Recent Advances in Measurement of Amylase Activity—A Comparative Study

Richard A. Kaufman1 and Norbert W. Tietz2

We evaluated four kinetic amylase procedures with respect to kinetics, analytical range, blank rates, reagent stability, reagent impurities, interfering substances, and intrinsic sensitivities. Each of the methods is shown to have its own unique advantages and disadvantages. A preliminary discussion of some alternative methods, in which glycosidic p-nitrophenyl α-oligosaccharides are substrates, is included.

Additional Keyphrases: kinetic enzyme assay • enzymatic methods • "kit" methods compared • economics of laboratory operation

The determination of α-amylase (1,4-α-D-glucan glucoamylase; EC 3.2.1.1) activity in serum is a widely accepted test for the diagnosis of acute pancreatitis and obstruction of the pancreatic duct. Among the various methods advocated over the years, perhaps the most reliable has been the classic saccharogenic assay (1). This method, however, is very time consuming, the preparation of reproducible starch solutions is difficult, and sample blanks are frequently high. Various amyloclastic methods in use are convenient, but lack reliability. Methods involving dye-labeled starches are also convenient, but have a limited analytical range, and their results do not always correlate well with those of established procedures, especially in the abnormal range (see Figure 1).

Turbidimetric or nephelometric techniques have been proposed but have not found wide acceptance. Although fast and relatively simple, they require the measurement of a relatively small change in absorbance (AA) and, as with the saccharogenic assay, the preparation of starch solutions poses difficulties. Some of these methods also require special instrumentation.

With the increasing popularity of kinetic methods for measuring enzymic activity, we suggested in 1972 (2–6) a new approach involving a coupled enzyme assay. The basic principles are shown in reactions 1 through 3.

\[
\begin{align*}
\text{α-amylase} & \quad \text{Starch} \rightarrow \text{maltose} + \text{maltotriose} + \text{dextrins} \\
\text{α-glucosidase} & \quad \text{Maltose} + \text{maltotriose} \rightarrow \text{glucose} \\
\text{glucose oxidase} & \quad \text{Glucose} + \frac{1}{2} \text{O}_2 \rightarrow \text{H}_2\text{O}_2 + \text{δ-gluconolactone}
\end{align*}
\]

(1) (2) (3)

For each mole of glucose oxidized, 0.5 mol of O₂ is consumed. Thus, the rate of removal of oxygen from the solution, measured with a Clark electrode, is directly related to the amylase activity in the sample.

A second technique, an extension of the above, involves the same principle as shown in the previous equations except for the indicator reaction shown in equation 4 and the following modification. Hydrogen peroxide formed from endogenous glucose during preincubation is decomposed by catalase (EC 1.11.1.6). At the end of the preincubation period azide is added to block catalase activity, and the following indicator reaction is used:

\[
\begin{align*}
\text{H}_2\text{O}_2 + \text{ABTS (reduced)} & \rightarrow \text{H}_2\text{O} + \text{ABTS (oxidized)} \\
\text{H}_2\text{O} + \text{ABTS (oxidized)} & \rightarrow \text{H}_2\text{O} + \text{ABTS (oxidized)} (A_{\text{max} 410 \text{ nm}})
\end{align*}
\]

The increase in absorbance at 410 nm is a measure of the amylase activity.

During the 10-min preincubation used in either method, endogenous glucose is removed while the lag phase is overcome (2–6).

Since the introduction of these techniques, several variations have been developed, some of which are commercially available in kit form. We have investigated four of these kinetic amylase procedures, giving close attention to analytical range, linearity, blank reactions, and interferences by glucose and other sample constituents, thereby extending the work of previous investigators (7–11).

Materials and Methods

Absorbance measurements were made with Acta CIII and Model 25 spectrophotometers (Beckman Instruments, Inc., Fullerton, CA 92634). The cell compartment in the Acta CIII instrument was maintained at 30 or 37 °C with Beckman Model 1818 and 1818 ACC thermocirculator attachments. To obtain kinetic data for the acc amylase procedure (Du Pont Instruments, Wilmington, DE 19888), we removed the respective reagents from the reagent pack and recombined them in the same proportions as used in the acc procedure; the reaction was monitored at 37 °C, with either a GEMSAEC (Electro-Nucleonics, Fairfield, NJ 07006) or an Aminco IIIA (American Instrument Co., Silver Spring, MD 20910) centrifugal analyzer.

