume, 20 ml; initial sample meniscus level (h_0), 35 cm above the outlet point. For tubing dimensions, see legend to Figure 1, as well as tubing description above.

The computer (Figure 1, #4), model 30-286, and the monitor (item #5) were manufactured by the IBM Corp., White

Plains, NY, and supplied as a Personal System 12. Crosstalk XVI (Microstuf, Inc., Roswell, GA) was used for data acquisition. Data analysis, including curve fitting, was done with Mathcad software (Mathsoft, Inc., Cambridge, MA).

The stock solution of collagen was a 1% solution of pepsin-treated porcine skin collagen in 3.3% sodium citrate, 0.19% sodium benzoate, pH 3.6; designated as Natural Soluble Collagen (Dermacol), it was produced by Pentapharm Ltd., Basel, Switzerland, and purchased from Centerchem, Inc., Stamford, CT. The stock solution was stored frozen. Collagenase from *Clostridium histolyticum* was provided by Sigma Chemical Corp., St. Louis, MO, as either the "crude" type IA product, with a specific activity of 320 or 550 units/mg (vs collagen), or the "high-purity" type VII product, with 1700 units/mg.

RESULTS AND DISCUSSION

The viscometer developed consists essentially of a flow meter in series with two capillaries, which provide resistance to flow. The basic design of the system is shown in Figure 1. The direction of liquid flow through the flowcell

(item #2) is upwards. One capillary is connected to the inlet of the flow cell, the other to the outlet. The first capillary connects the cell inlet to the reservoir (item #1) where the collagen and collagenase are initially mixed. The second capillary connects the cell outlet to a tube of relatively large diameter at the end of which the liquid leaves the system. The “force” driving liquid flow is the hydrostatic pressure resulting from the vertical difference between the meniscus in the reservoir and the outlet point.

The system parameters to be selected were: (1) the dimensions of the capillaries, and (2) the hydrostatic pressure. Determination of suitable capillaries was very time consuming, because the resistance of a capillary to flow varies inversely with the 4th power of its diameter (ref 2, p. 9, equation II. 1). Except for an initial calculation to estimate suitable capillary dimensions, the work was empirical. It was determined that the dimensions given in the Materials and Methods section and in the legend to Figure 1 would provide flowrates close enough to the desired range that selection of the hydrostatic pressure could be used for “fine tuning” (see below).

The rest of the system (Figure 1, items #3, 4, and 5) is electronic. Signals from the sensors of the flow cell are transmitted to the microprocessor of the flowmeter (#3), which calculates the flowrate. These data are then transmitted to the computer (#4), where they are stored for later analysis. The data are also displayed on the monitor (item #5) as they are collected.

The range of flowrates compatible with the flowcell used is ca. 0.3-5 ml/min. It was therefore necessary to choose a hydrostatic pressure which would provide flowrates falling within the permissible range for the most viscous and least viscous samples to be analyzed. The most viscous sample (DCSS) was the dilute collagen substrate solution which had not been exposed to collagenase. The least viscous sample was the same solution after all of the collagen had been digested by collagenase (Digest). On the basis of the data shown in Figure 2, a hydrostatic pressure $h = 35$ cm (dashed vertical line) was selected. This pressure provided flowrates for the experimental samples of ca. 1 to 4 ml/min, which is within the capability of the flow cell.

Flow rates (f) and effluent volumes (V) were then measured for standard solutions of glycerol at 22.5°C having viscosities (3-10 cP) covering virtually all of the viscosity range of our samples (Figures 3a and 3b). (A V vs time curve provides an alternative way of determining the flowrate, since the slope of this curve is the flowrate). When the initial value of $1/f$, obtained, in this case, from the curves in Figure 3b, was plotted against the known viscosity (Figure 4), the straight line relationship shown was obtained. The “known” viscosity of glycerol at 22.5°C for each of the concentrations used (Figure 3a) was obtained by interpolation from tabulated values.³

The decrease of flowrate (f) with time (Figure 3a) is due to the decrease in hydrostatic pressure (h) resulting from the drop in the level of the meniscus in the sample reservoir

