

VISCOMETRIC ASSAY OF COLLAGENASE ACTIVITY USING A STOP REAGENT TO TERMINATE ENZYME ACTION

ABSTRACT

A simple, precise viscometric assay procedure has been developed for the assay of true collagenase activity. The substrate is a commercially available pepsin-treated mammalian skin collagen. It comes as a solution which needs no treatment other than pH adjustment and dilution prior to its incorporation into the assay medium. Viscosity is measured after enzymatic action is terminated with a Stop Reagent. Any one of a wide variety of viscometers can be used, including a simple Ostwald-type viscometer. Other advantages of the procedure are also discussed. The temperature during incubation and viscometry should be controlled within $\pm 0.1^\circ\text{C}$. Such control is easily achieved with an inexpensive controller.

INTRODUCTION

Some 40 years ago, Gallop, Seifter, et al.^{1,2} developed a viscometric procedure for assay of collagenase activity. The substrate was a collagen preparation (ichthyocol) made from carp swim bladder. The procedure appears not to have been widely used, perhaps because preparation of the substrate and the assay itself are labor intensive. Simpler "collagenase" assay procedures utilizing synthetic peptides were also developed.^{3,6} However, while useful for screening purposes and during enzyme purification, these procedures cannot be relied upon to yield a measure of true collagenase activity.^{7,8}

In a previous publication from this laboratory,⁹ we demonstrated the suitability and advantages of using a commercially available pepsin-treated collagen from the skin of young pigs as substrate for the assay of true collagenase activity, and we subsequently reported¹⁰ on the design of a

capillary viscometer for monitoring the hydrolysis of the above substrate. We have more recently developed and herewith report a simple, precise assay procedure utilizing the pepsin-treated collagen in which the viscosity is measured after stopping the enzymatic reaction with a stop reagent. The advantages of this approach will be discussed.

MATERIALS AND METHODS

Incubation of enzyme with substrate was carried out in an Orbit Shaker Bath, model 3540, manufactured by LabLine Instruments, Inc., Melrose Park, IL. To achieve adequate control of temperature, the original temperature control unit was replaced with a digital RTD Temperature Controller and a 100 ohm RTD platinum probe purchased, respectively, from Cole-Parmer Instrument Co., Niles, IL, and Omega Engineering Co., Stamford, CT. Samples with a volume of ca. 20 ml were contained in 25 x 150 mm screw-capped test tubes. These were placed in plastic test-tube racks having holes 3 cm in diameter; the racks were fastened to the rotating platform of the Orbit Shaker-Bath and the platform was set to rotate at 100 rpm. When a volume of ca. 250 ml was to be incubated, it was contained in an Erlenmeyer flask, which was immobilized on top of the platform. Viscosity measurements were made with a size 200 Cannon-Fenske Routine Viscometer purchased from Cannon Instrument Co., State College, PA. An 8.0 ml aliquot of the sample was introduced into the viscometer. The temperature was controlled ($\pm 0.03^\circ\text{C}$) by a Cannon model M-1 Constant Temperature Bath.

The buffer was 50 mM in Tris, pH 7.00 ± 0.05 , and 5.0 mM in CaCl_2 . Pepsin-treated porcine collagen, produced by Pentapharm Ltd., Basel, Switzerland, was purchased from Centerchem, Inc., Stamford, CT, as Natural Soluble Collagen (Dermacol), a solution with the following composition: $1.0 \pm 0.1\%$ collagen, 3.3% sodium citrate, 0.19%

sodium benzoate, pH 3.6. The stock solution was frozen in 100 ml plastic bottles. After thawing, an equal volume of double-strength buffer was added, and the pH was adjusted to 7.0 ± 0.05 by the slow addition of NaOH while stirring. The resulting solution was diluted with an equal volume of buffer, giving a nominal collagen concentration of 2.5 g/l. This is referred to below as the Substrate Solution. Collagenase from *Clostridium histolyticum* was purchased from Sigma Chemical Corp., St. Louis, MO, as the "crude" type IA product, with a specific activity of 550 units/mg (vs collagen). The enzyme was dissolved in the buffer to yield a Collagenase Solution of the desired concentration.

The Assay Procedure

After pre-equilibration of the Collagenase Solution and the Substrate Solution to the desired incubation temperature (usually $22.0 \pm 0.1^\circ\text{C}$), 19 volumes of the latter were added to 1 volume of the former. After incubation for the desired time (usually 20.00 min), enzyme action was stopped by adding 1.30 ml 1.00 M glycine • HCl (the Stop Reagent)/20.0 ml reaction mixture. The viscosity of the resulting solution was measured as described above.

