COLORIMETRIC DETERMINATION OF AMYLASE*†

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A SIMPLE COLORIMETRIC method for the determination of amylase was devised because of the great importance of assays of this enzyme in clinical medicine and physiology. The significance of amylase in surgery has been abundantly demonstrated. Curiously, the conditions governing the determination of the activity of amylase have not been studied systematically, so that previous amylase methods have necessarily been more or less empirical and haphazard. The kinetics of this enzyme were examined critically by us and the results are included in this paper.

All methods for determining amylase have rested on one of three principles, the iodometric, saccharogenic or viscosmetric.

The iodometric principle was first advanced in quantitative form by Wohlgemuth in 1908.[1] Amylase activity was expressed in terms of the time required for a given enzyme sample to render a starch substrate colorless to iodine. Many modifications, based on this principle, have been used[2, 3] in an effort to reduce the subjective factor in color estimation. Since an excess of substrate must be present for optimal enzyme activity, an end-point which calls for total disappearance of the substrate is completely unsatisfactory. Most of the adaptations of this principle have, therefore, sought an end-point short of complete decolorization such as the point where the red color due to erythrodextrins takes the place of the blue of starch.[4] Redfern[5] introduced a glass color standard for comparison with starch-iodine colors before the achromic point is reached, but the technic is crude. The error of subjective color estimation has been the most important limitation of the iodometric principle.

The saccharogenic principle, now widely used,[4, 6, 7, 8] is based on measurement of the reducing groups produced by the hydrolysis of starch. Thus, by measuring the reducing power of the enzyme-substrate mixture before and after incubation one obtains a measure of enzyme activity. Reduction is measured by a copper method such as that of Shaffer and Somogyi[9] and results are expressed in terms of milligrams of glucose liberated. The accuracy of these methods is limited to materials in which initial reducing power is low. High blanks necessarily occur in diabetic urine and blood. Moreover, the preparation of the substrate in the valuable method of Somogyi[4] is inconvenient.

The viscosimetric principle, described by Davison,[10] depends upon the decrease in viscosity of the starch substrate as the average molecular size is

reduced by hydrolysis. Viscosity measurements are made in an Ostwald viscosimeter, the time required to reduce the viscosity by 20 per cent being taken as an expression of enzyme activity.[11] Elman and McCaughan[11] recommend this principle as the only means of measuring the full range of hydrolysis rather than the disappearance of substrate or the production of end products alone. The following sources of error are inherent in this method: the time of incubation is necessarily variable since viscosity change does not bear a linear relation to time; there is an initial change in viscosity which occurs with addition of the enzyme sample; further, the viscosity of the enzyme does not change during incubation.[12] Comparisons of the available methods have been made by several authors.[6, 12-16]

THEORY OF THE METHOD

Colorimetric methods are well suited to use in quantitative enzyme assays. If either the substrate or one of the reaction products is colored it may be measured directly in the photoelectric colorimeter. Also, reagents may be added to produce color with the substrate or a reaction product.

The blue color of the starch-iodine complex affords an opportunity to apply the second of these colorimetric procedures. Its depth with an excess of iodine was found by us to be strictly proportional to the amount of starch present.

A plot of the logarithm of the colorimeter reading against the arithmetic concentration of starch in the presence of a constant amount of iodine gives a straight line. By using this curve as a reference standard one may readily determine the amount of starch present in an unknown sample to within 0.02 mg.

Up to 4 mg. of starch with 4 ml. of 0.01 N iodine reagent will give colors which when diluted to 100 ml. can be conveniently read in the Evelyn photoelectric colorimeter, an instrument used in all of our work. A 660 millimicron filter eliminates all extraneous color such as that due to the excess iodine and to the dextrins formed in amylolytic hydrolysis. In this range a 100 per cent change in iodine concentration has only a slight effect on the color. In this way, measurement of the color given with iodine by aliquot samples of the starch-enzyme mixture before and after incubation permits enzyme activity to be expressed in terms of milligrams of substrate hydrolyzed.

Starches. The source and method of preparation of the starch influence its solubility, the stability of solutions, the initial reducing power, and the viscosity, as well as susceptibility to enzymatic hydrolysis.