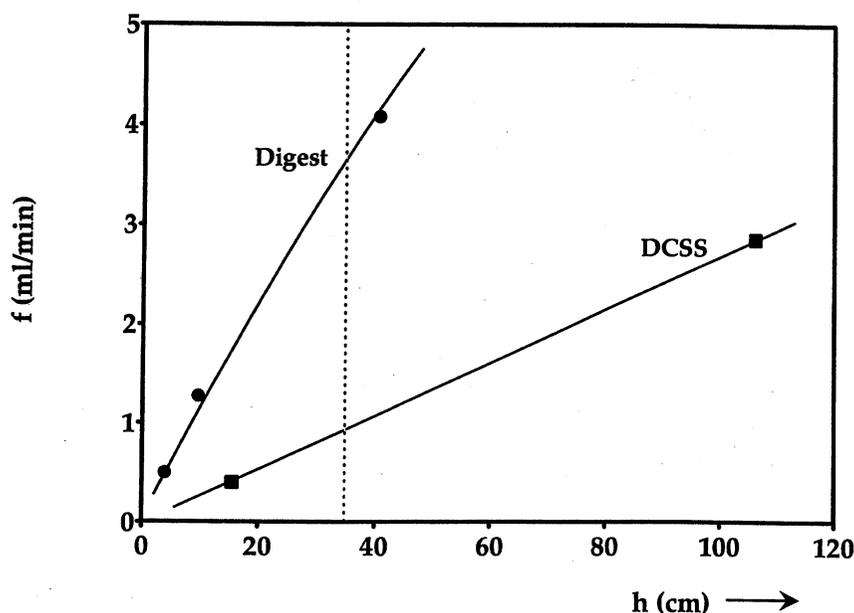


FIGURE 2. — Dependence of flowrate (f) on hydrostatic pressure (h) for the dilute collagen substrate solution before (DCSS) and after (Digest) thorough digestion with collagenase. Composition of DCSS: pepsin-treated collagen, 1.8 g/l; Tris, 0.25 M; citrate (sodium), 0.12 M; CaCl_2 , 9.8 mM; benzoate (sodium), 2.1 mM; pH, 6.8. The Digest was obtained by adding 0.95 mg collagenase to 30 ml DCSS and incubating at 23°C for >1 hour.

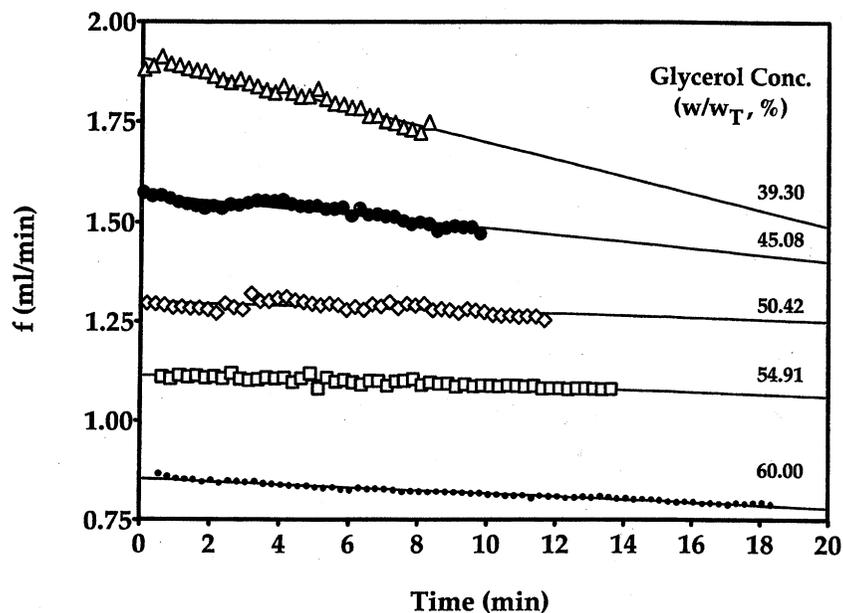


FIGURE 3a.— The flowrates (f) of standard solutions of glycerol in the viscometer. The decrease of f with time is due to the decrease in hydrostatic pressure. w is the weight of glycerol in an aqueous solution of (total) weight w_T .

