Factitiously Low Amylase Values

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When a commercial substrate was used in the determination of serum amylase activities of patients with acute pancreatitis, results much higher than those reported by the manufacturer with similar patients were found. A failure to quantitate all of the reducing groups liberated during incubation may account for the lower results. A modified saccharogenic method has been developed, which, by increasing the ratios of starch to enzyme and copper to reducing sugars, considerably extends the range of the determination. Unequivocally elevated serum and urine amylase values can be measured without repetition of all or part of the test and with good precision.

Recent studies of saccharogenic amylase methods (1, 2) stress the importance of using sufficient substrate to maintain maximum reaction velocity during incubation. This paper presents evidence that commonly used spectrophotometric copper reduction methods for the determination of reducing groups in postincubation mixtures have been misapplied, leading to factitiously low amylase values. A modified saccharogenic method, which permits analysis of most pathologic human sera or urines without repetition of either incubation or copper reduction steps and without sacrifice of precision in the normal range, is described.

Materials

Methyl and propyl p-hydroxybenzoates were obtained from Eastman Organic Chemicals Department, tris (hydroxymethyl) aminomethane ("Sigma 121" or Tris) from Sigma Chemical Co., soluble starch (Lintner) and D-glucose from Fisher Scientific Co., and D-maltose hydrate from Nutritional Biochemicals Corp. Krautman's Stable

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Buffered Starch and Krautman's Special Deproteinizing Reagent were purchased from U. S. Scientific Co., Brooklyn, N. Y. All other chemicals are ACS reagent grade.

Blood and urine specimens from hospitalized patients and Abnormal Clinical Chemical Control Serum (Hyland Laboratories) served as sources of high and normal amylase activity.

Solutions used in this study are as follows:

**Glucose or maltose standards.** Dissolve the desired quantity of D-glucose or D-maltose hydrate in 0.02 M benzoic acid-sodium benzoate buffer, pH 5.2.

5% Zinc sulfate (3)
0.3 N Barium hydroxide (3)
Somogyi copper reagent (3)
Arsenomolybdic acid reagent (3)
10% Sodium tungstate (Na₂WO₄·2H₂O)
0.667 N Sulfuric acid
Folin-Wu copper reagent (4)
Phosphomolybdic acid reagent (4)
Henry-Chiamori starch substrate (1)

1 M Tris-HCl buffer, pH 7.2 Flocculation was observed after several weeks at room temperature. The solution is stable at 4°. 1 M Tris-HCl buffers of other pH values were also prepared for running a pH-activity curve.

0.1 M Tris-HCl buffer, pH 7.2
0.1 M Phosphate buffer, pH 7.0 (1)

**Substrate A.** Heat 950 ml. of 0.1 M phosphate buffer, pH 7.0 to boiling. Add a slurry of 15 gm. of soluble starch, 1.35 gm. of methyl p-hydroxybenzoate, 0.27 gm. of propyl p-hydroxybenzoate, and 2 gm. of NaCl in 35 ml. of cold buffer and remove heat. Use an additional 15 ml. of cold buffer to transfer any remaining slurry. Stir, cool, and discard scum. The concentrations of preservatives are those used by Marsters et al. (5).

**Substrate B.** Prepare the same as A, but omit NaCl and substitute 0.1 M Tris-HCl buffer, pH 7.2 for phosphate buffer.

**Substrate C.** Prepare the same as A, but omit NaCl and substitute water for phosphate buffer. Store at room temperature and discard after one month.

**Methods**

In addition to modifications described later, the method of Folin and Wu (4), its Tonks modification (6), and the method of Nelson and
Somogyi as described by Annino (3), were used to determine reducing sugars.

In addition to modifications described later, the amylase methods of Henry and Chiamori (1), Krautman (7), and Somogyi (8) were used. With all three, reducing sugars were determined by the Folin and Wu method (4). In applying the Henry and Chiamori and Somogyi procedures, incubation was repeated with less enzyme solution if results over 700 units were observed; copper reduction was repeated with less deproteinized incubation mixture if results over 400 mg./100 ml. of glucose equivalent were observed. The Krautman procedure utilizes a starch substrate and deproteinizing reagent whose exact compositions are undisclosed. Whereas incubation and deproteinization are described in stepwise fashion, the directions state only that the deproteinized incubation mixture is subjected to a blood sugar determination "in the usual way." No limiting value beyond which either the copper reduction or incubation steps should be repeated is stated. In applying the Krautman method, unless otherwise indicated, only one incubation and one copper reduction was carried out per specimen.

