A VISCOSIMETRIC METHOD OF ESTIMATING ENZYME CONCENTRATION WITH SPECIAL REFERENCE TO AMYLASE

BY WILLIAM R. THOMPSON, CARL E. JOHNSON, AND RAYMOND
HUSSEY

(From the Department of Pathology, Yale University, New Haven)

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A viscosimetric method for the estimation of trypsin concentration was developed by Northrop and Hussey.¹ Since that time it has been employed for the estimation of trypsin and pepsin concentration by Hussey and Thompson,² and Northrop and Clark.³ Certain modifications of the original technique were employed in some of these investigations; but, owing to the fact that interest was focussed primarily upon the effects of radiations upon enzymes, some of these modifications have not been reported in detail.

The present purpose is to outline these modifications and to give the results of investigations whose object was to apply similar technique to the estimation of the concentration of amylase. This method depends upon the action of amylase upon a starch solution. The preliminary observations from which it was developed were made in our laboratory in 1923.⁴ Since that time, other workers⁵ have used a viscosimetric method of this type for the estimation of amylase concentration. However, in their published results the variation admitted in the mixing ratio with consequent variation in starch con-

¹ Northrop, J. H., and Hussey, R. G., *J. Gen. Physiol.*, 1922-23, 5, 353, 355.

² Hussey, R. G., and Thompson, W. R., *J. Gen. Physiol.*, 1922-23, 5, 647; 1923-24, 6, 1; 1925-26, 9, 309, 315, 217.

³ Clark, H., and Northrop, J. H., *J. Gen. Physiol.*, 1925-26, 9, 87.

⁴ Unpublished.

⁵ Davison, W. C., *Bull. Johns Hopkins Hosp.*, 1925, 37, 281. Maslow, H. L., and Davison, W. C., *J. Biol. Chem.*, 1926, 68, 75. O'Donovan, C., and Davison, W. C., *Bull. Johns Hopkins Hosp.*, 1927, 40, 238. Elman, R., and McCaughan, J. M., *Arch. Int. Med.*, 1927, 40, 58.

centration is objectionable, as is also the primary mixing of serum and substrate solution in the viscosimeter itself.

TECHNICAL PROCEDURES

The viscosimeters employed were of the Ostwald type. However, for the proposed work certain deviations from the usual design seemed advantageous, particularly the placement of the start and finish marks upon non-capillary tubing. The object of this was to prevent the formation of air bubbles. Two types of viscosimeters were used, which will be designated as the 5 ml. and the 10 ml. types, respectively, corresponding to the volumes of fluid for which they are designed. Those of the 10 ml. type were made in this laboratory and were adjusted to a 60 second water outflow time at 34°C. These viscosimeters were employed in previous work as well as in that to be reported at present. More recently a 5 ml. viscosimeter, otherwise of the same characteristics, has been employed with satisfactory results.

While measurements were being made, the viscosimeters were immersed in a vertical position so that the start line (upper mark) was between 3 and 4 cm. below the surface in a thermoregulated water bath, the temperature of which was restricted to an interval of 0.06°C. in the usual manner. In the case of the starch-amylase investigation this interval was $37.5^{\circ} \pm .03^{\circ}\text{C}$. A 10 gallon rectangular glass battery jar was used as a container. The water level of the bath was made sensibly constant without interference with thermoregulation by means of a connection with a reservoir *via* an automatic float-operated valve.

Preliminary observations were made to find a suitable temperature, hydrogen ion concentration, substrate concentration, autoclaving technique, and substrate-enzyme mixing procedure. Early in this work it became obvious that serious difficulty is to be encountered in any attempt to fix reproducible standards. Accordingly, as a temporary expedient only one lot of starch was employed in the present experiments (Baker's Soluble Starch, Lot number 13128).

A standard 7 per cent starch substrate solution was employed which was made in the following manner except that it was admissible to substitute any proportional set for the gravimetric and volumetric quantities involved.

7 gm. of Baker's Soluble Starch, Lot number 13128, were weighed out and suspended in 65 ml. of redistilled water in a Pyrex Erlenmeyer flask of approximately three or four times that volume. Then, with constant agitation to prevent overheating of the starch, the suspension was heated just to the boiling point; the top of the flask was covered with a double-layered unbleached muslin cap and the whole transferred to an autoclave wherein the temperature was rapidly raised to $120^{\circ} \pm 2^{\circ}\text{C}$. and so maintained for a period of 20 minutes. Then the solution was removed and cooled to a temperature between 50° and 70°C. and so maintained while 30 ml. of M/15 phosphate buffer (pH 6.7 according to Sørensen) was added, and enough redistilled water to make the total volume 100 ml., and the whole mixed and filtered through a single layer of unbleached dry muslin (previously

prepared by thorough washing with redistilled water), the temperature of all components being maintained between 50° and 70°C. throughout. The filtrate was collected in an Erlenmeyer flask, stoppered with a clean rubber stopper, and cooled, and maintained at $37.5^\circ \pm .03^\circ\text{C.}$ until used or discarded.