Glucose dehydrogenase (EC 1.1.1.47) was obtained from Beckman Microbes Division, Carlsbad, CA 92008; α-glucosidase was purchased from Boehringer Mannheim, Indianapolis, IN 46250; and mutarotase (EC 5.1.3.3) was purchased from Sigma Chemical Co., St. Louis, Mo 63178. Human hemoglobin was prepared from packed erythrocytes that had

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2 Nonstandard abbreviations used: G-6-PDH, glucose-6-phosphate dehydrogenase; PNP, p-nitrophenol; and ABTS, diaminonitromuconic acid.
The agastics, with Fig. of insert. due one-half and of nique. procedure decay glucose "Eskalab-Amylase" (8027) g/L amylase net mA/min "Eskalab-Amylase"

We determined amylase activity as described in the kit insert. Activity, by definition, is expressed in U/L, the amount of amylase activity that produces 1 μmol of maltose-min⁻¹·L⁻¹ of sample. Because 1 mol of maltose produces 2 mol of glucose, and hence 2 mol of NADH, the activity is thus defined as one-half of the amount of NADH formed. Endogenous glucose in the sample will be measured as well as the glucose produced due to amylase activity, potentially producing high initial absorbance readings.

Endogenous glucose up to 7 g/L was consumed in less than 7 min at 37 °C and in less than 6 min at 30 °C, i.e., well within the 25-min preincubation period. However, samples with high glucose values had a negative error in the measurement because NADH was unstable in the reagent (at 0.2 mmol/L the decay rate was -2.5 ΔmA/min, whereas at 0.07 mmol/L the decay was -1.0 ΔmA/min, both at 37 °C). High endogenous glucose concentrations produced a corresponding increase in the concentration of NADH and therefore a greater negative ΔmA/min change due to decay. Because this "negative" rate change in absorbance is continually being subtracted from the positive increase in absorbance due to amylase activity, the net absorbance is the sum of these two opposite rates. Thus, samples having high amylase activities and low or normal endogenous glucose concentrations will also have falsely low amylase values. Dilution of samples having glucose values >2.5 g/L is recommended by the manufacturer before assaying.

"α-Amyl-Harleco"  

The principle of this method (Harleco, Gibbstown, NJ 08027) is the same as in the Eskalab method, except that short-chain oligosaccharides (about 5 to 15 glucose residues) replace starch as the substrate. Again, we determined amylase activity (U/L) with this method according to the kit insert. Both endogenous glucose and glucose produced in the amylase reaction are measured, and samples in which glucose exceeds 3.0 g/L should be diluted. As much as 5.6 g of endogenous glucose per liter was fully metabolized within the preincubation period, and had no effect on measurements of amylase activity.

"Enzymatic Amylase DS"  
The Beckman Amylase DS procedure (Beckman Instruments Inc., Carlsbad, CA 92006) is more sensitive and displays better linearity of response to activity than their earlier and still-available method. The main difference between the two procedures is the substrate, which is maltotetraose in the DS method and starch in the previous method. An advantage of either procedure is the lack of interference by endogenous glucose. The sequence of reactions by which amylase activity is measured is as follows:

\[
\text{Maltotetraose} + \text{H}_2\text{O} \xrightarrow{\text{α-amylase}} 2 \text{maltose} \\
2 \text{Maltose} + 2 \text{PO}_4 \xrightarrow{\text{maltose phosphorylase}} 2 \text{glucose} + 2 \text{glucose} \xrightarrow{1\text{-phosphosphate}} 2 \text{1-glucose} \xrightarrow{\beta\text{-phosphoglucomutase}} 2 \text{glucose 6-phosphate} \\
2 \text{Glucose 6-phosphate} + 2 \text{NAD}^+ \xrightarrow{G-6\text{-PDH}} 2 \text{6-phosphogluconate} + 2 \text{NADH} + 2 \text{H}^+ \\
\]

Thus, for each molecule of substrate hydrolyzed, two molecules of glucose are ultimately measured, resulting in a twofold amplification. One unit of activity is defined as the amount of amylase activity per liter of sample that produces 1 μmol of NADH. Because 2 mol of NADH are produced for each mole of substrate hydrolyzed, the actual amylase activity (U/L) will be one-half of that obtained in the calculation suggested by the manufacturer.

