Differential Serum Amylase Determination by Use of an Inhibitor, and Design of a Routine Procedure

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We describe a new method for measuring pancreatic and salivary-type amylases in serum that requires no electrophoresis or chromatography. An inhibitor protein (from wheat) with a 100-fold greater specificity for human salivary than for human pancreatic amylase was used to analyze mixtures of the two enzymes. The concentration of pancreatic and salivary amylase was determined in 141 normal sera (72 men and 69 women). Statistically significant differences were found for serum pancreatic amylase between men and women, higher values being shown in women. No sex-related difference was found for the salivary component of serum amylase. With this method, the increase in serum amylase activity in pancreatitis was shown to be attributable to the pancreatic component. In mumps, the increase is attributable to the salivary component. In pancreatic insufficiency, serum pancreatic amylase activities were significantly lower than in the controls. Our method is simple and rapid; our results agree well with those of other authors who used chromatographic or electrophoretic methods.

Additional Keyphrases: variation, source of normal values, sex-related differences, tissue of origin, diagnostic aids, enzyme activity, isoenzymes of amylase

The gross similarity between salivary (S) and pancreatic (P) α-amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) caused early investigators to conclude that these two amylases were identical in humans (1, 2). Although more recent studies by more sensitive techniques have revealed differences in molecular weight and carbohydrate content, the two enzymes appear to have the same amino acid composition and exhibit the same action pattern (3). Consequently, the specific measurement of pancreatic amylase in the presence of salivary amylase in man has presented difficulties, and immunological methods of distinguishing between them have been unsuccessful. They can be separated by electrophoretic (4-6), electrofocusing (7), or chromatographic methods (8), but these techniques are tedious and time consuming.

Purification of a protein from wheat (Triticum aestivum) that is an α-amylase inhibitor with much greater (100-fold) specificity for human salivary than for human pancreatic amylase has been described (9). This paper describes the application of the inhibitor to the assay of pancreatic and salivary-type amylases in human serum.

Materials and Methods

Separation of Amylase Isoenzymes by Ion-Exchange Chromatography

A DE52-cellulose column (13 x 1 cm) was packed as prescribed by the manufacturers (Whatman, Maidstone, Kent, U.K.). Equilibration buffer was 10 mmol/liter tris(hydroxymethyl)aminomethane, pH 9.0, containing 10 mmol of NaCl and 3 mmol of CaCl2 per liter. Amylase solution, extracted from human pancreatic tissue and partially purified by gel filtration on Sephadex G-50 (9) was equilibrated by dialysis overnight against this buffer. After 2 ml of the solution, containing 33 U of amylase, was applied, the column was washed with four column volumes of eluting buffer, followed by an NaCl gradient (0–0.2 mol/liter), and 2.5-ml fractions were collected.

Preparation of Amylase-Free Serum

Amylase was removed from pooled normal human serum by adding 2 g of cross-linked starch (Starch "A"; Laing-National Ltd., Manchester, U.K.) to 100 ml of serum, with stirring (1–2 min). The mixture was then centrifuged (5000 × g, 10 min). The serum was decanted and stored in aliquots at −20 °C until required.

Standardization of Phadebas Blue Starch Method Against the Saccharogenic Method

Because salivary and pancreatic amylases hydrolyze insoluble starches at different rates (10, 11) standard curves were prepared for both enzymes. The enzymes were prepared by gel filtration of extracts of saliva and pancreatic tissue as described (9). S and P amylase in the concentration range 25–600 U/liter, were estimated...
by the Phadebas blue starch method as described by the substrate manufacturers (Pharmacia AB, Uppsala, Sweden), except that in the case of the purified enzymes albumin (7.4 μmol/liter) was incorporated in the reaction mixture for optimal enzyme activity (12). Each concentration analyzed by the blue starch method was also analyzed by the saccharogenic method of Robyt and Whelan (13), with soluble (Zulkowsky) starch (E. Merck, A.G., Darmstadt, Germany) as substrate and the Nelson copper reagents for reducing groups. Absorbance values at 620 nm obtained for the blue starch method were plotted against IUB units of enzyme (μmol of apparent maltose produced per minute) given by the saccharogenic method (Figure 1).