RESULTS AND DISCUSSION

Extensive measurements have been made of the viscosity of collagen solutions at pH 3.4.¹¹ Since many enzymes are virtually inactive at a pH so far below neutrality, the ability to measure the viscosity of collagen solutions at this pH suggested to us that we could (1) terminate the hydrolysis of collagen at neutral pH by lowering the pH to 3.4, and then (2) determine the extent of the hydrolysis which has taken place by measuring the viscosity of the resulting solution. However, in an early experiment, a heavy precipitate developed when we lowered the pH. The incubation medium we were using at that time contained 0.25 M Tris, 0.12 M citrate, and 10 mM CaCl_2 .⁹ The high Tris and citrate concentrations were suspected as possible causes of the precipitation.¹² When the incubation buffer was changed to 50 mM Tris, pH 7.0, 5.0 mM CaCl_2 , the solution remained perfectly clear after the reduction of the pH. The levels of citrate and benzoate introduced by the Substrate Solution did not give rise to precipitates.

To determine the time course of the hydrolysis, the outlined assay procedure (Materials and Methods) was followed, with an initial reaction mixture of 250 ml, a collagenase concentration during incubation of 50.6 U/ml, and an incubation temperature of $22.0 \pm 0.1^\circ\text{C}$. At times $T = 0, 1, 2, 4, 10, 20$ and 40 min a 20.0 ml aliquot was withdrawn

from the reaction mixture and added to 1.30 ml Stop Reagent. The viscosity (outflow time) was measured as described (Materials and Methods) at $22.0 \pm 0.03^\circ\text{C}$. A curve with the equation:

$$\eta = \eta_{\text{inf}} + \eta_1 \cdot \exp(-k_1 \cdot c \cdot T) + \eta_2 \cdot \exp(-k_2 \cdot c \cdot T) \quad (1)$$

was fitted to the data by non-linear least-squares analysis, using Axum software (MathSoft, Inc., Cambridge, MA). In equation 1, η is the measured outflow time ("viscosity") from the viscometer; η_1 , η_2 , k_1 , k_2 , and η_{inf} are parameters; c is the concentration of collagenase; and T is the time of incubation. The resulting curve is shown, together with the data in Figure 1a. The parameters of the fitted curve are

$$\eta_{\text{inf}} = 0.193, \eta_1 = 1.163, k_1 = 6.61 \times 10^{-4}, \\ \eta_2 = 0.871, \text{ and } k_2 = 1.543 \times 10^{-2}.$$

The asymptote η_{inf} is indicated, in Figure 1a, by the dotted line. The standard deviation of the data from the fitted curve is 0.040 min. Equation 1 thus described the data well, in agreement with van Hippel et al.¹³ and Mihalyi (ref. 14, p. 138). The three components of the fitted curve of Figure 1a, corresponding to the three terms on the right side of equation 1, are shown in Figure 1b, viz., the asymptote (η_{inf}), a slow exponential decay [$\eta_1 \cdot \exp(-k_1 \cdot c \cdot T)$], and a fast exponential decay [$\eta_2 \cdot \exp(-k_2 \cdot c \cdot T)$].

The success of the proposed assay procedure (Materials and Methods) depends on the relationship between the measured viscosity (outflow time), and the concentration (c) of collagenase during the incubation. To test this relationship the assay procedure was run with $c = 0$ to 200. U/ml. Incubation was at $22.0 \pm 0.1^\circ\text{C}$ for 20.0 min, and viscosity was measured at $22.0 \pm 0.03^\circ\text{C}$. Curve fitting was done as described above for the experiment of Figure 1. The results are shown in Figure 2a. The standard deviation was 0.016 min, i.e., 0.8% of the initial viscosity.

To facilitate comparison of runs made at different times, the data and fitted curve for each run are normalized with the equation

$$\eta_n = \frac{\eta - \eta_{\text{inf}}}{\eta_0 - \eta_{\text{inf}}} \quad (2)$$

where η = the measured outflow time for any sample, η_0 = the outflow time (from the fitted curve) for $c = 0$, and η_n = the normalized viscosity.

The normalized data and curve for Figure 2a are shown in Figure 2b.