In an extensive study of the metabolism of short-chain oligosaccharides by use of porcine pancreatic α-amylase, Roby and French (12) observed that 70% of maltotetraose was cleaved to two molecules of maltose and 30% was cleaved to glucose and maltotriose. If the amylase in serum would also cleave maltotetraose into glucose and maltotriose, then this portion of the activity would not be detected with the DS method. We examined the cleavage of maltotetraose by human salivary and pancreatic α-amylases with (a) the Beckman DS reagent and (b) the Beckman DS reagent to which was added glucose dehydrogenase (10 kU/L) and mutarotate (4 kU/L). Assay a measures the rate of production of β-glucose 1-phosphate, whereas assay b measures free glucose as well as glucose 1-phosphate. (Before performing the latter assay, we had to titrate with pyruvic acid the NADH formed from the oxidation of endogenous glucose present in the Beckman reagent, to regenerate NAD⁺.) A comparison of the rates of NADH formation between assays a and b indicated a ratio of about 2.1 for amylases from both sources, with the pancreatic enzyme giving a slightly higher value than the salivary enzyme. Thus the cleavage of maltotetraose by human pancreatic and salivary α-amylases appears to give predominantly two molecules of maltose.

Urine samples are known to contain the enzyme α-glucosidase at activities up to 35 U/L for normal individuals (13). Because α-glucosidase can slowly hydrolyze maltotetraose as

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6 These samples were supplied through the courtesy of Dr. David Bayse, Center for Disease Control, Atlanta, GA.
well as maltotriose and maltose, we investigated the possible interference by this enzyme. If maltotetraose were hydrolyzed by α-glucosidase to maltose, the latter would react with maltose phosphorylase to give glucose and glucose 1-phosphate, thus simulating amylase activity; hydrolysis of maltotriose to maltose and glucose would result in a similar interference. On the other hand, α-glucosidase could compete with maltose phosphorylase for the hydrolysis of maltose, which would result in a lower amylase activity. To examine the first mechanism we added 50 μL of α-glucosidase (100 U/L) instead of sample to 1.00 mL of reagent. The observed absorbance change (ΔA_{340 nm/min}) was insignificant, equivalent to about 1 U/L. To examine the second mechanism, we measured the amylase activity of a serum sample with and without 100 U of α-glucosidase per liter of the reaction mixture. Because there was no measurable difference in the ΔA_{340 nm/min} between the two measurements, we concluded that α-glucosidase does not significantly interfere.

"αca Amylase"

This procedure (Du Pont) was among the first to include the use of a well-defined amylase substrate, maltopentaose, which is hydrolyzed by amylase to give maltose and maltotriose. These two products are hydrolyzed by α-glucosidase to five molecules of glucose, which are measured by the hexokinase technique. Thus, for each molecule of substrate hydrolyzed by α-amylase, five molecules of glucose are ultimately measured, resulting in a fivefold amplification. Activity is expressed in U/L, i.e., that amount of amylase activity that hydrolyzes 1 μmol of maltopentaose-min⁻¹-L⁻¹ of sample. The calculation takes into account the fivefold amplification. Glucose interference is eliminated by passing the sample through a gel filtration column in the amylase pack header before assay. This treatment also removes other molecules of low molecular mass, some of which are potential inhibitors, as will be discussed.

**Results and Discussion**

A comparison of these methods (Table 1) makes it readily apparent that each approach has its distinct advantages and disadvantages. Amylase activity measurements have still not reached the highest state of sophistication, even though significant progress has been made. Some of the methods we outline later could potentially eliminate some of the deficiencies apparent in the four methods we examined.

**Sample volume.** The sample volume required for the methods varies from 10 μL for the Eskalab method to 100 μL for the Du Pont procedure. The relatively large amount of sample used in the latter method is apparently necessary because of the large reagent volume and the necessity of obtaining an adequate absorbance difference between the test and the excessively high reagent blank (Table 1 and Figure 2).

**Sample volume fraction.** The ratio of sample volume to
reagent volume differs greatly among the methods and reflects the differences in the inherent sensitivities of the individual methods (see below); it also reflects the need for small sample sizes with methods that detect endogenous glucose.