All incubations were at 40 ± 0.1°. Measurements for pH were made at 25° with a Radiometer pH Meter 22.* Spectrophotometric measurements were made with a Bausch and Lomb Spectronic 20 spectrophotometer† in 1/2-inch tubes. When absorbance readings exceeded 0.7, solutions were diluted with reagent blank or water and reread. Water was substituted for reagent blank as a diluent only after establishing that the slope and linearity of the standard curve remained unchanged and that the stability of the colored product was not adversely affected.

**Results**

*Determination of High Amylase Activities with a Commercial Kit*

This experiment was intended to clarify two puzzling features of the Krautman procedure. First, an incubation time of 45 min., instead of the usual 30 min., is used. In addition, the following note appears: "'The serum of 56 known acute pancreatitis patients yielded results from 189 to 300 units of amylase; 150 blood serums from known normal patients yielded results from 70 to 160 units.'" (7) Since the Krautman unit is calculated like the commonly employed Somogyi saccharogenic unit (8), one should anticipate results 50% higher by the Kraut-
Table 1. Comparison of Amylase Activities in Abnormal Patients' Sera
as Determined by Krautman and Henry & Chiamori Methods

<table>
<thead>
<tr>
<th>Serum</th>
<th>Glucose Conc. (mg-% Folin-Wu)</th>
<th>Amylase (mg-% Glucose Equivalent) Method A</th>
<th>Method B</th>
<th>Method C</th>
<th>C/A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>339</td>
<td>710</td>
<td>29</td>
<td>910</td>
<td>1.28</td>
</tr>
<tr>
<td>2.</td>
<td>145</td>
<td>1020</td>
<td>238</td>
<td>1480</td>
<td>1.45</td>
</tr>
<tr>
<td>3.</td>
<td>190</td>
<td>760</td>
<td>152</td>
<td>1150</td>
<td>1.51</td>
</tr>
<tr>
<td>4.</td>
<td>124</td>
<td>480</td>
<td>234</td>
<td>640</td>
<td>1.33</td>
</tr>
<tr>
<td>5.</td>
<td>105</td>
<td>1080</td>
<td>294</td>
<td>1450</td>
<td>1.34</td>
</tr>
<tr>
<td>6.</td>
<td>150</td>
<td>630</td>
<td>257</td>
<td>960</td>
<td>1.52</td>
</tr>
</tbody>
</table>

** Method A - Henry and Chiamori (1)
*** Method B - Krautman (6)
**** Method C - Single Krautman incubation, but reducing sugars assayed on smaller aliquots of filtrates to insure complete quantitation. (see text)

Parallel amylase determinations, by the methods of Henry and Chiamori and Krautman, were performed on 6 patients' sera in which high activity had previously been demonstrated amylaciastically. Results in Table 1 show much lower values by Krautman's method (Column B), however, especially in hyperglycemic sera; when the sugar determination was repeated on smaller aliquots of the same deproteinized incubation mixtures, the values shown in Column C were obtained. These exceed the Henry and Chiamori results by an average of somewhat less than 50%. Analysis of two of the same sera by the Somogyi method gave results in good agreement with those in Column A. Repetition of two of the Krautman determinations with different batches of commercially prepared substrate and deproteinizing reagent yielded practically the same values shown in Columns B and C.

Next a Hyland Abnormal Clinical Chemical Control Serum, having a labeled amylase activity of 925 Somogyi units per 100 ml., was incubated with Krautman's Starch in a series of test tubes. Krautman's Deproteinizing Reagent was added at 5-min. intervals from 0 to 60 min. and reducing sugars determined in the filtrates by the Folin and Wu procedure. Copper reduction was repeated on smaller amounts of filtrate when glucose equivalents greater than 400 mg./100 ml. were obtained. The resulting curve (Fig. 1), is linear to more than 800 mg./100 ml. Repetition with a different batch of the commercial substrate gave
virtually a superimposable curve. From these results and for reasons discussed later it seems reasonable to attribute the low amylase activities observed by Krautman to the procedure or procedures used for the determination of reducing sugars.