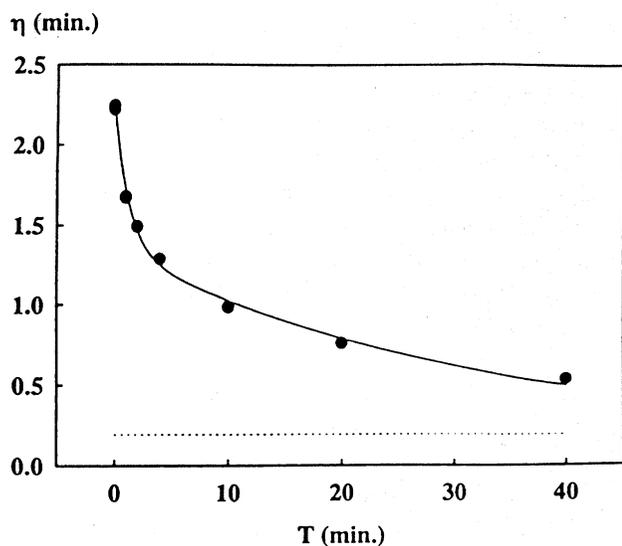


FIGURE 1a. — Time course of the hydrolysis of the substrate, a pepsin-treated mammalian skin collagen, by Clostridial collagenase, using the proposed assay procedure described in the Materials and Methods section. η (min) is the measured outflow time (in minutes) from the viscometer. The data are shown as black circles (\bullet). The curve is the best fitting double exponential decay curve. the dotted line is the asymptote approached by the curve as $T \rightarrow \infty$. The enzyme concentration was 50.6 U/ml (vs. collagen), the temperature $22.0 \pm 0.1^\circ\text{C}$.

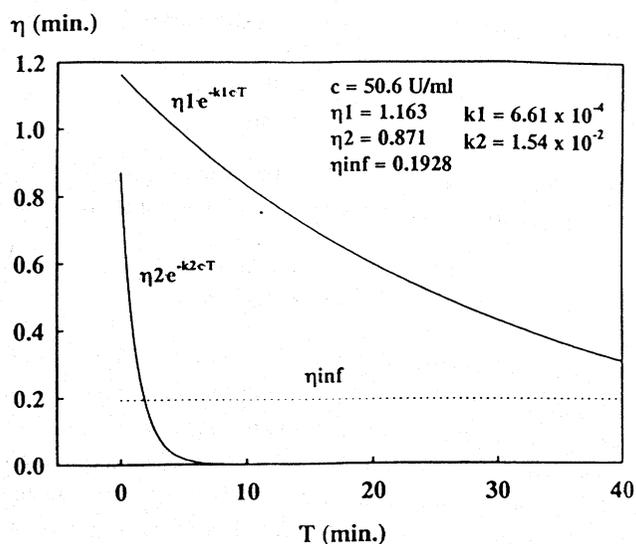


FIGURE 1b. — The three components of the fitted curve of Figure 1a. η_{inf} , the asymptote; $\eta_1 \cdot \exp(-k_1 \cdot c \cdot T)$, the slow exponential decay; $\eta_2 \cdot \exp(-k_2 \cdot c \cdot T)$, the fast exponential decay. The parameters of the components are shown.

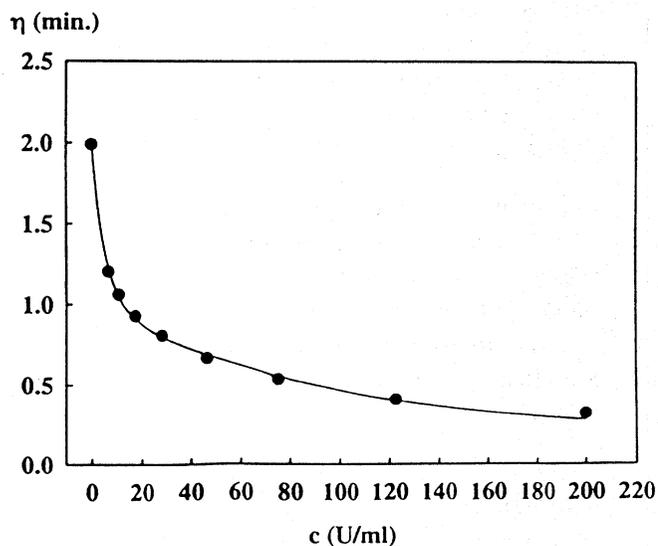


FIGURE 2a. — The dependence of the measured outflow time (η , minutes) on the concentration of collagenase. Incubation was at $22.0 \pm 0.1^\circ\text{C}$ for 20.0 minutes.