Substrate. Starch is generally considered the natural substrate of amylases and thus has been used in assay procedures for many years. It is rapidly hydrolyzed, as can be seen from the number of micromoles of products formed per minute per liter of specimen. With the Somogyi saccharogenic method, the upper normal value is 290 U/L vs only 80 U/L for the Du Pont method, in which maltopentaose is the substrate, and 55 U/L for the Beckman DS procedure, in which maltotetraose is used. (The Beckman insert gives 110 U/L as upper limit, but this figure is based on an inappropriate calculation of the activity.) The Harleco and Easkalab methods cannot be accurately compared in this scheme because hydrolysis products with longer chains than maltotetraose are not detected with these methods. Thus, the units given for these two procedures are lower than the actual enzyme activities, although this is partly offset by the variable amplification resulting from the \( \alpha \)-glucosidase hydrolysis of maltotriose and maltotetraose. The reasons for the greater apparent activity of the four methods we investigated are the multiplicative factor obtained and (or) the high molar absorptivity of NADH, which is used as an indicator of amylase activity. Starch, on the other hand, has the significant disadvantages of being an ill-defined substrate with limited stability and giving curvilinear kinetics in the coupled enzyme assay reviewed here. We have also observed that starch may inhibit \( \alpha \)-glucosidase.\(^7\)

The shorter-chain substrates used in the Du Pont and Beckman DS methods have the advantage of being well-defined and stable; furthermore, international units (U) of amylase activity can be calculated directly.

Reagent blank and reagent stability. Easkalab Method. The blank reaction rates at 37 °C for a freshly reconstituted reagent were as much as 1 ΔmA/min or 12 U/L (reference range for the method: 10-90 U/L), depending on the reagent lot. During storage, however, the blank rate decreases, becoming negligible at 40 h. This decrease appears to be due to the hydrolysis of small amounts of short-chain dextrins in the substrate, which are hydrolyzed by the \( \alpha \)-glucosidase in the reagent. This conclusion is supported by the observation that the blank absorbance increases slightly, presumably because of NADH formation from glucose oxidation.

The results of amylase measurements performed with sera exhibiting a wide range of amylase activities were essentially identical whether the reagent was freshly reconstituted, left at room temperature for 8 h, or stored at 4 °C for 48 h. This confirms the manufacturer’s claim.

Harleco Method. The reagent blank remains essentially constant during 24 h at 4 °C with a ΔmA\text{340 nm}/min of about 2 to 2.5, which is equivalent to 19-24 U/L (reference range for normal sera: 11-91 U/L). During storage the initial absorbance of the reagent blank increases from about 0.3 to 0.6 within 24 h at 4 °C or within 4 h at room temperature, mainly because of the formation of NADH from glucose oxidation.\(^8\) This increases the starting absorbance and narrows the analytical range of the method.

The reagent was stable for 24 h at 4 °C or at room temperature for 4 h, exhibiting before and after storage the same sensitivity with sera having various amylase activities. The manufacturer’s earlier claim, however, that the reagent could be frozen and thawed twice over a four-day interval could not be confirmed by us or others (7). After one thawing, the results were significantly lower (15–20%) than the results obtained with the same sera assayed with freshly reconstituted reagent. Harleco has deleted this claim in their most recent package insert.

Beckman DS Method. A minimal reagent blank (≤0.5 ΔmA\text{340 nm}/min) was detected, and remained constant if the reagent was stored for 24 h at 4 °C or left at room temperature for 8 h.

To investigate reagent stability, we assayed various dilutions of serum having high amylase activity by using reagents that were (a) freshly prepared, (b) left at room temperature for 8 h, or (c) stored at 4 °C for 24 h. The activity in all experiments was essentially identical, indicating good reagent stability under these conditions.

Du Pont Method. The reagent blank was rather large (about 0.06 ΔmA\text{340 nm}/min) and exhibited nonlinear kinetics over the entire 8-min interval that the reaction was monitored (see Figure 2). Speculation that this was due to contamination of the substrate with trace amounts of shorter-chain oligosaccharides (i.e., \( G_3 \) and \( G_4 \)) that were hydrolyzed during the preincubation and reaction periods has recently been confirmed by Du Pont.\(^6\) Sample measurements, however, are reproducible because the timing of the preincubation and measurement intervals, which is performed automatically, is extremely precise.