**Fig. 1.** Measurement of rate of liberation of reducing sugars from Krautman's substrate by a high-activity control serum.

**Copper Reduction Methods**

The copper reduction method used to analyze incubation mixtures should, without repetition on smaller aliquots, measure the sum of reducing groups liberated to the point at which their rate of formation decreases, glucose originally present in strongly hyperglycemic sera, and reducing sugars originally present in the substrate. Thus if zero-order amylolytic activity extends beyond 1000 units per 100 ml. of enzyme solution, a suitable sugar method should measure more than 1500 mg./100 ml. of glucose equivalent without repetition on smaller aliquots, while retaining precision in the range of low sugar concentrations. For the determination of urine amylase activities, an even wider range sugar method is desirable. The Folin-Wu and Nelson-Somogyi standard curves at high glucose concentrations are shown in Fig. 2. Leveling is observed near 400 mg./100 ml. in the original and modified Folin-Wu curves and below 800 mg./100 ml. in the Nelson-
Somogyi curve. None of these methods therefore covers the desired range.

The useful portions of the curves can be extended by increasing the ratio of copper to reducing sugar, either by using more copper or less sugar solution. The loss in sensitivity accompanying the latter alternative might be obviated by either omitting the final dilution (10) or by measuring absorbance at a higher wave length (11). Both possibilities were examined.

A Tonks curve was run using one-fifth the amount of standards and omitting the last dilution, making a final volume of 6 rather than 25 ml. A similarly modified Nelson-Somogyi curve was run (final volume 4 rather than 25 ml). Results shown in Fig. 3 indicate good linearity for the latter, slightly less so for the former. The modified Nelson-Somogyi curves were read at 490 (3) and at 540 m$\mu$ (10). The higher wave length provides greater sensitivity without sacrificing linearity and without appreciably elevating the absorbance of the blank. Additional glucose standards of higher concentration were run and no appreciable deviation from the Beer-Lambert law was observed below 4000 mg./100 ml. The added sensitivity afforded by measuring absorbance at 540 m$\mu$ permits a further reduction of the amount of sugar solution taken for assay by a factor of 2, thus extending the linear portion of the curve to over 8000 mg./100 ml. In Fig. 3, 4, and 5, the ordinate represents the product of the observed absorbance and the dilution factor.

Fig. 2. Appearance of glucose standard curves up to 2000 mg./100 ml as run by three commonly used blood glucose methods.
Next, the Nelson-Somogyi modification suggested by Teller (11) was tried. The volumes of glucose standards, Somogyi copper reagent, and arsenomolybdic acid reagent were in the ratio of 0.02:1:1 ml., in a final dilution of 25 ml. Spectrophotometric measurements at 660 mµ produced the curve in Fig. 4. The relationship between concentration and absorbance is virtually linear throughout the range. However, the sensitivity (change in absorbance per unit increase in concentration) is rather low. These results indicate that, although any of the Tonks or Nelson-Somogyi modifications described in this section might fulfill the previously noted criteria for suitability in the amylase determination, the procedure in which the final dilution step is omitted and spectrophotometric measurements are made at 540 mµ appeared most promising.

Development of Extended-Range Amylase Procedure

Next, application of the extended-range Nelson-Somogyi modification to the determination of reducing substances in deproteinized incubation mixtures was attempted. Tentative decisions concerning sub-
strate and buffer concentrations and the nature of the buffer and deproteinizing agents had to be made at this time. Since deviation from zero-order kinetics is anticipated near 700 amylase units in either the Henry and Chiamori or Somogyi incubation mixtures (1, 2), and since considerably higher activities are not infrequently observed, the ratio of starch to serum or urine was increased from 75 to 150 mg./ml., thereby approximately doubling the linear reaction range. Since the need for adequate buffering, particularly for urinary amylase determinations, has been demonstrated (1), Substrate A was used in early experiments. Alkaline zinc deproteinization was considered the method of choice since clearer filtrates are obtained (3), nonsugar-reducing substances in urine are effectively removed (11), and the required reagents are available in laboratories performing the Nelson-Somogyi procedure.