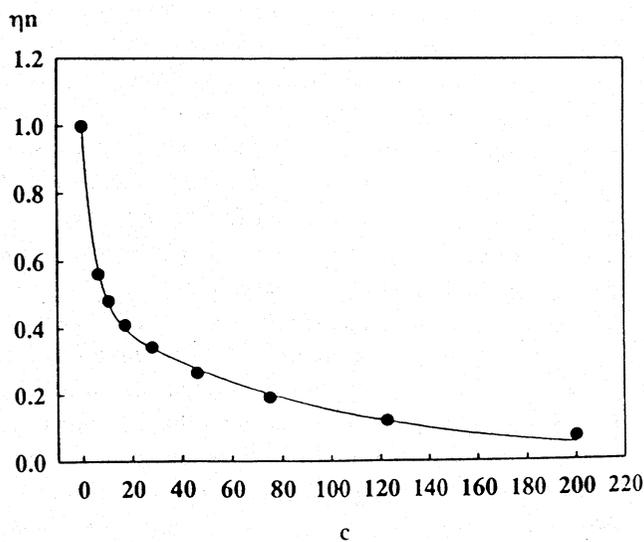


FIGURE 2b. — The dependence of the normalized viscosity η_n on the concentration of collagenase. The data are those of Figure 2a, normalized. For the curve fitted to normalized data, $\eta_{inf} = 0$; $\eta_n = 1$ at $c = 0$; the exponential decay constants (k_{1n} and k_{2n}) are the same as those of Figure 2a (k_1 and k_2); and the sum of the coefficients of the exponential terms is 1, $\eta_{1n} + \eta_{2n} = 1$.

For our purposes, specific viscosity can be defined as

$$\eta_{sp} = \frac{\eta - \eta_{inf}}{\eta_{inf}}$$

(Cf. Ref. 15, p. 700). The normalized viscosity therefore differs from the specific viscosity by a constant factor:

$$\eta_n = \left(\frac{\eta_{inf}}{\eta_0 - \eta_{inf}} \right) \eta_{sp}$$

η_{inf} is the viscosity (outflow time) of the solution (+Stop Reagent) after hydrolysis is complete ($T = \infty$). The solvent in our assay is the solution (+Stop Reagent) without collagen or its hydrolysis products. It is noted that, in equation 2 and in the definition of η_{sp} , we have used η_{inf} instead of the viscosity of solvent (Cf. ref. 15), because the contribution of the products of collagen hydrolysis to the viscosity of the solution cannot be assumed to be negligible.

To test the performance of the proposed assay procedure in the measurement of small concentrations of collagenase, the experiment described in connection with Figures 2a and 2b was repeated, except that the enzyme concentrations were reduced by a factor of 10. The normalized results are shown in Figure 3. The standard deviation was 0.7% of full scale (1). The sensitivity of the procedure is approximately 1 unit of collagenase per ml.

An important question concerning the proposed assay procedure is: how good does temperature control have to be? Temperature affects the measured viscosity in two ways: (1) indirectly, via its effect on the activity of the enzyme, and (2) directly, by its effect on the viscosity of the solution measured. Dealing first with the indirect effect: Figure 4a shows the relationship obtained for the dependence of the normalized viscosity (η_n) on the collagenase concentration during incubation at three temperatures, 20.0, 22.0, and 24.0°C. The viscosity measurements were all made at 22.00 ± 0.03°C. Let it now be assumed that an unknown yielded $\eta_n = 0.456$, as indicated by the dotted line segment 1 in Figure 4a. Let it further be assumed that the experimenter mistakenly thinks that the incubation temperature was 22.0, when it was, in reality, 24.0°C. From the 22° curve the experimenter concludes (vertical line segment 3) that the collagenase concentration during the incubation (c) was 25.00 U/ml. The 24° curve indicates that actually $c = 14.76$ U/ml (line segment 2). The 2° error in temperature therefore gave rise to an error of almost 70% [(25-14.76)/14.76] in the collagenase concentration of the unknown, or 35%/°C.

In the above analysis, it was assumed that $\eta_n = 0.456$. The error analysis can be generalized as follows: For the

normalized viscosity, η_{inf} (the asymptote) in equation 1 becomes 0 and $\eta_n = \eta_1 n \cdot \exp(-k_1 \cdot c \cdot T) + \eta_2 n \cdot \exp(-k_2 \cdot c \cdot T)$, considering η_n to be the independent variable, for any given value η_n , the 22° curve (in Figure 4a) yields a value c as the corresponding (dependent) collagenase concentration and the 24° curve yields a value c' as the corresponding (dependent) collagenase concentration. η_n ranges from 1, for which $c = c' = 0$, to 0 for which $c = c' = \infty$. (For $\eta_n = 0.456$, $c = 25.00$ and $c' = 14.76$). The above equation for η_n can be written as

$$f(\eta_n, c) = \eta_1 n \cdot \exp(-k_1 \cdot c \cdot T) + \eta_2 n \cdot \exp(-k_2 \cdot c \cdot T) - \eta_n = 0 \quad (3a)$$

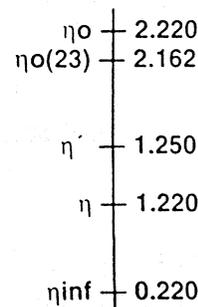
$$\text{and } f(\eta_n, c') = \eta_1 n \cdot \exp(-k_1 \cdot c' \cdot T) + \eta_2 n \cdot \exp(-k_2 \cdot c' \cdot T) - \eta_n = 0 \quad (3b)$$

For a given value of η_n , the corresponding values c and c' are the roots of $f(\eta_n, c)$ and $f(\eta_n, c')$. Knowing c and c' , the relative error (re) for the given η_n can be calculated: $re = (c - c')/c'$.

