THE PRECIPITATION OF GELATIN BY ETHANOL, AND ITS USE IN THE ESTIMATION OF PROTEOLYTIC ACTIVITY

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Summary

Within certain limits of gelatin concentration, pH, temperature, salt concentration, and ethanol concentration, the percentage light transmitted through precipitated gelatin solutions is an exponential function of the gelatin concentration. It is concluded that the mean micelle size of the precipitated gelatin is independent of the gelatin concentration within these prescribed limits.

Two methods for estimating proteolytic activity have been based on the precipitation of gelatin by ethanol. In the first method buffered solutions of gelatin, digested by the protease under standard conditions, are precipitated by an ethanol-buffer mixture, the decrease in turbidity as the result of proteolysis providing a measure of its activity. The relationship between the logarithm of the percentage transmitted light and the enzyme concentration approximated to a straight line over a considerable range of enzyme concentrations. The second method depends on the gravimetric determination of the amount of isoelectric gelatin rendered soluble in 80 per cent. ethanol by the action of the enzyme. Using solutions of protease from the mould *Aspergillus oryzae*, this value was found to be proportional to the amount of enzyme present if dilute solutions of enzyme were employed. The relationship between the amount of gelatin made soluble and the enzyme concentration varied according to the type of protease used.

These methods have been compared with other conventional methods for estimating proteolytic activity and for a number of proteases have been found to provide equal or greater sensitivity.

I. INTRODUCTION

Most samples of gelatin are polydisperse, owing to degradation in the course of their preparation. Thus by fractional precipitation Mosemann and Ligner (1944) obtained preparations having mean molecular weights of 16,000 and 89,000 respectively. It has also been shown by Briefer (1929) that the isoelectric point of gelatin depends on the manner of preparation. However, Jirgensons (1942) found by precipitation titration of degraded gelatin that the molecular weights determined in this way correspond approximately with those determined by cryoscopic methods, while fractionation of gelatin gives products having the same refractive index and isoelectric point (Straup 1931). It would appear, therefore, that the amino acid composition of the small gelatin molecules corresponds fairly closely with that of the larger molecules, although these molecules cover a wide range of molecular weights.

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If, as the data quoted above indicate, the complexity of gelatin is due mainly to variation in molecular weight, rather than to variation in amino acid composition and arrangement, information concerning the precipitation of gelatin with ethanol might provide a basis for following the digestion of gelatin by proteolytic enzymes.

A study has therefore been made of the relationship between gelatin concentration and light transmission under different conditions of pH, salt concentration, temperature, and concentration of the precipitant. Using these results, an optical method has been developed for determining protease activity.

For experiments requiring greater accuracy and reproducibility, it has been found advantageous to estimate the amount of gelatin precipitated gravimetrically, the precipitation with ethanol being carried out at the pH of minimum solubility of the gelatin. The sensitivity of this method has been compared with that of the turbidimetric method and other established methods for estimating protease activity.

II. Methods

(*a*) *Preparation of Gelatin Solutions and Method of Precipitation*

For these experiments a bulk lot of gelatin* was used. Buffered solutions were prepared by boiling a solution of succinic or acetic acid that had previously been partly neutralized with NaOH, and adding gelatin after removal from the burner. On cooling, the pH was adjusted with NaOH, using a glass electrode, the solution was made up to volume, and the pH checked. Dilute solutions were prepared by dilution with buffer of the same concentration and pH.

As it was hoped to use the information obtained for developing methods of protease estimation, rapid precipitation of the gelatin was employed. The precipitant was blown into the gelatin solution from a fast-flowing pipette, the jet of liquid striking the tube just above the surface of the solution, thereby causing rapid mixing.

(*b*) *Turbidimetric Experiments*

Changes in the turbidity of gelatin solutions were followed in an Evelyn type photometer using a green filter, or in a Coleman spectrophotometer at a wavelength of 535 mμ. Precipitation of the buffered gelatin solutions was carried out in 25 by 200 mm. test tubes standing in a constant-temperature water-bath, the precipitant mixtures being maintained at the same temperature as the gelatin. Unless otherwise stated, readings were taken 1 minute after precipitation in order to allow air bubbles to rise to the surface.