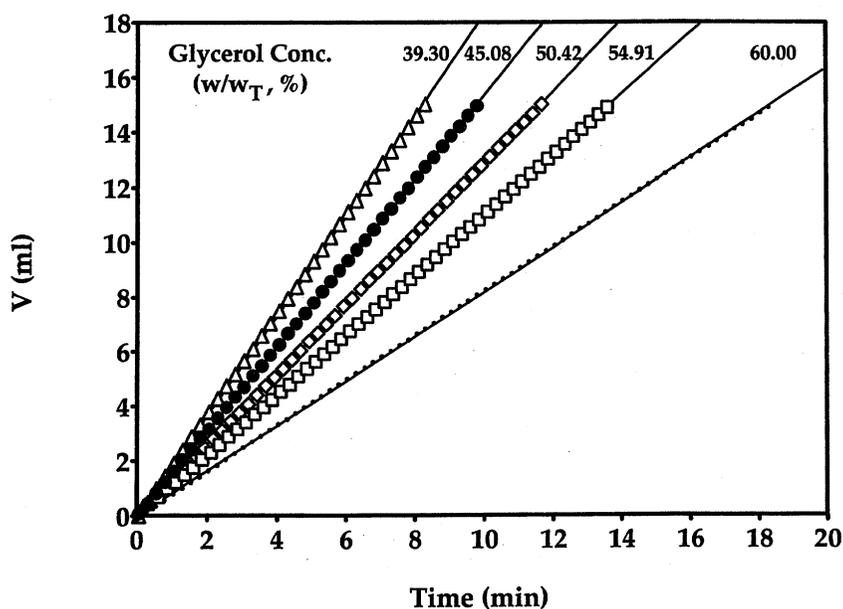


FIGURE 3b. — The effluent volumes (V) of the same (as in Figure 3a) standard solutions of glycerol. The initial flowrate (f_0) for each solution is the initial slope of the V vs T plot for that solution, and corresponds to the initial (extrapolated) value of f in Figure 3a.

(Figure 1, #1) as sample flows out of it. For many analyses it is necessary to know what the flowrate would be if the hydrostatic pressure were constant at its initial value (h_0). It is easily shown that the corrected flowrate at any time T

$$f_c = \frac{f}{1 - V/Ah_0} \quad (1)$$

V , the effluent volume (cm^3) at time T , is provided by the flowmeter; it is part of the data file. A is the internal cross-

sectional area of the cylindrical reservoir. For the apparatus described (Figure 1), $A = 4.57 \text{ (cm}^2\text{)}$ and $h_0 = 35 \text{ (cm)}$, so that $Ah_0 = 160 \text{ (cm}^3\text{)}$.

Figure 5 shows data obtained with a solution of collagen undergoing digestion by collagenase. Data (squares) were collected in clusters of 7 data points every 10 minutes. Within a cluster, flowrate measurements were taken (automatically) every 15 seconds. The curve shown is the

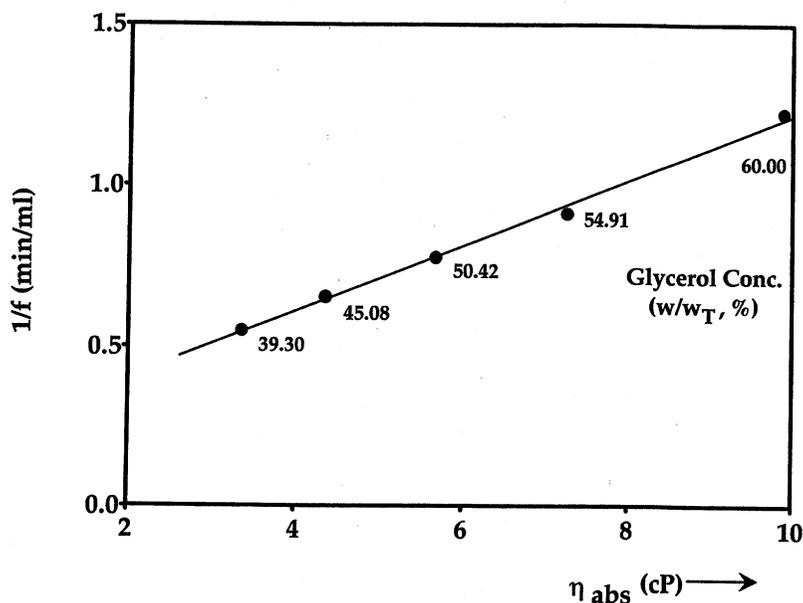


FIGURE 4. — The linear dependence of $1/f_0$, the reciprocal of the initial flowrate, on viscosity. The f_0 values are the initial slopes of the curves in Figure 3b.