The above calculations were made for 46 values of η_n in the range of $0.1 \leq \eta_n \leq 1$, using Mathcad software (Mathsoft Inc., Cambridge, MA). The results were plotted using Axum, and are shown in Figure 4b. The relative error varies between 0.27 (27%, at $\eta_n = 1$) and 0.70 (70%, at $\eta_n = 0.46$). Since this was for an error in temperature of 2°C, the relative error in the calculated concentration is thus 35%/°C. To ensure results with an error of <5% (absolute value), temperature control during incubation should therefore be within 0.1°C. Fortunately, this level of control is not difficult to achieve (See Materials and Methods).

The direct effect of temperature on the viscosity observed is shown in Figure 5a for an "enzyme blank," i.e., an assay solution containing substrate but no enzyme [such as the sample in Figure 2a for which $c = 0$ (and $\eta = 2$)]. As always, viscosity (outflow time) was measured after addition of the Stop Reagent. The slope of the line in Fig. 5a is -5.80×10^{-2} min/°C.

To illustrate the effect of an error in temperature control during the measurement of viscosity on the calculated concentration of collagenase, let it be assumed that the temperature control is set at 22.00°C and that it functions properly during standardization, yielding values $\eta_0 = 2.22$ (min) for the enzyme blank and $\eta_{inf} = 0.22$ for a totally hydrolyzed sample. Let it be



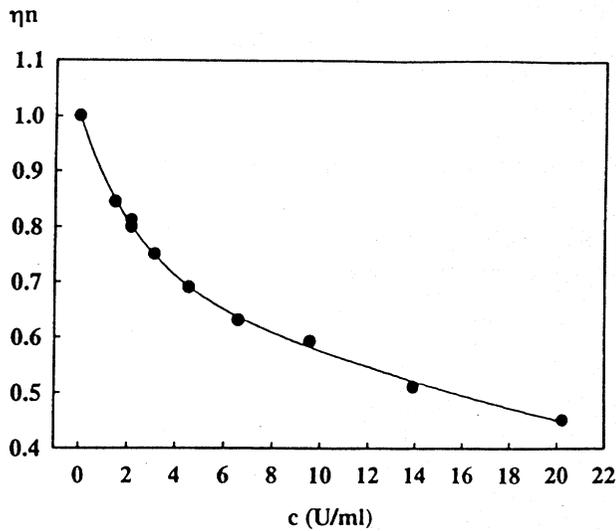


FIGURE 3. — The normalized viscosity as a function of collagenase concentration for low values of the latter. An enzyme concentration of 1 U/ml (vs. collagen) is detectable.

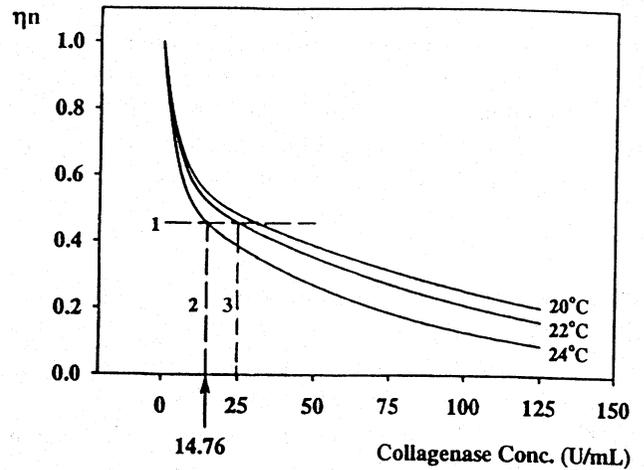


FIGURE 4a. — The effect of incubation temperature on the normalized viscosity. All viscosity (outflow time) measurements were made at $22.00 \pm 0.03^\circ\text{C}$. For line segment 1, $\eta_n = 0.456$. That normalized viscosity corresponds to an enzyme concentration of 14.76 U/ml at an incubation temperature of 24°C (line segment 2) and an enzyme concentration of 25.00 U/ml at 22°C (line segment 3).