We have found that high-grade potato starches are very soluble and uniform. Clear solutions give more intense colors with iodine per weight than any other starch examined and are of low viscosity, suggesting that this starch is composed of comparatively long, unbranched molecules. A soluble starch was, therefore, used throughout.

The Starch-Iodine Color. At neutrality the starch-iodine color is stable for many hours both in the dark and the light. We have confirmed the

finding of Swanson[17] that it gives a single peak absorption curve in the Beckman spectrophotometer having its maximum at 590 mu. The color disappears at alkaline pH values probably because the iodine is displaced from the starch molecule by hydroxyl ions. Dialysis of the complex removes the iodine completely in a few minutes, color being restored on addition of more iodine which is evidence that the bond between starch and iodine is a loose one.

KINETICS OF STARCH HYDROLYSIS BY AMYLASE

The kinetics of starch hydrolysis by amylase were investigated in order to outline proper conditions for an assay procedure. Fresh human urine was the source of enzyme.
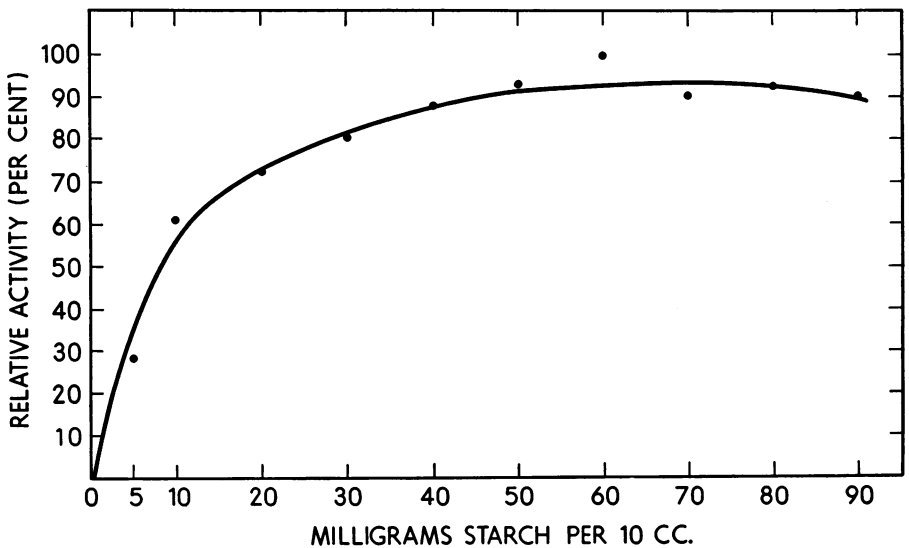

FIG. 1.—The effect of substrate concentration on the rate of enzymatic hydrolysis of starch. Progressive amounts of starch were incubated for on hour with a constant amount of enzyme and phosphate buffer at pH 7 and 37° C.

Effect of Substrate Concentration. A plot of substrate concentration against reaction velocity yields a typical curve (Fig. 1) showing that maximum velocity, or saturation of the enzyme with substrate, was reached at about 45 mg. per 10 ml. We therefore chose a substrate concentration of 80 mg. per 10 ml. for all subsequent work. In this range change in substrate concentration has a minimal effect on the velocity.

A quantitative expression of enzyme-substrate affinity which helps to characterize enzyme systems is afforded by the Michaelis and Menten constant,[18] most accurately found by plots of the reaction velocity with varying substrate concentrations according to the method of Lineweaver and Burk[19] (Fig. 2). Values for starch cannot be expressed in terms of molecular weight and so have been expressed in terms of milligrams per 10 ml. The

Michaelis and Menten constant for the starch employed by us, using a single enzyme concentration, was 7.14 mg. per 10 ml. This agrees well with the value of 7.9 mg. per 10 ml. found by Hanes[20] who used a saccharogenic method.