Estimation of Serum Pancreatic Amylase Activity

Procedure. Into each of two 10-ml glass centrifuge tubes were added, in the order given, 0.3 ml of phosphate buffer (pH 6.9, 50 mmol/liter) containing 50 mmol/liter NaCl and 0.5 mmol/liter CaCl₂, followed by 0.2 ml of serum. The mixture was shaken gently by hand and then 10 μl of inhibitor solution (described below) added to one tube and the contents mixed again. All tubes were pre-incubated at 20 °C (room temperature) for 25 min. This allows for maximum inhibition of salivary amylase in the tube containing the inhibitor. The reaction mixtures were then diluted to 4.1 ml with distilled water and both tubes were analyzed for amylase activity by the Phadebas blue starch method in a 15 min (37 °C) incubation procedure. Albumin (10 mg) was substituted for serum in the blank tubes. Absorbances at 620 nm were measured with a Cecil CE 272 spectrophotometer (Cecil Instruments Ltd., Cambridge, U.K.). Amylase values for both tubes were read off the standard curve shown in Figure 1. In the case of amylase values greater than ~250 U/liter (see Results) the serum was diluted with albumin (100 g/liter) before analysis, and the appropriate dilution factor applied. The value for total serum amylase (T) was obtained from the tube without inhibitor added. Amylase remaining in the presence of the inhibitor (Ar) was obtained from the absorbance values given by the tube containing the inhibitor and calculated from Figure 1. The fraction of amylase uninhibited, Ar/T, is proportional to the P/S ratio of the sample, as illustrated by the P/S standard curve.

Construction of P/S standard curve. Purified salivary and pancreatic amylase were mixed in phosphate buffer (as above) to give P/S ratios in the range 0.1–8.0. Enzyme activities ranged from 50 to 200 U/liter (pancreatic) and 20 to 400 U/liter (salivary). These mixtures were made up immediately before use. They were analyzed for amylase in the presence and in the absence of inhibitor as follows. To duplicate 10-ml glass centrifuge tubes the following additions were made, with gentle shaking by hand between each addition: 0.2 ml of phosphate buffer (composition as above), 0.1 ml of amylase free serum, and 0.2 ml of amylase mixture (P + S). Inhibitor solution (10 μl) was added to the tube for pancreatic amylase assay. The tube without added inhibitor is used to find the total amylase value. Amylase-free serum, 0.1 ml, was included in the reagent blank. The 25-min pre-incubation, 15-min incubation, and absorbance readings were all done as above. Amylase activities were calculated for the solution in the presence and absence of inhibitor. The fraction of amylase uninhibited (i.e., that remaining in the presence of inhibitor) was calculated for each tube. This value was plotted against the known P/S ratio (Figure 2) and is seen to be proportional to the P/S ratio (see Results).

Calculation of serum pancreatic (P) amylase. Total serum amylase, T, is made up of salivary plus pancreatic, i.e.

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**Fig. 1.** Standardization of the blue-starch method against the saccharogenic method (13) with pancreatic (P) amylase and salivary (S) amylase.

**Fig. 2.** Standard curve obtained by mixing purified pancreatic and salivary amylase in P/S ratios in the range 0.1–8 and analyzing the mixtures for amylase activity in the presence and absence of inhibitor (0.5 μg per tube).