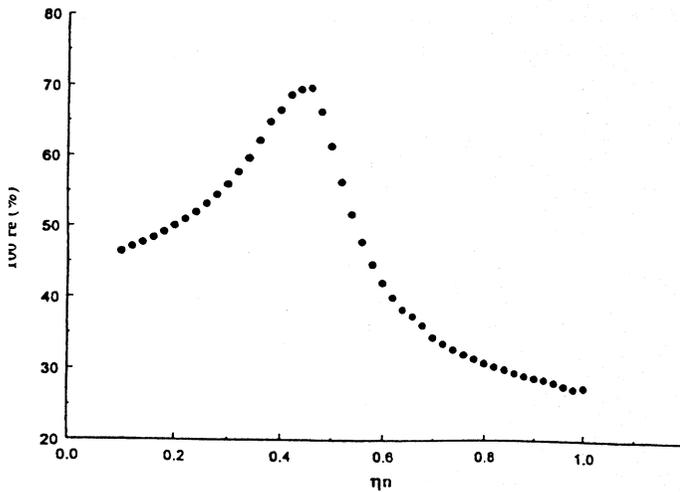


FIGURE 4b. — The relative error (in percent) for a 2°C error in incubation temperature at various values of the normalized viscosity.

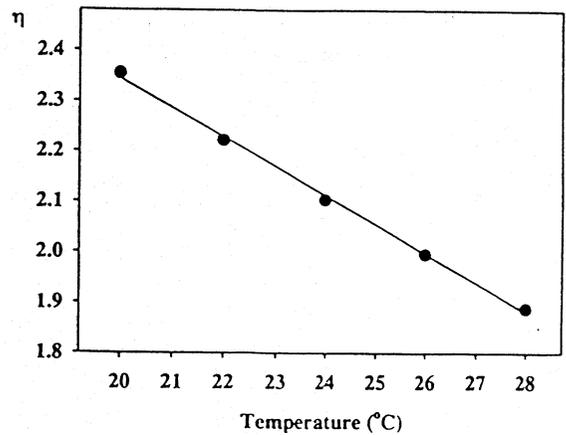


FIGURE 5a. — The dependence of outflow time on the temperature of the solution being measured. The solution was an "enzyme blank," i.e., it contained the usual concentration of substrate (and all other assay components), but no enzyme.

assumed further that the subsequent measurement of the viscosity for an unknown sample yielded a value of 1.22, but that, due to a malfunction of the control unit (of which the investigator was unaware), the temperature during the last measurement was actually 23.00°C. From the value of η_0 and the slope of the line in Figure 5a we infer that, if the enzyme blank had been measured at 23°, a value

$$\eta_0(23) = 2.220 - 0.058 = 2.162$$

would have been obtained. Now, the normalized scale is defined by $\eta_0 = 2.220 \rightarrow \eta_{0n} = 1$ and $\eta_{inf} = 0.220 \rightarrow \eta_{inf n} = 0$. On normalization of the remaining values we obtain $\eta_0(23) = 2.162 \rightarrow \eta_0(23)_n = 0.9710$, and $\eta = 1.220 \rightarrow \eta_n = 0.500$. The outflow times (in minutes) are summarized in the adjacent illustration. Assuming that $\eta_n/\eta_n' = 0.9710$ for any sample, we can conclude that, if the temperature control unit had not malfunctioned, the investigator would have obtained a normalized viscosity for the unknown of

$$\eta_n' = \eta_n / 0.9710 = 0.515$$

Referring to equation 3a, we can write

$$f(c) = \eta_1 n \cdot \exp(-k_1 \cdot c \cdot T) + \eta_2 n \cdot \exp(-k_2 \cdot c \cdot T) - 0.500 = 0 \quad (4a)$$

$$\text{and } f(c') = \eta_1 n \cdot \exp(-k_1 \cdot c' \cdot T) + \eta_2 n \cdot \exp(-k_2 \cdot c' \cdot T) - 0.515 = 0 \quad (4b)$$

Using the known values of the parameters at 22°C ($\eta_1 n = 0.4132$, $k_1 = 0.262$; $\eta_2 n = 0.5868$, $k_2 = 0.012$), the root of equation 4a is $c = 17.454$, and that of equation 4b is $c' = 15.526$. The relative error is therefore $re = (c - c')/c' = 0.124$ (12.4%).

This analysis can be generalized to any observable value of η (at 23°C) as follows [still assuming the same values of η_{inf} and η_0 (at 22°C) and the validity of Figure 5a]: Instead of equations 4a and 4b, we then have

$$f(\eta_n, c) = \eta_1 n \cdot \exp(-k_1 \cdot c \cdot T) + \eta_2 n \cdot \exp(-k_2 \cdot c \cdot T) - \eta_n = 0 \quad (5a)$$

$$\text{and } f(\eta_n', c') = \eta_1 n \cdot \exp(-k_1 \cdot c' \cdot T) + \eta_2 n \cdot \exp(-k_2 \cdot c' \cdot T) - \eta_n' = 0 \quad (5b)$$

where $\eta_n = 0.9710 \cdot \eta_n'$.