Effect of Enzyme Concentration. A linear relation between enzyme concentration and rate of hydrolysis is the most important prerequisite of any enzyme assay. Although the change in starch-iodine color is theoretically produced by only the initial steps in starch hydrolysis, this linear relation (Fig. 3) proves it to be an adequate measure of enzyme concentration.
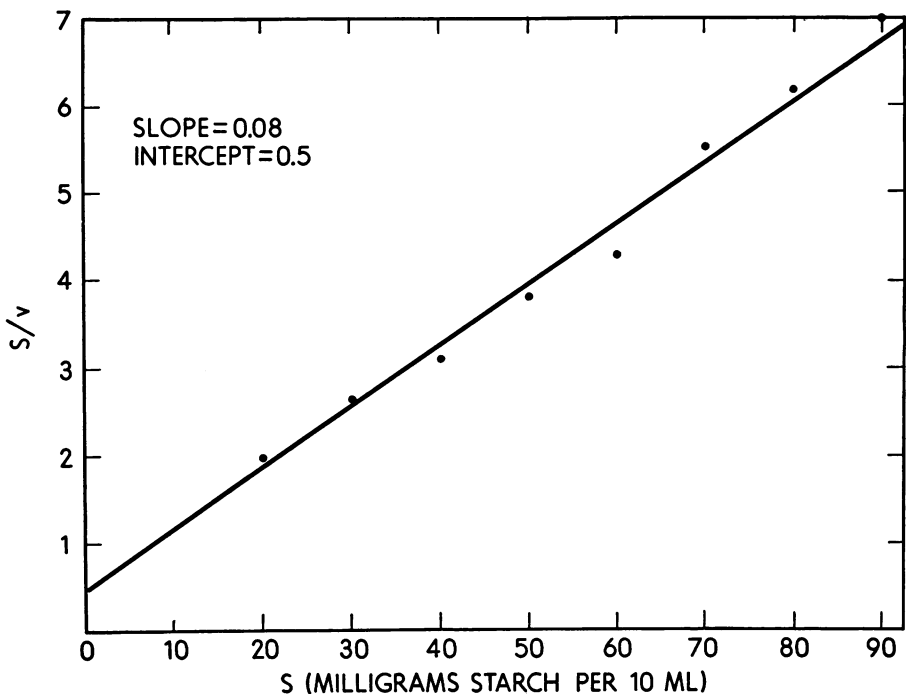

SLOPE=0.08
INTERCEPT=0.5

S/v

S (MILLIGRAMS STARCH PER 10 ML)

FIG. 2.—Data from Fig. 1 plotted according to Lineweaver and Burk. Substrate concentration, S, in mg. per 10 ml., is plotted against S divided by the velocity of hydrolysis, v, expressed as milligram of starch hydrolyzed per hour. From the intercept and slope of this curve the Michaelis and Menten constant was calculated to be 7.14 mg./ml.

Effect of Time. The time course of hydrolysis (Fig. 4) is linear, equal amounts of starch being hydrolyzed in equal times until the enzyme is no longer saturated with respect to substrate. The prediction from Figure 3 that the limit of saturation will be reached when about 35 mg. of the 80 mg. of starch is hydrolyzed is confirmed experimentally by the sharp decrease in velocity at this point. For this reason enzyme preparations are diluted so that not more than 35 mg. (44%) of the starch will be hydrolyzed in the allotted time. Kjeldahl[21] advised a 40 per cent limit of hydrolysis and Evans,[16] 30 percent.

pH Optimum. With some variation previous workers have found the pH optimum for amylase to be around neutrality.[22, 23, 24] At 37° C. we find the optimum to be at pH 7.4 (Fig. 5). Amylase, therefore, appears to be one of the few enzymes commonly found in the serum which exerts maximum activity at precisely the normal pH of blood.