(*c*) *Gravimetric Experiments*

An Oertling air-damped balance was used for weighing the centrifuge tubes used in these experiments. The order of accuracy was ± 0.2 mg. Details of procedure are provided in the experimental section.

* Supplied by Davis Gelatine Pty. Ltd., Sydney.

III. Experimental and Results

(*a*) *The Precipitation of Gelatin*

Aliquots (2 ml.) of solutions having differing gelatin concentrations were precipitated by 10 ml. of ethanol at 40°C. and the light transmission measured after 1 minute. The gelatin was at a pH of approximately 5.0 and contained no added buffer. From Figure 1 it is apparent that a straight-line relationship exists between the gelatin concentration and the logarithm of the percentage light transmitted up to concentrations of about 0.4 per cent. gelatin, at which point the slope of the curve changes sharply. As would be expected, the gelatin concentration required for the appearance of measurable turbidity increased with decreasing concentrations of ethanol in the final mixtures.

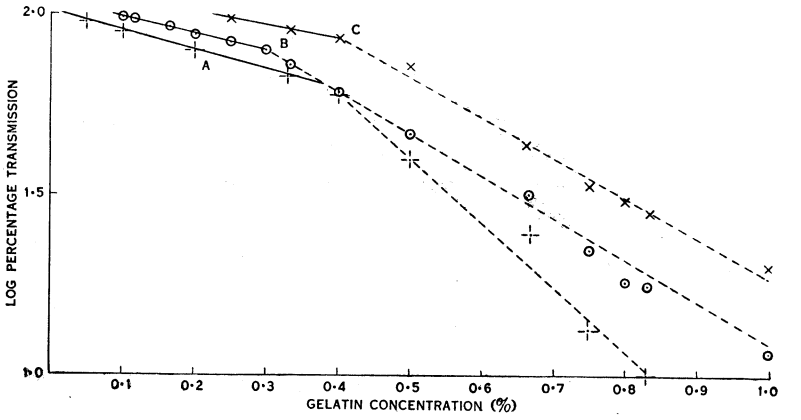

Fig. 1.—Relationship between percentage transmission and gelatin concentration.
A, 2 ml. gelatin solution + 10 ml. ethanol.
B, 5 ml. gelatin solution + 10 ml. ethanol.
C, 6 ml. gelatin solution + 10 ml. ethanol.

The transmission values of precipitated gelatin suspensions were found to change with time (Fig. 2) and, in general at low gelatin concentrations, the transmission values decreased with time whereas at high gelatin concentrations there was a steady increase in transmission. At intermediate concentrations the transmission has been found to decrease to a minimum, then increase steadily.

The effect of pH on the precipitation was followed by precipitating 0.3 and 0.75 per cent. gelatin solutions in 0.1M succinate buffer, with five volumes of ethanol, over a range of pH values. The relationship between pH and log percentage transmission is shown in Figure 3. In each case an atypical pH-precipitation curve was obtained. With 0.3 per cent. gelatin a single minimum value was obtained indicating maximum precipitation at pH 4.9, whereas with 0.75 per cent. gelatin two minima were obtained, at pH 4.3 and 7.5 respectively.

Probably this is related to the fact that at pH values near the isoelectric point a non-linear relationship exists between gelatin concentration and log percentage transmission for high gelatin concentrations.

The range of gelatin concentrations giving a rectilinear relationship between log percentage transmission and gelatin concentration varies considerably with pH, the most limited range being at the pH of minimum solubility. Using a different batch of gelatin, it has been shown that the limiting gelatin concentrations giving this straight-line relationship at pH values of 3.5, 4.0, 4.5, 5.0, 6.0, 7.0, and 8.0 were 1.2, 0.8, 0.5, 0.4, 0.8, 1.0, and 1.2 per cent. respectively. The turbidity produced by a particular sample of gelatin under standard conditions of concentration, temperature, pH, etc. will of course depend on the extent of degradation of the gelatin.