In any series of enzyme digestions used in a viscosimetric method of studying their progress it is the custom in this laboratory to follow the procedure of Northrop and Hussey¹ in fixing the ratio, R , of volume of enzyme solution to volume of substrate solution to be mixed. In the case of the starch-amylase digestions of the present report $R = 1/5$. In any case the digestion procedure is as follows.

A definite volume, V , of substrate solution is measured into a suitable flask (of capacity at least $4V$) which is carefully stoppered and maintained in a thermostat such as has been described. At a known instant of time a volume RV of enzyme solution is added and the whole thoroughly mixed. The time on a chronometer to the nearest thousandth of an hour is noted at the beginning and at the end of this transfer and the mean of these, τ_o , is computed. A proper portion of the mixture is transferred to each viscosimeter to be employed, care being taken to avoid undue temperature change and the formation of air bubbles. An attachment is made to each viscosimeter in such manner that by means of a definitely maintained partial vacuum system and a three-way stop-cock the liquid can be raised at will and then allowed to flow through the capillary tube and the outflow time between marks observed with the aid of a calibrated stop-watch. A sequence of such observations is made and the time in hours to the nearest thousandth is read and recorded which corresponds to the instant of passing the upper mark (start line), in each instance employing the same chronometer as for the estimation of τ_o . For the i -th observation for a given digestion and a given viscosimeter let τ_i be the time so observed, and let s_i be the time in seconds observed for the outflow. Then with s_i we associate t_i defined by the relation

$$(1) \quad t_i = \tau_i - \tau_o + \frac{s_i}{7200}$$

Obviously, t_i is the interval of time (in hours) elapsed from the mean time of mixing enzyme and substrate (τ_o) to the mean time of observation. The number-pair (t_i, s_i) will be called a D -point and all such points for a given digestion in a given viscosimeter will be said to form a set, D . These points may be charted on coordinate paper (with s as ordinate and t as abscissa) and a smooth curve drawn. By convention this curve is to begin at the s axis and extend continuously as a decreasing function of t with continuous negative but increasing first derivative. The point of intersection of this curve and the s axis may be designated by $(0, s_o)$.

The original procedure of Northrop and Hussey¹, working with trypsin and pepsin gelatin digestions, involved the graphic estimation of s_o by extrapolation. Hussey and Thompson² succeeded in developing a system (unpublished but employed in their published work) of estimation of s_o by a sequence of measurements

in the same viscosimeter of the outflow time of a blank consisting of a mixture prepared in the same way as the digestion mixture with the exception that a conventional solution devoid of enzyme was substituted for the enzyme solution (e.g., 0.85 per cent saline).

The importance of s_0 is obvious in view of the following remarks. A number, ϕ , is taken such that $0 < \phi < 1$, and such that there exists a point of every D -set to be used having its ordinate less than S where

$$(2) \quad S = \phi \cdot s_0,$$

and the smooth curve mentioned above has one and only one point with S as ordinate. Then let T be the abscissa of this point. The point, (T, S) , may be called the *threshold crossing point*; the line, $s = S$, the *threshold*, and T the *threshold intercept*.

Now, let Q be defined by

$$(3) \quad Q = \frac{1}{T}$$

where T is as defined above, an approximation of the time (in hours) required for the standard change in viscosity.

It has been shown that for several enzyme-substrate systems Q is at least a fair approximation of enzyme concentration in arbitrary units so chosen that for unit enzyme concentration $T = 1$. In all preceding work and in that reported in the present communication the arbitrary value, $\phi = 0.8$, has been assigned corresponding to 20 per cent change in viscosity.

By the procedure of estimating s_0 for a given digestion curve just before the digestion is started, one of the greatest difficulties due to the personal element in curve plotting is eliminated. Accordingly, the curve is always drawn through the point, $(0, s_0)$, so estimated. However, this point is not called a D -point, but is associated with the D -set.

To decrease further the differences in curve plotting technique due to differences in individual judgement, the following curve plotting rules may be adopted.

The minimum number of D -points shall be five, exclusive of rejected points if any. Not more than three D -points shall be used which have abscissae exceeding T and none shall be used whose abscissae exceed $1.5T$. If (t_i, s_i) is a point of the D -set and there exists a point on the curve having the same ordinate, s_i , let t_i' be its abscissa and let $d_i = t_i - t_i'$ and otherwise let $d_i = t_i$. Now, let N be the number of D -points (unrejected). Then unless

$$(4) \quad |\sum d_i| \leq 0.002 \cdot N \cdot T$$

(where the summation is over the N points) the curve is rejected as a representation of the points and another drawn, and so on until this condition is satisfied, which is always possible. Now, let λ be defined by

$$(5) \quad \lambda = \frac{\sum |d_i|}{N}.$$

Then, if $\lambda > \theta \cdot T$, where θ is a constant previously fixed for all work of a given type, the whole digestion experience represented by this D -set is discarded. In experiments so far completed the value, $\theta = 0.032$, was used. This was arbitrarily fixed but was taken equal to four times the mean value of λ (in hours) obtained from a long experience of similar curve plotting. As a matter of personal experience not more than once in a hundred instances was discarding so indicated.

