THE DETERMINATION OF PEPTIC ACTIVITY: AN EXAMINATION AND APPLICATION OF THE GATES METHOD OF PROTEOLYTIC ENZYME TITRATION.

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Despite the numerous methods for the determination of proteolytic enzymes, there are few, if any, which incorporate all the advantages of accuracy, simplicity, and brevity, and which require only a small sample for a determination. In 1927 Gates (1) published a preliminary report of a proteolytic enzyme titration possessing these desirable features, and demonstrated his method at the 1928 meeting of the American Societies for Experimental Biology held at Ann Arbor. This method has been extensively investigated in this laboratory and found to be most valuable for rapid and accurate determination of peptic activity. The principles of the method as outlined by Gates have been retained. However, the authors have to some extent modified the manipulative details, and have supplemented the outline of the procedure as presented in the preliminary report to include an accurate means of quantitative estimation of peptic activity.

Principle of Method.

The Gates method involves the digestion of the gelatin layer of a photographic film. The surface of a film is composed of a uniform layer of gelatin in which silver is evenly dispersed. Upon reduction of the film by exposure and subsequent development of the film, an almost opaque substrate results. Digestion of the gelatin liberates reduced silver with a progressive change from opacity to transparency, depending in degree upon the extent of the digestion. Proteolytic activity is determined by an estimation of the difference in the intensity of the light penetrating the film before and after digestion.
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Procedure.

Preparation of Films.—The choice of films is optional. Of the two types tested, Eastman's commercial and commercial-ortho, the latter was found to be the more sensitive for the estimation of peptic activity. A film 8 inches by 10 inches is a convenient size to use. To insure complete and uniform reduction, the films are exposed to Roentgen rays, 25,000 volts, 10 milliamperes, at a target range of 24 inches for a period of 2 minutes. A pile of six films can be exposed at the same time. The films are then developed and fixed in plain sodium hyposulfite, no hardener being used. As the opacity of the film will depend upon the time that the film is allowed to remain in the developer, it is well to standardize this procedure. The opacity of the film depends entirely upon the extent of the reduction of the silver. The amount of gelatin on the films is not influenced by exposure or development. Therefore a dark film will favor more accurate results, inasmuch as the greater the opacity, the greater will be the change in penetrability of light through the film per unit of gelatin digested. On the other hand, if the films are allowed to become too dark it will be impossible to measure the slight amount of light penetrating. Films developed until the red light in the developing room is not discernible through them give optimal results. Rewashing of the developed films with distilled water and again drying before use is advantageous in order to "set" the film so that subsequent wettings are without effect. After development the large films are cut into smaller units, $\frac{3}{4}$ inch by 1 inch, to be used for digestive tests.

Digestion of Films.—The films are digested in cells, made by mounting rings of No. 14 copper wire, $\frac{3}{4}$ inch in diameter, upon glass squares 1 inch by 1 inch by means of paraffin. This forms a small chamber, the capacity of which is approximately 0.5 cc. The cell is so filled with the solution under examination that the meniscus of the fluid projects above the copper ring. The film to be digested is now placed on top of this cell, the gelatin layer downward and in intimate contact with the enzyme solution. Inasmuch as the film forms the roof of the digestion chamber, the silver, when liberated, will fall to the bottom, thus not interfering with the course of the reaction. Another glass square is placed over this film, and the digestion cell, now complete, is firmly held
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together with an ordinary spring clothes-pin, and is immersed in a constant temperature water bath for a carefully timed interval. This digestion cell, when carefully constructed, will be absolutely water-tight, allowing no dilution of the enzyme solution when placed in a water bath. A carefully controlled temperature is most important because the temperature coefficient of the reaction is extremely high. A water bath, constant to 0.02° has been found to be the most satisfactory means of temperature regulation. At the conclusion of the digestion the films are carefully washed free of enzyme by immersion in the water bath and dried in a current of air.

Temperature and pH Regulation.—If the digestion were allowed to proceed at optimal temperature, the minute amount of gelatin present on the film would be completely removed in a few minutes. To prolong the time of digestion and reduce the error in timing, the films are digested at 25°. At this temperature the gelatin remains hard and firm and at the completion of the reaction presents a smooth, even appearance. Even at this low temperature the films would be completely digested in the 10 minutes by the concentration of pepsin normally found in gastric juice. This offers an opportunity for the dilution of the specimen to be determined with a buffer solution, thus bringing all the samples analyzed to the same pH and reducing still more the amount necessary for a single determination. In a series of peptic digestions carried out at acidities ranging from pH 1 to 4, a pH of 2 was found to be most favorable. Sörensen's glycine-NaCl-HCl buffer solution (2) is the choice at this high acidity.