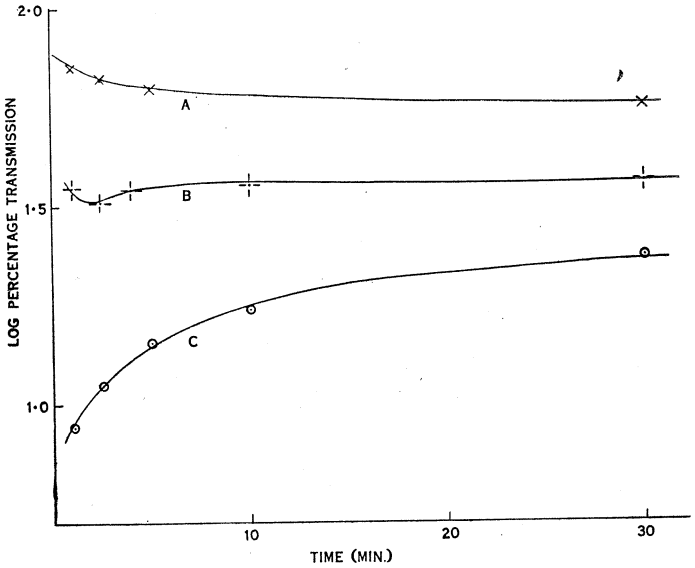

Fig. 2.—Variation in log percentage transmission with time.
A, 2 ml. 0.2 per cent. gelatin + 10 ml. 80 per cent. ethanol.
B, 2 ml. 0.5 per cent. gelatin + 10 ml. 80 per cent. ethanol.
C, 2 ml. 1.0 per cent. gelatin + 10 ml. 80 per cent. ethanol.

Gelatin is known to retain some of its gel structure in solution at temperatures less than about 35°C. The precipitation of a dilution series of gelatin solutions in 0.1M succinate buffer at pH 7.0 was therefore carried out at a number of temperatures, both the gelatin solution and the precipitant being maintained at the experimental temperature. From Figure 4 it is apparent that at 30°C. the range of gelatin values giving a straight-line relationship between concentration and log percentage transmission was less than at 40°C.

The possible influence of uneven local ethanol concentrations was also investigated by precipitating aliquots (2 ml.) of the gelatin solutions with mixtures (10 ml.) of ethanol and 0.1M succinate buffer at pH 7.0, which

contained 80 and 90 per cent. ethanol to give final ethanol concentrations of 66.7 and 75.0 per cent. respectively. Aliquots (5 ml.) of the gelatin solutions

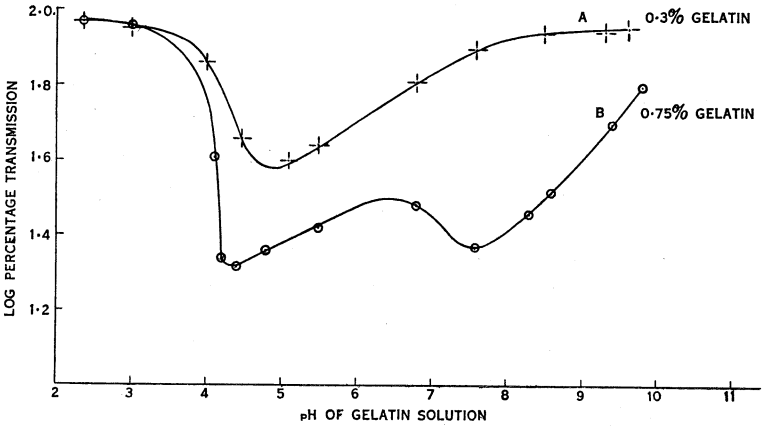

Fig. 3.—Variation in log percentage transmission with pH.
A, 2 ml. aliquots of 0.3 per cent. gelatin precipitated with 10 ml. ethanol.
B, 2 ml. aliquots of 0.75 per cent. gelatin precipitated with 10 ml. ethanol.

were also precipitated with 10 and 15 ml. of undiluted ethanol, to give the same final ethanol concentrations. Since the precipitation of 2 ml. of 2 per cent.