Analytical range and reaction rate linearity. The Easkalab method was linear to about 600 U/L (ΔA/min = 0.05) at 30 °C as well as 37 °C. The kinetics was claimed to be linear for 15 min beyond the 25-min preincubation period; however, we

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\(^{7}\) Kaufman, R. A. and Tietz, N. W., unpublished observations.

\(^{8}\) Experiments showed that adding pyruvate and lactate dehydrogenase to the reagent resulted in an immediate decrease in absorbance at 340 nm, supporting the suggestion that NADH was formed during storage.

Hickey, M. E., A discussion of the kinetics of the \( \alpha \)-amylase method. \textit{aca Update} 1, 2 (1978).
observed linearity for only 5 to 10 min (Figure 3). The degree of nonlinearity differed with different sera, becoming more evident in the samples having high activity. Thus the reaction rate does not always follow zero-order kinetics.

The Harleco method was linear to at least 1000 U/L (0.10 ΔA 340 nm/min), which agrees with the manufacturer's claim. The reaction rate curves, however, show a slight upward curvature, which becomes more evident with samples with increased amylase activity (Figure 4). Thus the reaction rate, as with the Eskalab procedure, does not strictly follow zero-order kinetics.

The Beckman DS method was linear to at least 600 U/L (0.18 ΔA 340 nm/min), as claimed by the manufacturer. Zero-order kinetics were observed at all amylase activities within this range (Figure 5).

We found the analytical range of the Du Pont method to extend to at least 600 U/L (ΔA 340 nm/min = 0.38); however, we found a significant decrease in the ΔA 340 nm/min with time, similar to that observed with the blank (see Figure 2).

The linear analytical ranges for all four methods are summarized in Table 1.

Reagent purity. Each of the reagents, except those for the Du Pont method, were tested for contamination with lactate dehydrogenase (EC 1.1.1.27), α-hydroxybutyrate dehydrogenase (EC 1.1.1.30), and glucose phosphate isomerase (EC 5.3.1.9). (In the Du Pont procedure the substrates for these enzymes are removed, so no interference would be expected from them.) To detect dehydrogenase activity, we added NADH and either pyruvate or acetoacetate to the reagent and compared the ΔA/min observed with that of the reagent to which only NADH had been added (enough to raise the absorbance by 1 absorbance unit). Glucose phosphate isomerase activity was tested by adding fructose to the reagent and comparing the ΔA/min with the reagent blank rate. For these experiments, an aqueous aliquot of the respective metabolites (pyruvate 1 mmol/L, acetoacetate 40 mmol/L, and fructose 30 mmol/L) equal to the respective sample size for each procedure was added to the normal reagent mixture at the assay temperature suggested by the manufacturer.

None of the rates were affected by the addition of acetoacetate; however, after addition of pyruvate to the Beckman DS reagent, there was a precipitous drop in the absorbance at 340 nm. The change in absorbance corresponded to the nanomoles of pyruvate added, indicating that the reagent contained a significant amount of lactate dehydrogenase activity. Others have made similar observations with the earlier Beckman amylase reagent (14) and more recently with the Beckman DS reagent (15). A positive interference by fructose was observed with the Harleco reagent only, confirming a similar observation by Porter and Roberts (7); fructose equivalent to 600 mg/L increased the blank rate by approximately 2 ΔmA/min (21 U/L).

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Fig. 3. Reaction rate curves of Eskalab amylase method
Test conditions as in Fig. 2.

Fig. 4. Reaction rate curves of Harleco amylase procedure
Test conditions: 30 °C; rest as in Fig. 2.

Fig. 5. Reaction rate curves of Beckman DS amylase procedure
Test conditions as in Fig. 2.
Interferences. Ten serum samples, selected without conscious bias, were supplemented with pyruvate (1 mmol/L), acetocetate (10 mmol/L), hemoglobin (1.0 g/L), or fructose (300 mg/L) and then assayed by each of the methods. Pyruvate had a negative interference in the Beckman DS and Harleco procedures, because of endogenous lactate dehydrogenase in the sample and lactate dehydrogenase contamination in the Beckman DS reagent. The degree of interference depended on the pyruvate concentration, the lactate dehydrogenase activity (in sample and reagent), and the amylase activity (NADH produced by amylase activity is required for pyruvate reduction). This interference was not observed in the Eskalab method, probably because of the small sample size, the long preincubation, and the low reaction pH. In the Du Pont method pyruvate is removed by the gel-filtration column in the pack header.