Effect of Temperature. Roberts[2] found a flat range from 30 to 35° C. for the optimum of amylase activity. Kjeldahl[21] reported an optimum temperature of 48° C. and O'Donovan[24] described a "marked reduction" in activity after the enzyme was heated to 58° C. for 30 minutes. The temperature-
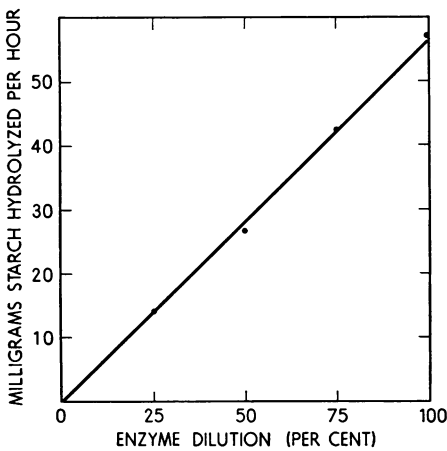

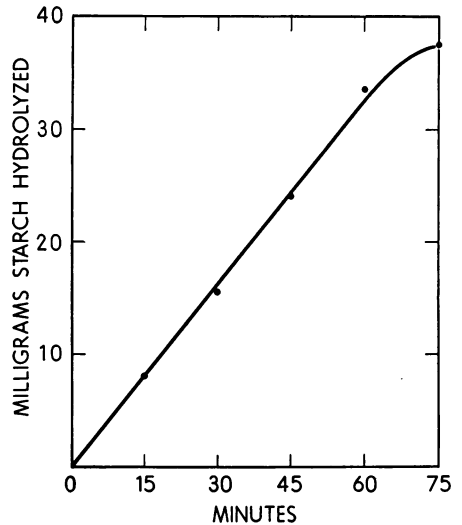FIG. 3.—Effect of enzyme concentration on the rate of hydrolysis.

FIG. 4.—Time course of hydrolysis. The velocity decreases when the enzyme is no longer saturated with substrate.

activity curve, determined by our method (Fig. 6), puts the optimum temperature at 55° C.

Activators and Inhibitors. Activation of amylase by chloride ion has been observed by several investigators.[3, 22, 25-29].

The activity of dialyzed saliva is increased 3.8 times with sodium chloride in 3.42 millimolar final concentration. We have tested no smaller concentrations; however, the effect of increasing concentrations of sodium chloride is shown in Table 1. Wohlgemuth[30] observed activation in as low concentrations as 0.16 millimoles per liter (0.00097 per cent). The normal blood salt concentration of 105 millimoles per liter makes addition of salt unnecessary in an assay of undiluted sera. With dilution, however, we have found that the activity of serum falls off more than the calculated amount because of the fall in concentration of the chloride ion activator.

Enzyme samples with activity greater than that required to hydrolyze 35 mg. of starch per ml. of enzyme per hour must be diluted. To preserve

maximum activity dilution is performed with 0.9 per cent sodium chloride.

Activation of amylase by other substances, such as whole blood and tissue extracts, has been studied by Wohlgemuth[31] and O'Donovan.[24] This activity has been at least partly traced to proteins and amino acids by Rockwood[32] who also was the first to quantitatively determine the inhibiting effect of fluoride ion.[27] We have made use of the fluoride effect to inhibit the enzyme at the end of the incubation period, since acid inhibition precipitates the proteins in the enzyme sample carrying some color out of solution. A combination of fluoride ion inactivation, reduction to room temperature, and dilution are sufficient to stop the reaction. Fluoride ion is included in the Iodine Reagent described below.

TABLE I

*Effect of Sodium Chloride Concentration on
Amylase Activity of Dialyzed Saliva*

Final Concentration of Sodium Chloride		Relative Amylase Activity in Percent
Millimoles per liter	Percent	
0	0	26.4
0.00342	0.02	100.0
0.00684	0.04	99.0
0.01710	0.10	83.2
0.03420	0.20	85.4
0.06840	0.40	85.8
0.13680	0.80	59.8
0.25650	1.50	76.2
0.42750	2.50	48.0
0.85500	5.00	42.1
1.71000	10.00	14.1

METHOD

Reagents

STARCH SOLUTION. A 2% stock solution is freshly prepared for each series of determinations. Weigh approximately 2 gm. of starch* in a 100 ml. beaker and suspend this starch in about 25 ml. of distilled water. Bring about 60 ml. of distilled water to a boil, pour rapidly into the starch suspension and transfer the contents quantitatively to a 100 ml. volumetric flask using hot water for washing. On cooling make the flask to volume with distilled water. This procedure yields 100 ml. of a 2% starch solution which is dissolved and almost water clear.