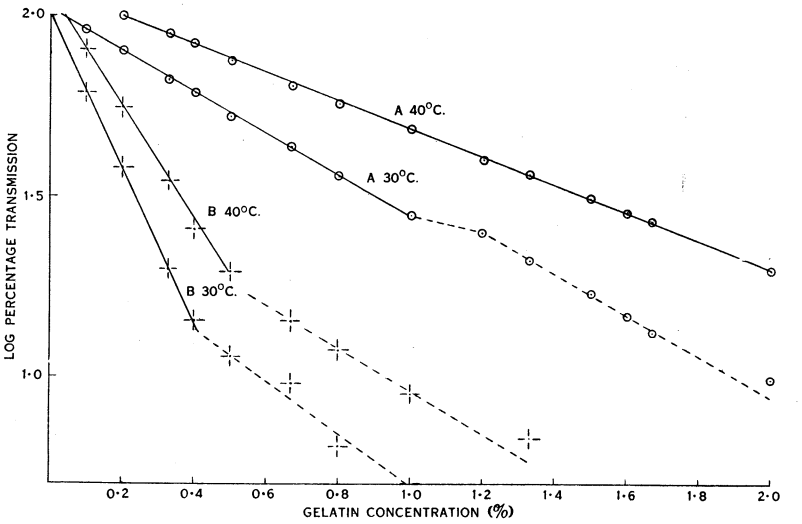

Fig. 4.—Effect of temperature on the precipitation of gelatin with ethanol.
A, 2 ml. gelatin solution + 10 ml. 80 per cent. ethanol at 40 and 30°C.
B, 5 ml. gelatin solution + 15 ml. ethanol at 40 and 30°C.

gelatin by 10 ml. of 80 per cent. ethanol is equivalent to precipitation of 5 ml. of 1 per cent. gelatin by 10 ml. of ethanol, it might be expected that the

slope of curve *C* in Figure 5 would be twice that of curve *D*. The ratio of the slopes of curves *C* and *D* is in fact 1.8. Similarly, instead of the ratio of 1.5, which would be expected for the slopes of curves *A* and *B*, the experimental value is 1.35. There is no evidence that the use of buffer-ethanol mixtures improved the approximation to a rectilinear relationship between gelatin concentration and log percentage transmission, nor was the range of final gelatin concentrations giving this relationship increased. It does, however, permit variation of the working range of transmission values for a particular initial gelatin concentration.

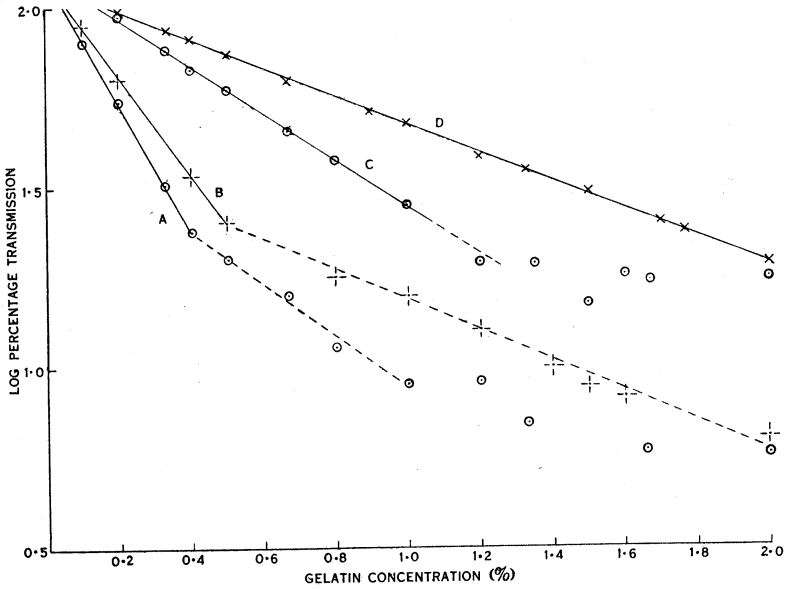

Fig. 5.—Effect of dilution of the precipitant with buffer on the concentration range of the straight-line relationship between log percentage transmission and concentration of gelatin before precipitation.
A, 5 ml. gelatin solution + 15 ml. ethanol.
B, 2 ml. gelatin solution + 10 ml. 90 per cent. ethanol.
C, 5 ml. gelatin solution + 10 ml. ethanol.
D, 2 ml. gelatin solution + 10 ml. 80 per cent. ethanol.