As a prelude to attempting actual incubations, a glucose standard curve was run in the presence of substrate and deproteinizing reagents. Centrifugation of mixtures of 0.1 ml. of glucose standards, 1 ml. of Substrate A, 2 ml. each of 5% zinc sulfate and 0.3 N barium hydroxide, and 4.9 ml. of water gave clear supernatants. Halving the amounts of deproteinizing reagents resulted in somewhat turbid supernatants. Mixtures of 1 ml. each of supernatants, water, and Somogyi copper reagent were heated in a boiling water bath for 15 min.
Addition of 1 ml. of arsenomolybdic acid reagent produced clear solutions. Spectrophotometric readings were made at 540 mμ. The resulting curve showed a slight upward concavity and about 13% decrease in slope compared with that obtained with aqueous standards. Repetition with Substrate B produced full color and a linear curve, but a very high blank. Using Substrate C and replacing 0.1 ml. of water with an equal volume of 1 M Tris-HCl buffer, pH 7.2, a curve of identical shape and slope was obtained, but with a much lower blank. Therefore, in subsequent work, buffer and substrate were prepared and stored separately. Another standard curve was run in the presence of 1.8 instead of 1 ml. of Substrate C. Although good linearity and a low blank were obtained, some turbidity was noted in the final colored solutions.

A maltose standard curve, run in the presence of Substrate C, tris buffer, and deproteinizing reagents, is shown in Fig. 5. Unlike Henry and Chiamori's concave curve (1), a nearly straight line was produced throughout the range. Anhydrous maltose, as compared to an equal weight of glucose, is capable of reducing 47% of the amount of cupric reagent under the described conditions. In another experiment in which the reaction time for the copper reduction was varied, 15 min. in an actively boiling water bath was found to be adequate for maltose. This result does not rule out the necessity for prolonging the heating cycle to oxidize higher molecular weight sugars (2); nevertheless the 15-min. reaction time was adopted and has proved satisfactory when applied to incubation mixtures.

![Graph](image-url)  
**Fig. 5.** Maltose standard curve run in presence of substrate and deproteinizing reagents.
Tubes containing 1 ml. of day-old Substrate C, 0.1 ml. of 1 M Tris-HCl buffer, pH 7.2, and 0.1 ml. of commercial control serum having a labeled activity of 925 Somogyi units, were incubated at 40° for times varying from 0 to 90 min. At zero time and at 10-min. intervals, the reaction was stopped by zinc deproteinization. Quantitation of reducing sugars by the modified Nelson-Somogyi technic described above produced the plot shown in Fig. 6. The liberation of reducing groups proceeded at a constant rate to about 1400 mg./100 ml. of glucose equivalent, as expected (1). Thereafter the rate decreased sufficiently slowly to permit measurement of activities up to 3000 mg./100 ml., with an error of less than 8%. Upon repetition of this experiment with 45-day-old substrate, which showed no evidence of mold or bacterial decomposition, the same initial velocity was observed, although the rate began to drop sooner (at about 1000 mg./100 ml.) and more steeply. The activity loss may possibly be attributed to a decrease in concentration of available substrate, resulting from micelle formation (13). It is recommended, therefore, that the substrate not be stored for longer than one month.