Hanson and Yasmineh (14) and Lorentz (10) found that the keto acid problem can be overcome by replacing NADP+ with NADPH+, because NADPH reacts less readily with lactate dehydrogenase and keto acids. We have found that adding semicarbazide also effectively eliminates keto acid interference. The semicarbazones form relatively rapidly (≤5 min at pH 6.6 and 37 °C), and the semicarbazide (at 40 mmol/L) does not appear to affect amylase activity.

Fructose in the sample gave a positive interference with the Harleco procedure because of glucose phosphate isomerase in the reagent. Acetocetate and hemoglobin did not interfere with any of the assays.

NADH stability. The stability of NADH in the respective reagents varied among the four procedures, being least in the Eskalab reagent (see earlier comments on endogenous glucose under Eskalab procedure). In the Harleco reagent, the NADH was moderately stable (at 0.2 mmol/L, ΔM A/min was −0.8).

The NADH decay in the Beckman DS reagent depended on the amylase activity in the sample, but was small in relation to the NADH produced in the reaction; thus the effect on the results had no clinical significance. In the Du Pont reagent, the decay of NADH (at 0.2 mmol/L) was also −0.8 ΔM A/min, which is within the error of the method.

Log phase and total analysis time. The lag phases are similar for the methods (4 to 5 min), except in the Eskalab procedure, where it is 25 min. This long lag phase in the Eskalab method contributes to its longer total analysis time (Table 1).

Sensitivity. The absorbance change per unit of enzyme activity (ΔM A·min·L−1·L−1) is lowest for the Eskalab method (0.082) and highest for the Du Pont procedure (0.596). The relative intrinsic sensitivities for the four methods were calculated by assaying the amylase activity (ΔM A/min) of six sera by each of the four methods, and then “normalizing” these values by calculating the ΔM A/min that would be obtained if the assay methods all had the same sample/volume ratio. By these criteria, the Beckman DS method was least sensitive and was arbitrarily set to 1.0. The Eskalab procedure had a relative sensitivity of 1.3, the Harleco method 2.0, and the Du Pont method 3.9. However, depending on the isoenzyme composition of the individual samples, these figures varied slightly, as expressed by the range given in Table 1. The isoenzyme bias became very apparent when we assayed an Ortho abnormal control serum (Ortho Diagnostics, Raritan, NJ 08869), which contains porcine pancreatic amylase.

Substrate–isoenzyme bias. The relative bias of the individual methods was investigated by determining the activity ratio obtained with purified samples of human salivary and human pancreatic amylases. Although the salivary enzyme appeared to give slightly faster hydrolysis rates than the pancreatic enzyme with the Eskalab and Beckman DS methods (ratio for both, 1.1), as compared to the Du Pont and Harleco procedures (ratio for both, 0.9), this difference is of little clinical significance. The Somogyi saccharogenic method (1), which we also used for comparison, gave a ratio of 0.9.

Precision studies. Day-to-day and within-day precision studies were determined for each method with Ortho normal and abnormal control sera. The results are tabulated in Table 2.

The Beckman DS procedure gave the smallest standard deviation (SD) and coefficient of variation (CV), probably because of the combination of the relatively large sample size (50 mL), large ΔA/min, and low reagent blank. Conversely, the Eskalab procedure had the least precision and largest CV, possibly because of the small volume (10 μL) and small ΔA/min.

Cost per test. The reagent cost per test currently ranges from $0.93 for the Beckman DS method to $2.45 for the Du Pont procedure. These figures, however, are somewhat misleading. The Du Pont procedure, although the most costly in terms of reagents, requires the least operator intervention and therefore has a relatively low labor cost. On the other hand, with the Beckman DS method the use of 1.0 mL of reagent is assumed, but many spectrophotometers require a larger sample size, which increases the cost for this procedure.

Methods Involving Chromogenic Substrates

The assays we evaluated are complex and involve several coupled-enzyme reactions. In addition, the indicator reaction in them all involves production of NADH, which is subject to a series of potential interferences, as has been shown. Furthermore, the Harleco and Eskalab methods measure endogenous glucose and glucose produced in the amylase reaction, which often leads to unacceptably high absorbance readings and may require predilution of the sample even for samples with normal amylase activity.