(*b*) *Estimation of Protease Activity Turbidimetrically*

The concentration of gelatin in the buffer substrate for this estimation will depend on the sample of gelatin used, the sensitivity of the photometer used, and the pH of precipitation. In general the gelatin concentration was such that on precipitation under the standard conditions the percentage transmission was between 10 and 20.

(i) *Reagents.*—The buffer substrate consisted of 2 per cent. gelatin (or any other suitable concentration) prepared in 0.1M succinate buffer at pH 7.0 without boiling the gelatin. The precipitant contained four volumes of ethanol

mixed with one volume of 0.1M succinate buffer at pH 7.0. As crystals of succinate appear after standing for long periods at room temperature, the precipitant is prepared immediately before use.

(ii) *Procedure.*—Aliquots (2.0 ml.) of buffer substrate in 25 by 200 mm. test tubes and a stoppered flask of precipitant were allowed to equilibrate in a water-bath at 40°C. The enzyme solution (0.2 ml.) was added to the substrate, which was shaken and incubated for 1 hour at 40°C. Precipitant (10 ml.) was then blown into the tube, the contents of which were transferred to a cuvette and the light transmission measured 1 minute after precipitation. The cuvette was rinsed with water and 80 per cent. ethanol after use, and drained on absorbent paper. When assaying a series of enzyme solutions it is convenient to use several cuvettes matched with the control cuvette. A single blank tube containing succinate buffer (2.2 ml.) and precipitant (10 ml.) may be used where very dilute purified enzyme solutions are being used. Otherwise a separate blank containing succinate buffer (2.0 ml.), enzyme (0.2 ml.), and precipitant (10 ml.) is required for each test.

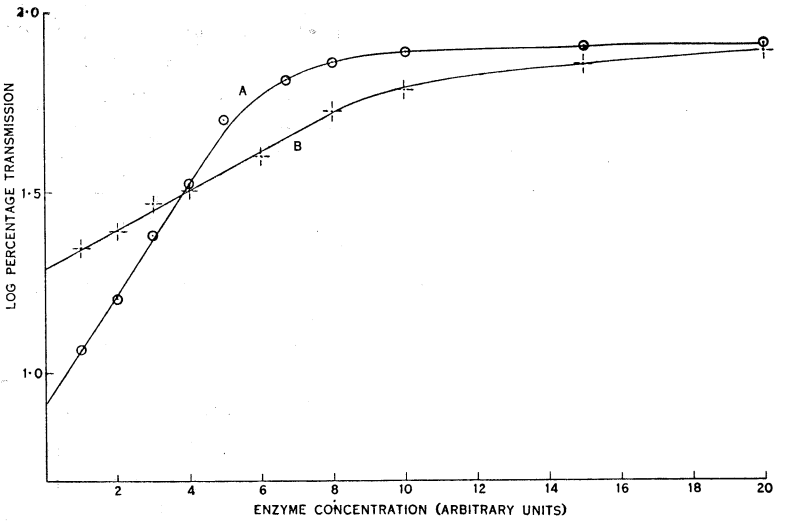

Fig. 6.—The turbidimetric estimation of protease activity. Relation between percentage transmission and concentration of mould protease.
A, 30 seconds after precipitation.
B, 30 minutes after precipitation.

The activity of the protease solution was obtained by comparison of the transmission value with a standard dilution curve for the particular protease being tested. In Figure 6 log percentage transmission is plotted against concentration of protease from *A. oryzae* expressed as arbitrary units. Graph *A* was obtained using a period of 30 seconds between precipitation and reading the turbidity, whereas for graph *B*, a 30-minute period was used. In each case a straight-line relationship was obtained over a considerable range of enzyme

concentrations, but the slope of the lines differed. The curves flatten when no visible precipitate is formed with the addition of ethanol-buffer mixture, the transmission at this stage being less than 100 per cent. This may be due to differences in the density of the test and blank solutions with a consequent difference in light focusing, or it may be due also in part to absorption of light by the gelatin solution.