Measurement of Light Penetrability.—To measure the light penetrating before and after digestion, a Klett or other Duboscq type of colorimeter is used. The films are read against a gelatin-silver suspension prepared by dissolving the gelatin layer from two films in about 2 cc. of hot water, filtering through filter paper to remove any large heavy particles, and suspending this black silver emulsion in sufficient glycerol to fill the cup of the colorimeter. The viscous properties of the glycerol will prevent any settling of the silver within the course of several hours. Below the cup of the colorimeter containing this gelatin-silver suspension is placed one of the films from which the gelatin and silver have been removed. The other cup contains glycerol to which has been added the same
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proportional amount of water as that present in the gelatin-silver suspension just described; below it is placed the film that is being measured. Very accurate determinations of the light coming through the unknown film can be made by regulating the depth of the suspension on the opposite side of the colorimeter until the light intensities match. The films are carried between two thin layers of sheet brass, lacquered flat black in order to avoid internal reflection, and having a hole of ½ inch diameter centrally bored to allow exposure of the digested area of the film. The film carriers are clamped to the rack of the colorimeter by means of a simple paper clip in such a way as to allow the exposed portion of the film to be directly under the cup.

The films are read before and after digestion and the difference in the reading is a measurement of the proteolytic activity of the solution used in their digestion. It will be found that despite careful exposure and development the films will vary in their initial readings. To exclude any variable, it is best when making a series of determinations, to pick a group of films having the same initial reading.

Sensitivity.—An indication of the sensitivity of this method can best be demonstrated by a typical digestion curve showing the variation in films acted upon by different concentrations of a commercial enzyme solution. Fig. 1 shows the course of a representative digestion, the concentration of enzyme being variable and the time constant.

As can be seen from Fig. 1, this method is sensitive to but slight changes in concentration and can be used for the quantitative estimation of extremely dilute solutions of pepsin. The curve in Fig. 1, because of its logarithmic character, suggests that the underlying reaction is monomolecular in character. A plot of the reading difference against the logarithm of the enzyme concentration (Fig. 2) supports this view.

In view of the facts that (a) the temperature of the reaction is low, and the time of reaction short, thus minimizing enzymatic decomposition, (b) the amount of substrate is so small that except in very extreme dilutions there is a relatively large predominance of enzyme and therefore less inhibition due to the end-products of the reaction, and (c) due to the flat nature of the substrate the reaction surface is at all times practically constant, one would
expect according to Northrop (3) an approximation to this monomolecular reaction.

**Determination and Calculation of Unknown Solutions.**—The results obtained when films are digested with varying dilutions of a pepsin solution of undetermined proteolytic activity, although accurate, are merely comparative. As has been stated, films will vary in their initial readings. Also the silver suspension, by means of which the light intensity is measured, will vary with the films. It is impossible, therefore, directly to measure an unknown solution and express the results in absolute units because the

![Graph](http://www.jbc.org/)  
**Fig. 1.** Change in opacity of films due to peptic digestion. The differences on the abscissae are expressed in mm.

standard for comparison is not constant. An unknown peptic activity can only be determined by comparing it with solutions of known digestive power run under the same conditions of test. In this way accurate determinations of proteolytic activity of the unknown solutions can be made and directly compared with that of a known standard. Varying dilutions of Armour's commercial pepsin 1:10,000 are used as a standard. All solutions are adjusted to the same pH by dilution with a buffer solution and are run in a manner similar to those illustrated in Fig. 1, the concentration of enzyme being the variable. At the same time, with films of the same initial reading, and with the same silver suspen-
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In order to measure all the films, digestions of the unknown samples are run. The digestion curve is plotted from the values obtained from the known solutions. The values of the unknown solutions are then interpolated from this curve and are expressed as per cent of the known solutions.

As illustration, the determinations of two samples of gastric juice, secreted in response to a toast and tea test meal are shown. These samples vary appreciably in peptic activity. Six dilutions of Armour's commercial pepsin 1:10,000 ranging from a 1.0 to 0.03 per cent serve as the standards. The digestion curve obtained from the readings of these standards is shown in Fig. 3, where enzyme concentrations are plotted on the logarithmic scale of semilogarithmic paper. The concentrations of the unknown samples may thus be read directly.

As can be seen from Fig. 3, a 50 per cent dilution of unknown Sample 1 was equal in strength to a 0.26 per cent solution of standard 1:10,000 pepsin. Thus the concentration of enzyme in the undiluted sample is equivalent to the concentration of enzyme in a 0.52 per cent solution of 1:10,000 pepsin. Expressing the

![Fig. 2. Change in opacity of films due to peptic digestion. The differences on the abscissa are expressed in mm.](http://www.jbc.org/figure/2)
pepsin value of a 1.0 per cent solution of 1:10,000 pepsin optionally as 1,000 units, then we can express the concentration of the unknown as 520 units.