The change from phosphate to Tris buffer improved the quantitation of reducing groups. Before using Tris in the amylase assay, additional data had to be obtained, namely the determination of pH of
maximum activity, comparison of reaction rates in the two buffer systems, and measurement of buffering capacity. A pH-activity curve was run by incubating 0.1 ml. of highly active serum for 30 min. at different pH's with 1 ml. of Substrate C and 0.1 ml. of 1 M Tris-HCl

buffer. The resulting curve (Fig. 7) is skewed upward and has a rather broad maximum centering about pH 7.2. In another experiment several sera and strongly acidic or alkaline urine specimens were mixed with 1 ml. of Substrate C and 0.1 ml. of 1 M Tris-HCl buffer, pH 7.2; the pH values of the incubation mixtures were measured before and, in some cases, after incubating for 30 min. at 40°. The pH values of mixtures of equal volumes of supernatants after deproteinization, water, and Somogyi copper reagent were also recorded in several instances. Data summarized in Table 2 indicate that buffering is probably adequate under the most extreme conditions likely to be encountered in routine work, despite the pH of incubation being somewhat more remote from the pK of the Tris than from the phosphate system. In another experiment, initial velocities were determined with the same serum, with freshly prepared starch substrate, and with a constant chloride concentration, in the presence of either Tris or phosphate buffers of the same molarity. In measuring reducing groups, internal glucose standards were used to compensate for the decreased color developed.
in the presence of phosphate. Lineweaver-Burk plots of the two sets of data displayed identical slopes and intercepts, within the limits of experimental error. Finally, four batches of Hyland control sera having labeled activities between 600 and 1000 Somogyi units and four sera from patients without pancreatic disease were assayed using Substrates A and B. No significant differences were observed. Higher activities with Tris have been reported with amylolastic (14) and turbidimetric (15) procedures.

The ability of zinc sulfate to stop the amylase reaction was confirmed by incubating 0.1-ml. aliquots of highly active control serum at 40° with 0.1 ml of Tris-HCl buffer, pH 7.2, 1 ml of Substrate C, and 2 ml of 5% zinc sulfate for times ranging from 0 to 30 min. Deproteinization was completed and reducing sugars measured by methods described previously. No amylase activity was detectable.

Selected Method

A routine saccharogenic amylase determination, evolved from foregoing experiments is summarized in stepwise fashion:

1. Pipet 1 ml of Substrate C and 0.1 ml of 1 M Tris-HCl buffer, pH 7.2, into four 15-ml centrifuge tubes and place in a 40° water bath.
2. Add 0.1 ml of water to the first tube (RB) and 0.1 ml of 200 mg./100 ml. glucose standard to the second (St) and mix.
3. Add 0.1 ml of serum or urine to the third tube (X), mix, and incubate all four tubes for 30 min. at 40°.
4. Add 2 ml. of 5% zinc sulfate to all 4 tubes and mix.
5. Add 0.1 ml. of serum or urine to the fourth tube (C) and mix.
6. Add 4.8 ml. of water and 2 ml. of 0.3 N barium hydroxide to all tubes, shake vigorously, let stand for 10 min., and centrifuge for 10 min. at moderate speed.
7. Pipet 1 ml. each of supernatant, water, and Somogyi copper reagent into test tubes and mix.
8. Cover tubes with marbles and heat in an actively boiling water bath for 15 min.
9. After cooling tubes for 2 min. in a cold water bath, add 1 ml. of arsenumolybdic acid reagent to each tube, mix, let stand for 3 min., and transfer to spectrophotometer cuvets.
10. Measure absorbance of each at 540 m\( \mu \) against water. When readings exceed 0.7, dilute with a measured amount of water, remeasure the absorbance, and multiply absorbance by dilution factor. Spectrophotometry should be carried out without unnecessary delay. About 3% darkening occurs between 3 and 15 min. after addition of arsenumolybdic acid reagent. Calculation is as follows.

\[
\frac{A_X - A_0}{A_{st} - A_{nn}} \times 200 = \text{units amylase activity per 100 ml. serum or urine}
\]

These units are equivalent to Somogyi saccharogenic units.

For accurate work, if a result of over 1400 units is obtained, repeat the entire determination using a more dilute enzyme solution. Serum or urine dilutions should be made with normal saline if the diluted specimens are to be stored. Otherwise, water may be used, since buffer contains sufficient chloride to permit the enzymatic reaction, to proceed at maximum velocity.

The preparation of a precalibrated glucose standard curve, run in the presence of substrate and deproteinizing reagents, is convenient and justified by the excellent day-to-day reproducibility of the quantity \( (A_{st} - A_{nn}) \). A control serum of known high amylase activity should be run frequently.