In some cases it is desirable that the overall time for each estimation be short as, for instance, in the chromatography of enzyme solutions. The incubation time may then be reduced to 1 minute and the incubation temperature increased to 45°C. Under these conditions care must be taken that enzyme solutions are not inactivated prior to estimation and that there is no appreciable evaporation from the substrate solutions.

(*c*) *Estimation of Protease Activity Gravimetrically*

As the turbidimetric technique is not readily applicable to enzyme solutions containing considerable amounts of precipitable material or coloured components, a more general method for protease estimation has been developed.

(i) *Reagents.*—For the buffer substrate 20 g. of gelatin were added with stirring to a hot solution of glacial acetic acid (5 ml.) and 40 per cent. NaOH (10 ml.) in distilled water (900 ml.). The gelatin solution at approximately pH 5.0 was cooled and the pH adjusted with NaOH to the required value. The volume was adjusted to 1 l. and the pH checked. The precipitant was ethanol containing HCl or NaOH according to the pH of the gelatin used. When using a pH of 7.0 the precipitant contained 2.0 ml./l. of 5N HCl.

(ii) *Procedure.*—Aliquots (2 ml.) of buffer substrate in tared 15 ml. centrifuge tubes were equilibrated in a water-bath containing distilled water at the required temperature, and enzyme solution (0.5 ml.) was added. The tubes were shaken and incubated for 1 hour, then cold precipitant (10 ml.) was blown in to give rapid mixing. The tubes were allowed to stand in an ice bath for 15 minutes for completion of flocculation and were then centrifuged at 3000 r.p.m. in an angle centrifuge for 10 minutes. The clear supernatant was discarded, and the tubes inverted on absorbent paper for 5 minutes. They were then dried at 90-100°C. for 4 hours, cooled, and weighed. A control test was performed for each enzyme solution after heating in a boiling water-bath for 10 minutes. The difference between the weight of precipitate in the test and control tubes is a measure of the amount of gelatin rendered soluble under the conditions of the test. Since the accuracy attained using this method depends largely on accuracy in pipetting, it is advisable to hold the buffer substrate solution at 40°C. during pipetting. Increasing the time of standing before centrifuging, or the time of drying, has been shown to have no measurable effect on the results.

In Figure 7 the relationship between enzyme concentration and the amount of gelatin rendered soluble is shown for the crude protease of *A. oryzae*,

crystalline trypsin (Armour), and crystalline chymotrypsin (Armour), the enzyme solutions being prepared in 0.1M phosphate buffer at pH 7.0. With the mould protease, and to a lesser extent with the other enzymes, the relationship is approximately linear for low enzyme concentrations, and it is possible therefore to obtain a curve relating the amount of enzyme present to the experimental values. The unit of enzyme activity is taken to be that causing a reduction of 1 mg. in the precipitable gelatin at low enzyme concentrations. The curves of Figure 7 are calibration curves obtained in this way.