In a similar manner a 50 per cent dilution of Sample 2 is equivalent in proteolytic activity to a 0.116 per cent 1:10,000 pepsin solution. The undiluted sample is equal in strength to a 0.232 per cent solution, and the concentration of pepsin is 232 units. Thus proteolytic activity is expressed in very comprehensive terms; namely, as a definite concentration of an accurate and easily reproduced standard enzyme solution.
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Preparation of Standard Solutions.—The standard solution of 1.0 per cent strength is made at frequent intervals, 0.25 gm. of dry commercial pepsin being dissolved in 25 cc. of a buffer solution and thus brought to the optimal pH. Other dilutions are made from this 1 per cent solution. Inasmuch as only 1 cc. of the standard solution is necessary to make an entire series of varying dilutions, this 25 cc. will serve as a standard for about a month, during which time there is no demonstrable loss of activity if kept in the cold. It is estimated that a 4 ounce bottle of commercial pepsin will furnish material for a referable standard for many years. Armour’s commercial pepsin has been found over a period of 2 years to lose not over 10 per cent of its digestive power (4). All determinations over a long period of time can thus be made referable to the same standard and accurate comparative values obtained.

DISCUSSION.

All measurements of proteolytic activity involve either (1) an estimation by chemical or physical means of the amount of substrate digested per unit time or (2) a measurement of the time required to digest a unit of substrate. In either case, however, the accuracy of the method is dependent upon the accuracy with which one can measure the amount of protein that disappears or the unaffected residue. In the case of the method here developed for the quantitative estimation of peptic activity, the amount of substrate digested is so slight that, if the extent of hydrolysis were to be measured by any of the common chemical methods, it would be inappreciable. Yet with the digestion of this minute amount of substrate there is a change from opacity to transparency, permitting a wide range of differential readings. This extreme sensitivity in measuring proteolysis makes Gates’ method valuable in the quantitative estimation of extremely dilute enzyme solutions, and also in the qualitative demonstration of the slightest proteolytic activity. Thus proteolytic activity, that might normally escape detection by methods in common use, can be accurately measured.

The time-saving element of the method is another of its valuable and important features. Cells can easily be assembled and placed in the water bath at 1 minute intervals. A 50 per cent dilution of
normal gastric juice will give the optimal amount of digestion in 10 minutes. Thus thirty films can be digested in the course of 1 hour. By running triplicate checks, ten unknowns can be determined in 1 hour's time plus the time required to run the six standards and for the reading of the films.

The time that the films should be allowed to digest varies with the samples. The digestions should proceed until the films are from 25 to 50 per cent digested. This allows greatest accuracy in the reading of the films and at the same time gives results that fall on the steepest portion of the digestion curve illustrated by Fig. 1. However the digestion periods should be at least 10 minutes in length so that no appreciable error due to variation in timing results. Therefore, in the determination of solutions having a strong proteolytic activity, these solutions should be diluted until the digestion is optimal in 10 minutes. This can be ascertained in a preliminary digestion series in which the concentration of the unknown is the variable. In this preliminary trial, after a little experience one can easily estimate without the accuracy of instrument reading the dilutions at which to run the digestions. In the determination of a more dilute sample the digestion period is proportionally lengthened. At the same time a more dilute series of standards is used. With experience one learns just what dilution of standards to run with various digestion periods so as to run the gamut from opacity to transparency of the standard films and thus plot an accurate standard digestion curve.

Lastly, this method has the feature of requiring only a very minute volume of sample for a determination. The capacity of the digestion chamber is 0.5 cc. All samples are diluted at least to half strength so that the maximum volume required for duplicate determinations is 0.5 cc. of the original solution to be tested. In the case of more concentrated enzyme solutions, the required amount is less, inasmuch as the dilution of the sample must be greater. This is of great value in fractional analysis of gastric samples where a greater portion of the sample is used for the determination of other constituents.

Although all references in this paper are to the procedure for the determination of peptic activity, the method is equally applicable to the determination of proteolytic enzymes acting in alkaline medium. The only essential change that must be made concerns the buffer solution employed for dilution of the samples.
SUMMARY.

1. A technique for the quantitative estimation of gastric proteolytic activity based upon the Gates method is presented.
2. The accuracy, advantages, and applications of this technique are discussed.

The authors wish to express to Dr. Frederick L. Gates their appreciation of his thoughtful criticisms of the manuscript of this paper; they also appreciate his generosity in consenting to publication of it before supplementing his preliminary paper by a more extended report of his own work.

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