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* The starch which we have found most valuable is Takamine Soluble Starch manufactured by Takamine Laboratories, Clifton, New Jersey.

Iodine Reagent. This is a solution of 0.01 Normal Iodine containing Potassium Iodide (0.3%) and Potassium Fluoride (5%). Weigh 13 Gm. of Iodine into a weighing bottle. Dissolve, according to the method described by Van Slyke,[33] about 30 Gm. of pure potassium iodide in about 250 ml. of water and transfer the iodine quantitatively from the weighing bottle to a 1 liter volumetric flask washing with the iodide solution. Add the remainder of the iodide solution to the liter flask and dilute the contents to the mark. This 0.1 N stock solution is standardized by titration with a standard sodium thiosulfate solution using a starch indicator; a deviation of 0.005 N is permitted. To make the final iodine solution 50 Gm. of potassium fluoride is weighed out and added to a 1000 ml. flask containing 100 ml. of the stock iodine solution and the contents diluted to the mark.

This reagent is kept in a cool place in the dark or in a darkened bottle. Slow loss of iodine will occur but is not noticeable by titration with sodium thiosulfate for 3-4 weeks. If few determinations are to be made, it should be prepared in smaller quantities.

Phosphate Buffer. 0.04 M Sorensen's phosphate buffer, pH 7. Dissolve 3.471 Gm. anhydrous Na_2HPO_4 and 2.118 Gm. KH_2PO_4 in 1 liter of water.
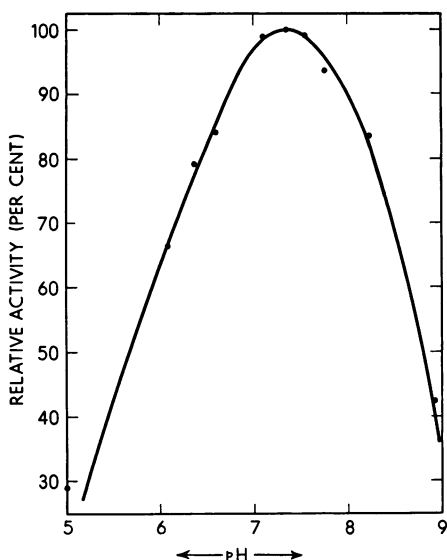

Fig. 5.—Effect of pH on the rate of starch hydrolysis by amylase. Activity is expressed in terms of per cent of the maximum.

The Standard Starch-Iodine Curve. Into each of a series of 100 ml. flasks containing 4 ml. of the iodine reagent is delivered progressive amounts of starch from 0.1 to 4.0 mg. Each flask is made to the mark and a sample of the contents is read in the Evelyn colorimeter in standard colorimeter tubes with a 660 millimicron filter.

The colorimeter readings plotted logarithmically against starch content will give a straight line which is used as the standard curve in all determinations. The concentration of the starch solution is checked daily before each test: Dilute 1 ml. of freshly prepared starch reagent to 100 ml. and compare the colorimeter reading of 5 ml. of this diluted starch in a 100 ml. volumetric flask containing 4 ml. of iodine reagent with the standard curve corresponding to 1 mg.

The Assay Procedure. Determinations are done in duplicate, tubes being incubated at 37° C. for one hour. For each sample to be assayed deliver 5 ml. of phosphate buffer and 4 ml. of starch solution into rimless test tubes and

stopper. For each tube prepare two 100 ml. flasks, each containing 4 ml. of the iodine reagent. Bring the test tubes to constant temperature in a stirred thermostatic bath at 37° C. Add 1 ml. of enzyme solution, appropriately diluted, from a blowout pipette to each tube; restopper the tube and invert to mix. Then immediately remove 0.5 ml. and transfer to one of the volumetric flasks; swirl the contents to assure maximal inactivation of the enzyme by fluoride ion.