A single D -point at a time may be rejected under the following conditions.

Let D_j be one of the D -points (tentatively excluded) and let λ' be defined by

$$(6) \quad \lambda' = \frac{\sum |d_i| - |d_j|}{N - 1},$$

Then, if $N-1 > 5$ and $|d_j| > 4\lambda'$, the point may be rejected. The remaining points are treated then as if the point D_j had never existed. Obviously, the process may be repeated as long as the conditions given above are satisfied.

EXPERIMENTAL RESULTS

The demonstration of the relation between enzyme concentration and T , the time required for a prescribed change to take place, may be called the *critical experiment* in any attempt to evolve a viscosimetric method of the type originally described by Northrop and Hussey.¹ In the case of the present amylase investigation such an experiment was made with amylase solutions bearing a known interrelation by dilutions from a common stock solution.

Four such experiments were performed in each of which three amylase solutions, designated by A , B , and C , respectively, were employed. In each instance the Solution A was a 0.005 per cent pancreatin (Parke Davis) solution in 0.85 per cent saline made by allowing some of the pancreatin powder and the proper amount of saline to lie in contact for $\frac{1}{2}$ hour (the quantities being such that a 0.1 per cent suspension was formed), followed by filtration of the mixture and dilution of a portion of this with saline to twenty times its volume. Solution B was a portion of A diluted to twice its volume with saline, and in the same manner C was formed from B . In each experiment two digestion curves were obtained for each of the solutions used. The values of T so obtained are given in Table I together with the mean of each pair, designated by α , β and γ , respectively.

However, as it is apparent that differences in amylase concentration exist between solutions prepared according to the same procedure at different times, a quantity, J , which depends upon all the observations of a given experiment, is employed as a basis of comparison between these experiments; where J is defined by

$$(7) \quad J = \frac{4\alpha + 2\beta + \gamma}{6}.$$

TABLE I

Soln.		Exp. 1	Exp. 2	Exp. 3	Exp. 4
A	1	0.389	0.429	0.353	0.426
	2	0.390	0.498	0.378	0.426
	α	0.390	0.459	0.366	0.426
B	1	0.981	0.971	1.100	1.030
	2	0.890	0.954	0.954	0.964
	β	0.904	0.963	1.027	0.997
C	1	1.782	1.717	1.589	1.870
	2	1.629	1.736	1.572	1.701
	γ	1.706	1.727	1.581	1.786
J		0.846	0.915	0.850	0.914

TABLE II

	Exp. 1	Exp. 2	Exp. 3	Exp. 4	mean	a.d.	A.D.
α'	0.461	0.501	0.430	0.466	0.467	.020	.010
β'	1.068	1.053	1.208	1.092	1.105	.051	.026
γ'	2.016	1.887	1.860	1.954	1.929	.056	.028

Accordingly, we define α' , β' , and γ' for any given experiment by

$$(8) \quad \alpha' = \frac{\alpha}{J}, \beta' = \frac{\beta}{J}, \text{ and } \gamma' = \frac{\gamma}{J}.$$

The values obtained for these variables in the four separate experiments are given in Table II together with the means, $m(\alpha')$, $m(\beta')$, and $m(\gamma')$.

If a reciprocal relation as in (3) held exactly between amylase concentration and T , and the relative concentrations of A , B , and C were 4:2:1, respectively, then we should find $m(\alpha') = 0.5$, $m(\beta') = 1$, and $m(\gamma') = 2$. The differences between these values and those observed may be compared with the precision measure (A.D.) of each, which may be found in Table II also. Accordingly, wherever differences of such relative magnitude may be neglected, we may employ the method as if the reciprocal relation held exactly.

DISCUSSION

In the past a fairly high precision in the estimation of enzyme concentration in the case of trypsin and pepsin has been attained by resorting to a large number of separate estimations and employing the mean of these. It is a matter of prime importance to restrict evaporation of solutions employed and to maintain uniformity in technique. Enzyme solutions should be protected from undue irradiation (*e.g.*, with sunlight) and stored in a refrigerator or low temperature bath when this is admissible. Changes in observers should be made frequently to detect personal bias particularly in curve plotting. The rules given above are obviously arbitrary. Their object is to reduce the effects of personal differences to a point of negligibility. Obviously, however, there are many other possible systems equally, if not more effective in this respect; but most of these which readily suggest themselves offer much more difficulty in application. Obviously, also, certain of these refinements may be neglected in conformity with tolerances which are relatively large.

SUMMARY

Certain technical modifications of the viscosimetric methods as first employed by Northrop and Hussey have been presented. Most of these have been employed with satisfactory results in the irradiation studies of Hussey and Thompson over a period of several years. These are in turn applied to a method of estimation of amylase concentration.