Each value of η_n' yields a root c' , the correct calculated concentration, and each corresponding value η_n yields a root c , the erroneous calculated concentration. The difference $\Delta c = c - c'$ is plotted against η_n' in Figure 5b (○). The relative error re (□) is also plotted against η_n' . It should be noted that for $\eta_n' = 0$, $c = \infty$, and for $\eta_n' = 1$, $c = 0$. Remembering that the analysis was done assuming a temperature error of 1°C, we can conclude that, except at a very low enzyme concentrations, temperature control to within 0.1°C is adequate to yield concentration values with

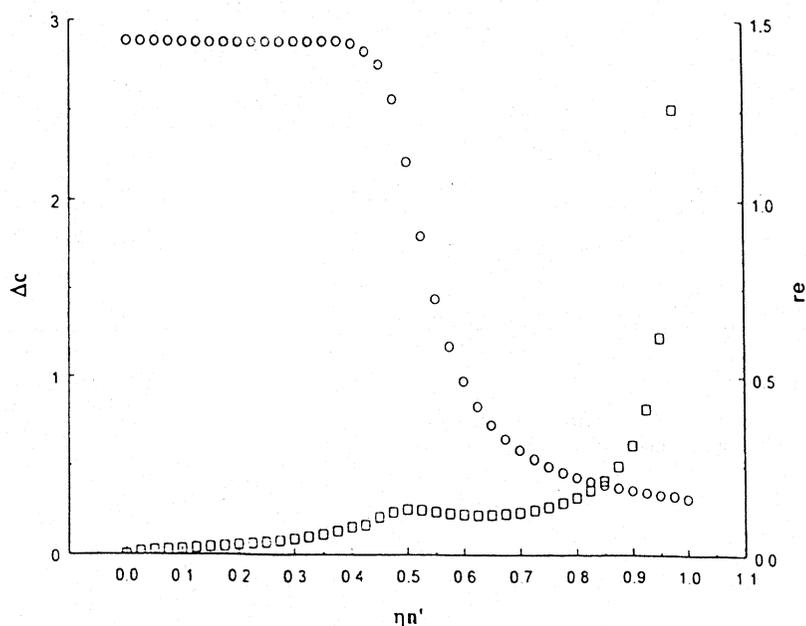


FIGURE 5b. — The effect of a 1°C error in temperature during the measurement of viscosity on the collagenase concentration (c) inferred from the measurement. Δc (○) is the resulting error in collagenase concentration, re (□) is the relative error. These are plotted as functions of the correct normalized viscosity η_n' . Note that $\eta_n' = 0$ corresponds to an enzyme concentration of ∞ , and $\eta_n' = 1$ to the absence of enzyme.

an error <5%. Viscosity baths available commercially provide control with a precision of ca. 0.03°C.

In the procedure of Gallop, Seifter, et al.,^{1,2} outflow time was measured periodically during the incubation of the substrate with the collagenase, i.e., while the viscosity of the reaction mixture was decreasing as a result of the hydrolysis of the collagen. [The incubation time (the time elapsed since the mixing of enzyme and substrate) for a given outflow time from the viscometer was taken to be the midpoint between the incubation times at the beginning and end of the outflow.] In the procedure of Mozersky et al.,¹⁰ flow rates, whose reciprocal is a measure of viscosity, were also measured while the reaction between enzyme and substrate was proceeding. By uncoupling the incubation of enzyme with substrate from the viscosity measurement, the procedure described in the present paper achieves the following advantages: (1) the precision of the measurements of both incubation time and viscosity are improved, (2) the incubation time can be as short as desired, (3) numerous samples can be incubated simultaneously (with time-staggering), (4) any one of a wide variety of instruments can be used for the viscosity measurement, from the very sophisticated to the simplest, (5) the viscosity measurements can be made at any convenient time, and (6) the viscosity measurement on any sample can be repeated, if necessary.

CONCLUSION

A viscometric procedure for the assay of true collagenase activity is presented (Materials and Methods) in which the viscosity is measured after termination of the enzymatic reaction with a Stop Reagent. The substantial advantages of the procedure are documented. They include excellent precision and simplicity of operation.

ACKNOWLEDGMENT

The authors are pleased to acknowledge the outstanding technical assistance of Mr. Benjamin Louis Martin in this investigation.