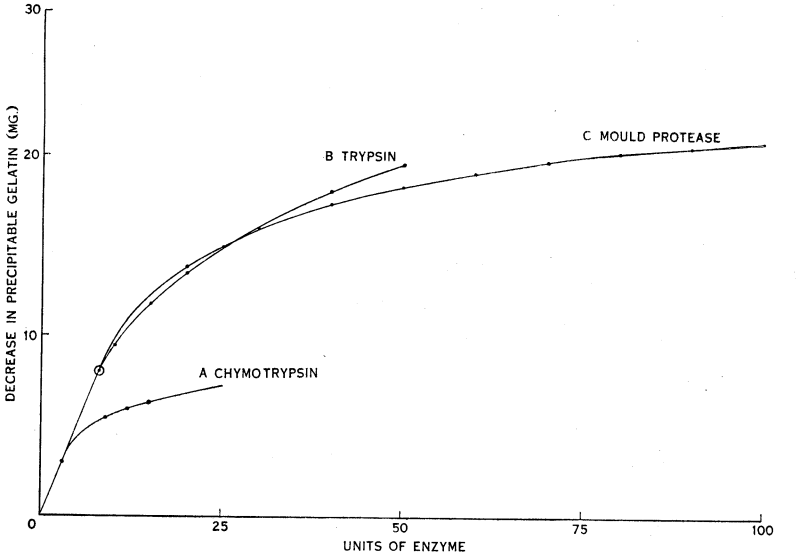

Fig. 7.—Calibration curves of the gravimetric estimation of protease activity. The relation between gelatin hydrolysed (mg.) and enzyme concentration for *A*, chymotrypsin; *B*, trypsin; and *C*, crude mould protease.

As would be expected, different samples of gelatin provide slightly different dilution curves for each enzyme solution. The dilution curves for the mould protease using Davis gelatin and Difco gelatin are shown in Figure 8 to be similar in shape, but there is a slight difference in absolute values. In experiments using this method a bulk stock of gelatin is therefore used, all calibration curves having been obtained with this material. It is possible to incorporate reducing agents or traces of metal ions in the buffer substrate where necessary.

The high buffer capacity of acetate at the pH of maximum precipitation of the gelatin used provides adequate protection against changes in pH as the result of addition of enzyme solutions of high buffer capacity and widely varying pH. When aliquots (2 ml.) of gelatin substrate solution at pH 7.0 were precipitated with aliquots (10 ml.) of ethanol containing different amounts of 5N HCl, maximum precipitation occurred with 1.8 ml. HCl per litre, but the variations in the amounts of gelatin precipitated were within experimental

error over the range 1.3-2.5 ml. HCl per litre. A titration curve of the buffer-substrate solution may be used to determine the amounts of acid or alkali required in the precipitant for maximal precipitation of the substrate as the pH of the buffer substrate varies from the point of maximum precipitation.

The foregoing method may be adapted to use the colorimetric biuret test, the gelatin precipitate being dissolved in water and estimated by the procedure for biuret estimation of proteins (Robinson and Hogden 1940).

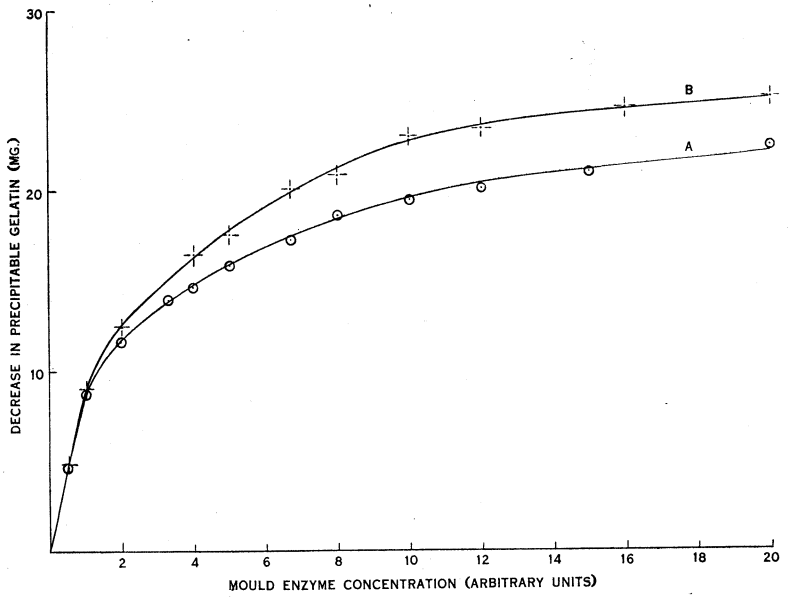

Fig. 8.—Dilution curves obtained for mould protease using *A*, Davis gelatin and *B*, Difco gelatin.

(*d*) *Comparison of Sensitivity of Methods of Protease Estimation*

In order to compare the sensitivity of these techniques for protease estimation with other well-known methods, solutions of crystalline trypsin, chymotrypsin, and pepsin (Armour); crystals containing protease from *A. oryzae* (Crewther and Lennox 1950); and non-crystalline papain (Parke-Davis Products) were diluted 1/10, 1/100, 1/1000, and 1/10,000 and estimated by the viscosity reduction of gelatin (Lennox and Ellis 1945), haemoglobin digestion (Anson 1938), acetone titration (Linderstrøm-Lang 1927), and by the gravimetric and turbidimetric techniques. The results shown in Table 1 indicate that the last two methods compare favourably with other methods of protease assay.