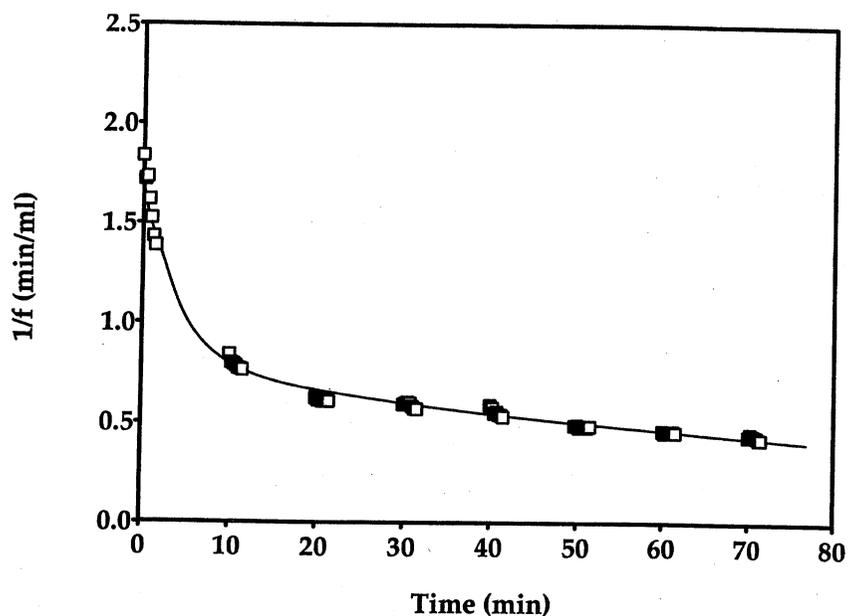


FIGURE 5. — Time course of the hydrolysis of pepsin-treated collagen with Clostridial collagenase as observed with the viscometer. Conditions of incubation: collagen, 2.2 g/l; Tris, 0.25 M; CaCl_2 , 9.7 mM; citrate (sodium), 0.12 M; benzoate (sodium), 2.9 mM; collagenase, Sigma type VII, 173. units (vs collagen)/ml; pH, 6.8; temperature, 21° C.

best-fitting (by non-linear least squares analysis) double exponential decay curve; its equation is

$$\frac{1}{f} = 0.3889 + 0.9312e^{-0.37137T} + 0.5140e^{-0.031947T} \quad (2)$$

The curve is very similar to curves we had obtained with the laborious measurements made with an Ostwald type (Cannon-Fenske) viscometer (Ref. 1, Figures 2 and 3).

In the above experiment, in which the sample was incubated for 70 minutes, the reservoir containing the enzyme and substrate would have run dry long before the end of the experiment. To circumvent that, the solution in the effluent collection vessel was continuously pumped back into the reservoir with a peristaltic pump. Incubation could therefore continue indefinitely, and the hydrostatic pressure remained nearly constant.

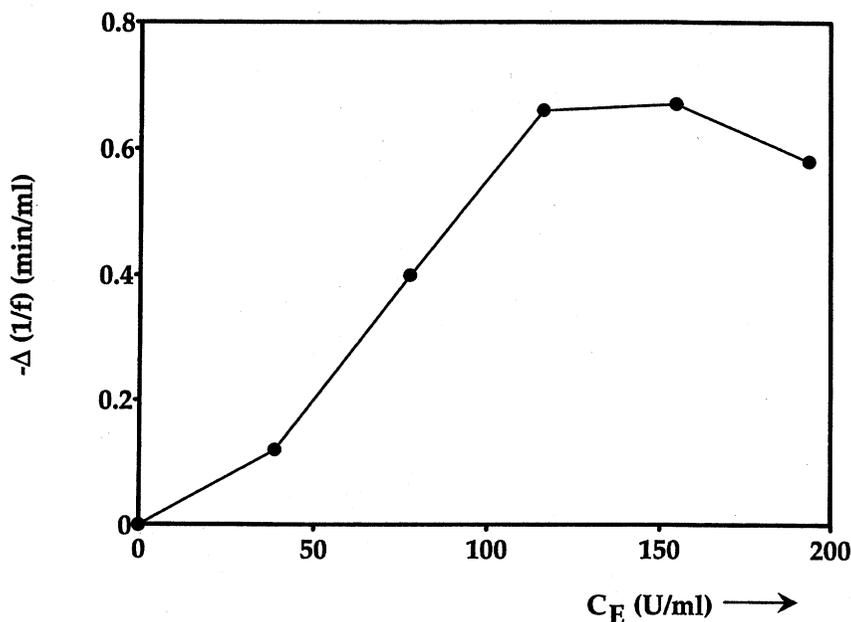


FIGURE 6. — The decrease in “viscosity” ($1/f$) of a solution of collagen after 2 minutes digestion with various concentrations of collagenase. Conditions of incubation: as for Figure 5, except that the collagenase was Sigma type IA, with a specific activity of 550 units/mg. The enzyme concentration varied (in 5 equal steps) from 0 to 194 units/ml.