REFERENCES

1. Gallop, P. M., Seifter, S., and Meilman, E.; Studies on Collagen I. The Partial Purification, Assay, and Mode of Activation of Bacterial Collagenase. *J. Biol. Chem.* **227**, 891-906, 1957.
2. Seifter, S., and Gallop, P. M.; Collagenase from *Clostridium histolyticum*. In *Methods in Enzymology* (Colowick, S. P., and Kaplan, N. O. eds.) Academic Press, New York, 5, pp. 659-665, 1962.
3. Grassmann, W., and Nordwig, A.; Quantitativer Kolorimetrischer Test auf Kollagenase, *Z. Physiol. Chem.* **322**, 267-272, 1960.
4. Nagai, Y., Sakakibara, S., Noda, H., and Akabori, S.; Hydrolysis of Synthetic Peptides by Collagenase. *Biochim. Biophys. Acta.* **37**, 567-569, 1960.
5. Wünsch, E., and Heidrich, H. -G. Zur quantitativen Bestimmung der Kollagenase. *Z. Physiol. Chem.* **333**, 149-151, 1963.
6. Van Wart, H. E., and Steinbrink, D. R.; A Continuous Spectrophotometric Assay for *Clostridium histolyticum* Collagenase. *Anal. Biochem.* **113**, 356-365, 1981.
7. Harper, E., and Gross, J.; Separation of Collagenase and Peptidase Activities of Tadpole Tissue in Culture. *Biochim. Biophys. Acta.* **198**, 286-292, 1970.
8. Heidrich, H. G., Prokopová, D., and Hannig, K.; The Use of Synthetic Substrates for the Determination of Mammalian Collagenases. *Z. Physiol. Chem.* **350**, 1430-1436, 1969.
9. Mozersky, S. M., and Bailey, D. G.; Measurement of Collagenase Activity in the Tannery and in the Laboratory. I. The Assay Medium. *JALCA* **90**, 357-364, 1995.
10. Mozersky, S. M., and Bailey, D. G.; 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. *JALCA* **91**, 263-269, 1996.
11. Kahn, L. D., and Witnauer, L. P.; The Viscometric Behavior of Solubilized Calf Skin Collagen at Low Rates of Shear, *J. Biol. Chem.* **241**, 1784-1789, 1966.
12. Courts, A.; Effect of Citrate and of pH on the Structure of Collagen and Gelatin. *J. Soc. Leather Trades' Chem.* **47**, 213-220, 1963.
13. Von Hippel, P. H., Gallop, P. M., Seifter, S., and Cunningham, R. S., An Enzymatic Examination of the Structure of the Collagen Macromolecule. *J. Amer. Chem. Soc.* **82**, 2774-2786, 1960.
14. Mihalyi, E.; "Application of Proteolytic Enzymes to Protein Structure Studies." CRC Press 1972.
15. Swindells, J. F., Ullman, R., and Mark, H.; Determination of Viscosity. In *Physical Methods of Organic Chemistry*, Vol. I, Part I, 3rd edition (Weissberger, A. ed.) Interscience Publishers, New York, pp. 689-726, 1959.

DISCUSSION

Steve Yanak, Buckman Laboratories: Can you briefly explain the difference between Oswald, Cannon Fenske, and Brookfield viscometers.

Oswald is a general term that includes the Cannon-Fenske viscometer that I showed a picture of earlier, the piece of glass capillary apparatus that I have used. There are a whole variety of sophisticated viscometers which include rotary viscometers such as the Brookfield. It doesn't matter which type of viscometer you use but the simplest kind, of course, is based on the glass capillary.

Steve Yanak, Buckman Laboratories: Do you feel that it is safe in the packing house where you want to analyze some hides for collagenase activity? Did your method describe an extraction process or solution preparation where you could do it right there?

I have that worked out but I didn't describe anything in the paper. I would be happy to supply that information if you want it.

Ann Stanley, BLC: I also would be interested in that information. I have a question related to the halophiles when you are actually testing those. Do you make a preparation of the halophiles from bacteria or do you extract the enzyme? I didn't catch that part of the presentation.

We are not sure that they are producing them because we have not been able to pick it up yet, but these enzymes are producing collagenases presumably into the medium. So that what we do is spin down the bacteria at the end of the culturing operation and work with the supernatant.

Ann Stanley, BLC: You work with the medium that the bacteria were grown in?

Yes.

Bill Marmer, Eastern Regional Research Center: Just to reiterate. Sam is really showing you a very simple technique that could be applied in the packing house or in the tannery and we are very willing to work with anybody who wants to try this technique. We invite collaboration of that sort. We already have some. We invite others to participate too.