IV. Discussion

The fact that a straight-line relationship can be obtained between log light transmission and gelatin concentration for solutions of gelatin precipitated with ethanol suggests that the mean micelle size of the precipitated gelatin does

not vary appreciably with gelatin concentration. Bischoff and Desreux (1951) have demonstrated a similar constancy of mean micelle size for precipitated polymers, as the concentration of precipitant varies.

The two methods for protease estimation described in Section III are both based on the familiar principle of estimating the amount of substrate rendered non-precipitable as a result of enzyme action. When using gelatin as substrate, this involves the following considerations.

High molecular weight gelatin, which has been carefully prepared, can be readily broken down by boiling in water at physiological pH into smaller units, which are also present in considerable amount in commercial gelatin preparations. It is possible therefore that the high molecular weight components of commercial gelatin will comprise smaller units linked by readily hydrolysable peptide bonds, which may be more susceptible to the attack of certain proteases than are peptide bonds linking other amino acid residues. It is possible also that certain hydrogen bonds present in the larger components are absent from the degradation products.

TABLE 1

COMPARISON OF SENSITIVITY OF METHODS OF PROTEASE ASSAY

Enzyme	Maximum Dilution Showing Significant Activity				
	Viscosity Reduction of Gelatin	Haemoglobin Digestion	Acetone Titration	Gravimetric Method	Turbidimetric Method
Trypsin 0.5 mg./ml.	1/100	1/100	1/10	1/1000	1/100
Chymotrypsin 0.5 mg./ml.	1/100	1/10	1/10	1/100	1/10
Pepsin 0.5 mg./ml.	1/1	1/100	—	1/10	—
Mould protease 0.3 mg./ml.	1/100	1/100	1/10	1/1000	1/100
Papain 2.0 mg./ml.	1/100	1/10	1/10	1/100	1/10,000

It is to be expected therefore that, for any one enzyme, the sensitivity of the protease methods described above will depend on the concentration of ethanol used and the conditions of precipitation. Thus enzymes acting preferentially on components of the gelatin having molecular weights such that they are precipitated under the experimental conditions but may be rendered soluble by the hydrolysis of a single peptide bond, will be more readily detected than enzymes acting on the larger or smaller components of the gelatin, and therefore causing little decrease in the precipitable material present. It might be expected therefore that the gravimetric and turbidimetric methods would show

variable sensitivity to different proteases, as the threshold of molecular weight is different in the two methods. This is to some extent demonstrated in Table 1 where considerable variation is shown in the sensitivity of the two methods to the action of various proteases. Similarly the viscosity reduction of gelatin, which is probably related particularly to hydrolysis of the higher molecular weight components of gelatin, need not necessarily be sensitive to the same enzymes as the gravimetric method, which is concerned chiefly with the lower molecular weight fraction. Evidence that this is so will be presented in a later publication.

It will be appreciated that the turbidimetric method is limited to a rather narrow range of pH, it being necessary to vary the substrate concentration according to the pH used. The nearer the pH approaches the isoelectric point of the gelatin used, the lower will be the maximum concentration of gelatin it is possible to use. On the other hand, the sensitivity of the method would increase accordingly. At high pH the range of transmission values corresponding with 0-2 per cent. gelatin would be limited to higher values, and it should be possible to maintain sensitivity by using undiluted ethanol as precipitant.

The use of gelatin as substrate for protease estimation has the disadvantage that no standard preparation of gelatin can be obtained. On the other hand, its high solubility and the ease with which it is attacked by proteinases without prior denaturation are points in its favour. The process of denaturing a pure protein such as haemoglobin probably introduces substrate heterogeneity. The use of a substrate requiring no denaturation also avoids the possible effect of the denaturant on the enzyme itself.

V. Acknowledgment

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