Independent duplicate analyses were performed with a precision of \( \pm 11\% \) at approximately 70 units per 100 ml. and \( \pm 6\% \) at approximately 200 units per 100 ml., at the 5% level of confidence.

**Discussion**

In numerous papers on saccharogenic methods, the merits and disadvantages of various procedures for determining reducing groups
have been discussed with respect to reducing equivalence and reaction times required with different sugars. Possible inadequacies of these methods at high sugar concentrations have not been studied thoroughly, except in the case of a titrimetric copper reduction procedure (16). In addition to previously described experimental findings with a commercial amylase kit, several reports have appeared which arouse the suspicion that reducing sugars in amylase incubation mixtures are not always measured completely by the original and Tonks-modified Folin-Wu procedures.

In one series of 23 sera, subjected to parallel saccharogenic and amylolastic assays which should have yielded roughly equivalent results, maximum values of 400 and 1070 units, respectively, were found (17). In two other series, in which the amylase activities of large population samples with known pancreatic disease were measured saccharogenically, the highest reported values were 372 and 440 Somogyi units (18, 19). The range of maximum values in those and the Krautman series, 300 to 440 units, corresponds well with plateaus in the Folin-Wu and Tonks curves shown in Fig. 2, allowing for preincubation concentrations of reducing sugars.

Enzyme inhibition, insufficient substrate, or sampling techniques may all be possible causes for the low values observed in the series mentioned above. Inhibitors have now been shown to be absent from at least several batches of Krautman's substrate. Since Somogyi's incubation conditions provide a high enough ratio of starch to serum to permit maximum velocity to about 700 units (1, 2), insufficient substrate can be ruled out as a possible explanation for the low maximum values observed in three of the series (17–19). From the large size of the samplings of patients with known pancreatic disease (7, 18, 19), some higher values should have been anticipated. Furthermore a literature search failed to reveal any large study of pancreatitis patients exhibiting such low maximum amylase activities, when results were obtained by amylolastic methods. Such discrepancies are too large to be explained by differences between Somogyi saccharogenic and amylolastic units.

Extended-range amylase procedures, utilizing spectrophotometric copper reduction methods, have appeared previously. Teller (11) used a fivefold dilution of deproteinized incubation mixture to avoid cloudiness in the final colored solutions. Apparently he was unaware that, by diluting, he extended the range of his sugar determination fivefold, since he prepared still higher dilutions before carrying out
copper reductions on highly active specimens. The proposed method has several advantages over Teller's, including a somewhat more sensitive sugar quantitation, a higher starch-to-serum ratio, and better buffering. Teller's method has been criticized (20) because the deproteinized incubation mixtures are cloudy, and the degree of cloudiness differs markedly between incubated and control supernatants. Although this finding has been confirmed, it is not felt to be an important disadvantage, since cloudiness disappears from all solutions in the final dilution step. Furthermore, when Teller's procedure is modified by using a well-buffered (Tris or phosphate) substrate, the final colored solutions remain clear, although the turbidity of the deproteinized solutions is thereby increased (21).

A recently published micro procedure (22) has significantly extended limits of both incubation and sugar quantitation, but the latter is accompanied by a corresponding loss of sensitivity. The presence of sodium citrate during incubation may be another objectionable feature of that method (23). β-Amylase [sic] (24) activities in serum are claimed to be measurable with a single incubation and sugar determination to 5000 mg./100 ml. of glucose equivalent by the method of Friedman. This is surprising in view of the concentrations of substrate and copper employed. Andersch (25) and Natelson (10) have published methods which extend the limiting value measurable by single incubation and copper reduction to a point intermediate between that of the Somogyi (2, 8) or Henry and Chiamori (1) procedures and our present one.

An amylase result at least 4-6 times the upper limit of the normal range is important in distinguishing acute pancreatitis from acute abdominal diseases which might require emergency surgical treatment, and which are also capable of causing some elevation in amylase activity (26). Use of a procedure which requires repetition, totally or in part, to measure diagnostically significant values is unnecessarily time consuming. Use of a procedure which completely fails to measure such values renders the test virtually useless.

References