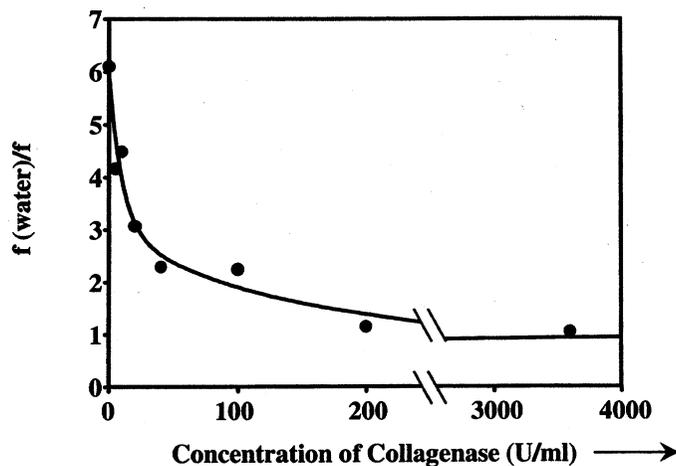


FIGURE 7. — The dependence of “viscosity” [$f(\text{water})/f$] on enzyme concentration. Conditions of incubation: collagen, 2.4 g/l; Tris, 48. mM; CaCl_2 , 4.8 mM; citrate (sodium), 27. mM; benzoate (sodium), 3.1 mM; collagenase, Sigma type IA, 0 to 200 units/ml; temperature, 22° C; time (except for the last sample), 20 minutes. The last sample had an enzyme concentration (c) of 200 units/ml and was incubated for 6 hours; this is equivalent to a 20 minute incubation of a sample with $c = 3600$ units/ml, as plotted (last point). For the equation of the curve fitted to the data, see text.

Equation 2 is a special case of the more general equation

$$\eta = \eta_0 + \eta_1 e^{-k_1 c T} + \eta_2 e^{-k_2 c T} \quad (3)$$

where η is the viscosity of the collagen-collagenase solution at any time T after mixing enzyme and substrate, η_0 is the viscosity after all of the collagen has been hydrolyzed, and c is the concentration of collagenase. The second and third terms on the right side of equation 3 are the contributions to the viscosity, over and above η_0 , of

those components of the collagen which undergo rapid and slow hydrolysis by collagenase. Initially ($T = 0$) these contributions are η_1 and η_2 , respectively. The rate constants for the hydrolyses are k_1 and k_2 , respectively.

It is easily shown that the initial slope of a plot of η (or $1/f$) vs T is proportional to the enzyme concentration (c). This is true in spite of the fact that the decay of η is a double exponential, not a single exponential, function of time. The use of initial slope values makes it unnecessary to resolve

the data into two decay curves (+ a constant term). The initial slope can be estimated from data collected early in the incubation. When this was done for various collagenase concentrations, using an incubation time of 2 minutes, the results shown in Figure 6 were obtained. This indicates that, under favorable circumstances, collagenase concentration can be estimated from early flowrate measurements.

From equation 3 it is apparent that the dependence of the viscosity (η or $1/f$) on enzyme concentration (c), at a given time, is of exactly the same form as its dependence on time (T), at a given enzyme concentration; c and T appear only as the product cT . Knowledge of η vs c is what is needed for an assay of collagenase activity. To obtain the data plotted in Figure 7, the flowrate (at $h = h_o = 35$ cm) was measured after 20 minutes incubation of each of a number of samples containing different concentrations of collagenase. f (water)/ f , the ratio of the flowrate of water to the flowrate of the collagen-collagenase solution, is used here instead of $1/f$ as a measure of the viscosity (η) of the sample. The best fitting curve (shown) has the parameters (eq. 3) $\eta_o = 0.937$, $\eta_1 = 2.94$, $k_1 = 5.0 \times 10^{-3}$, $\eta_2 = 2.07$, $k_2 = 3.80 \times 10^{-4}$.

CONCLUSION

Design parameters for a flowmeter-based system for capillary viscometric assay of collagenase activity are presented (Figure 1 and related text). The advantages of the system are: (1) simplicity, (2) assuming the availability of a suitable flowmeter and a computer for data collection, the system is inexpensive, and (3) measurements and data collection are automatic.

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