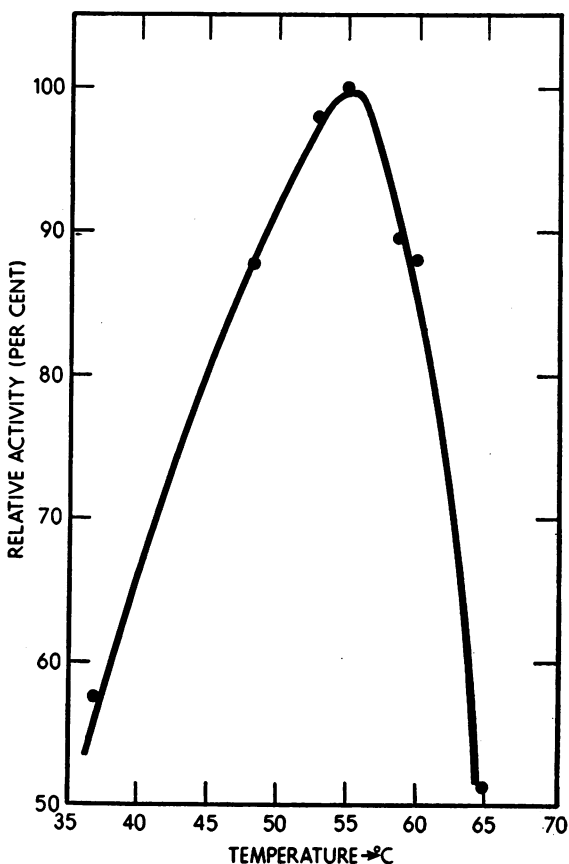Another 0.5 ml. sample is taken after one hour of incubation.and treated

FIG. 6.—Effect of temperature on amylolytic hydrolysis.

similarly. The contents of the flasks are diluted to the mark with distilled water and an appropriate quantity is transferred to colorimeter tubes and read in a colorimeter with a 660 mu filter.

From the standard curve the amount of starch present in the aliquot removed before and after incubation is found. The initial aliquot is 1/20 of the total incubating volume and the final 1/19. Therefore, to find the

675

changes of total starch present in the incubating tube the initial figure is multiplied by 20 and the final by 19.

Results are expressed as units. One unit is that amount of enzyme which hydrolyzes 1 mg. of starch in 1 hour at 37° and pH7 in phosphate buffer provided that not more than 44% of the starch is hydrolyzed. In this paper units are expressed per 1 ml. of enzyme. Preparations of low activity may be incubated longer and a corresponding factor employed. Strong preparations with activity above 35 units must be appropriately diluted with 0.9 per cent sodium chloride as described above.

RESULTS

Normal Values. It has been reported[34] that normal human serum amylase levels vary as much as 200 per cent from one individual to another.

In a series of 50 normal persons values by the present method ranged from 9.2 to 34.9 units. The average of these figures was 18 units; standard deviation ±5.32. No variations with sex or age were found. Multiple analyses of a given individual, examined at different times, gave more constant results, usually remaining within 10 units of one another.

Random urine samples varied in activity from 24.3 to 76.2 units with an average of 52.8. Dehydration, by limiting fluid intake, causes a rise in urinary amylase concentration which closely parallels the rise in urine specific gravity. Diuresis has the reverse effect. The dependence of amylase excretion on renal function has been repeatedly demonstrated. [7, 25, 28, 35, 36] Our limited experience with nephritic specimens supports the findings of these authors, that amylase activity is increased in the serum and decreased in the urine with decreased renal function.

Serum amylase levels were studied more thoroughly in one specific disease, diabetes mellitus. Much work has been reported on this subject using all amylase methods. Opinion has extended from that of Myers and Killian[7] who observed a significant increase in the serum, to that of Somogyi[37] who found a decrease in 33.4% of 382 diabetic sera to a level equalled by only 4.1% of a similar normal group. With our method we have been able to confirm the observation of Somogyi that the amylase values in diabetes mellitus are usually abnormally low. Thirty-six diabetic sera had an average activity of 13.8 units with a standard deviation of ±3.61.

SUMMARY

A simple accurate colorimetric method for the determination of amylase is described and the kinetics of starch hydrolysis by amylase as measured by this method are presented. The finding of a reduction in serum amylase in diabetes has been confirmed as an example of an application of this technic.

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