Though these methods represent significant methodological advancements in the measurement of amylase activities, efforts will be continued to develop a system in which amylase activity would be measured more directly, thus eliminating many of the potential interferences and inconveniences. Most such efforts have centered around the use of p-nitrophenyl glycosides, which produce free p-nitrophenol (PNP), either through direct hydrolysis by amylase or through a coupled reaction involving α-glucosidase and β-glucosidase (EC

Table 2. Precision Data for Four Amylase Procedures

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Precision within-run</th>
<th>Precision within-run</th>
<th>Day-to-day</th>
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<td></td>
<td>&quot;Ortho Normal&quot;</td>
<td>&quot;Ortho Abnormal&quot;</td>
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<tr>
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<td>Mean, U/L</td>
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<td>468.6</td>
<td>459.0</td>
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<td>20.4</td>
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<td>CV, %</td>
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<tr>
<td>Harleco</td>
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<td>CV, %</td>
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<tr>
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<td>CV, %</td>
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<tr>
<td>Du Pont aca</td>
<td>Mean, U/L</td>
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<td>400.6</td>
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<tr>
<td></td>
<td>SD, U/L</td>
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<td>18.4</td>
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<tr>
<td></td>
<td>CV, %</td>
<td>4.6</td>
<td>0.9</td>
<td>2.4</td>
</tr>
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</table>

n = 10 for each determination.
Hall has proposed a kinetic assay involving p-nitrophenyl α-maltoheptaoside synthesized enzymatically from α-cyclodextrin and p-nitrophenyl α-glycoside.

Although no detailed data on any of these methods are currently available, these procedures appear to offer distinct advantages over the NADH-coupled enzyme reactions. Among the methods involving p-nitrophenyl glycosides, the most convenient may be those that allow for direct release of PNP by amylase action without the involvement of glucosidases. Additional experiments will be required to evaluate further the merits of these various procedures, particularly the relative specificity towards the pancreatic as opposed to the salivary amylase. The only apparent disadvantage of either of these methods is that PNP at pH 7.0 is only about 50% in the quinoid form; this will necessitate strict control of the pH of the reaction mixture. Nevertheless, these methods offer advantages of simplicity over present methods, and decades of frustration with amylase activity measurements may finally come to an end.

References


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Fig. 6. Reaction rate curves of amylase assay with p-nitrophenyl maltotrioside

Test conditions: 0.50 mL reagent containing phosphate buffer (100 mmol/L, pH 6.9), p-nitrophenyl maltotrioside (about 15 mmol/L, the substrate preparation containing some maltotriose and small amounts of other unidentified contaminants); serum about twofold upper reference range.

3.2.1.21). In 1958, Jansen and Wydeveld (16) synthesized p-nitrophenyl α-maltoside, which they used to determine amylase activity in a two-point assay by quantitating the amount of PNP released. Gillard et al. (17) utilized the same substrate in the continuous-monitoring technique for measuring salivary amylase; this substrate, however, had inadequate sensitivity for serum amylase measurements (16). Simultaneously with us, several researchers attempted to improve this assay by using glycosides with longer carbohydrate chains. Wallenfels et al. (18) prepared a series of glycosicid p-nitrophenyl α-oligosaccharides with the aid of microbial enzymes; they showed that amylase hydrolyzes PNP most rapidly from the maltotriose glycoside, which is highly selective for pancreatic over salivary amylase. For our investigations we synthesized p-nitrophenyl α-maltotrioside chemically, using modifications of procedures for preparing α-glycosides (19, 20). An example of an assay with this substrate is given in Figure 6. Note the minimal lag phase at the beginning of the reaction as well as after the addition of 10 μL of saliva.

Driscoll et al. (21) and Gargiulo et al. (22) synthesized a mixture of glycosidic p-nitrophenyl oligosaccharides (predominantly G4 to G10) and proposed a two-point assay based on the following reaction:

\[
\text{PNP-(glucose)}_{4-10} \rightarrow \text{PNP-(glucose)(4-10)-n} + (\text{glucose})_{n}
\]

(9)

\[
\text{PNP-(glucose)}_{1-3} \rightarrow \alpha-\text{and } \beta-\text{glucosidase} \rightarrow \text{PNP + glucose}
\]

(10)

In this procedure, amylase hydrolyzes the longer-chain p-nitrophenyl glycosides to shorter-chain p-nitrophenyl glycosides, which are then hydrolyzed by α- and β-glucosidases to produce free PNP.