Each point is the mean of at least 5 analyses. The bars indicate ±2 SD.
The ratio P/S is known from Figure 2

\[ T = P + S \] (1)

Let \( P/S = R \)

\[ \therefore S = P/R \] (2)

Substituting P/R for S in equation 1 we get

\[ T = P + P/R \]

\[ = P(1 + 1/R) \]

\[ \therefore P = \frac{T}{1 + 1/R} \] (3)

Since \( P/S = R \),

\[ P = \frac{T \times P/S}{1 + P/S} \] (4)

Salivary amylase activity is then found by substituting the value obtained for \( P \) in equation 1, i.e.

\[ S = T - P \]

**Calculation of pancreatic amylase at extreme values.**

At \( P/S \) values greater than 5 and less than 0.2 the standard curve is nonlinear (Figure 2). When serum P/S values occur outside these ratios the pancreatic amylase activity may be calculated as follows. Pancreatic amylase and salivary amylase standards are included in each batch of analysis. The standards are selected to be as near as possible to the activities of the unknown sera. The analysis is performed as described and the values of the individual sera with and without inhibitor are read off Figure 1. Amylase remaining in the presence of the inhibitor (Ar) is made up of the fraction of salivary amylase uninhibited (0.1, i.e., 10% activity) plus the fraction of pancreatic amylase uninhibited (0.8, i.e., 80% activity) (See Figure 3 and Inhibitor Units, below), i.e.,

\[ Ar = 0.1S + 0.8P \] (5)

or 10 Ar = S + 8 P (6)

Since \( T = S + P \)

\[ 7P = 10Ar - T \] (6-7)

\[ P = \frac{10Ar - T}{7} \] (8)

Since \( T \) and Ar have been found by analysis, the pancreatic component of serum may be calculated by equation 8. The salivary component of serum is again obtained by difference (\( T - P \)). The inhibitor preparation used may differ slightly in the degree of inhibition of S and P amylase standards from that described above. For example if the fraction of S amylase and P amylase uninhibited is 0.12 (12%) and 0.83 (83%), respectively, then equation 5 should read:

\[ Ar = 0.12S + 0.83P \]

and the equations above solved for \( P \) accordingly.

These calculations are based on the following assumptions: (a) that the degree of inhibition is constant over the expected range of \( S \) and \( P \) at fixed concentration of inhibitor; (b) that the degree of inhibition of \( P \) is not affected by the presence of \( S \) and vice versa, i.e., that the inhibition of \( S \) is independent of the presence of \( P \); and (c) that the total serum amylase consists of pancreatic and salivary type amylases only. The validity of these assumptions will be discussed below.

**Inhibitor Units**

The inhibitory activity of the inhibitor, prepared as described (9), was calculated as follows. Inhibitor solution (10–50 \( \mu l \)) was added to 0.3 ml of phosphate buffer (as above) containing 2 mg of albumin, followed by 0.2 ml of salivary amylase solution (42–52 mU) in the above buffer. Control tubes were set up without the inhibitor. Pre-incubation, incubation, and absorbance values were found as described for amylase assay above. The quantity of inhibitor that diminished the salivary amylase activity by 50% was calculated, because the plot of salivary amylase activity vs. inhibitor concentration is linear in the region of 50% reduction of enzyme activity (9). The inhibitor unit is defined as the quantity of inhibitor required to reduce the activity of 2.0 U of amylase by 50%, i.e., 1 unit of inhibitor inhibits 1.0 U of S amylase under these conditions. Based on a specific activity of 245 units of inhibitor per milligram of protein as obtained in the purification procedure (9), 0.1 \( \mu g \) inhibitor caused 50% inhibition of S amylase, while 10 \( \mu g \) inhibitor were required to cause the same degree of inhibition of \( P \) amylase. This showed the inhibitor to be 100 times more specific for \( S \) than for \( P \) amylase (i.e., the salivary/pancreatic inhibitor-activity ratio = 100).

In utilizing the inhibitor for differential amylase assay described above, a quantity of inhibitor should be chosen that causes maximal (~90%) inhibition of S amylase with the least inhibition (~20%) of \( P \) amylase. This corresponds to about 122 milliunits (or 0.5 \( \mu g \), calculated on specific activity of 245). This inhibitor
concentration should be then tested to ensure that the degree of inhibition is constant over the expected range of S and P amylase concentrations as in Figure 2. It is not necessary to obtain a preparation of specific activity as high as 245 for use in the differential amylase assay. Of vital importance, however, is to obtain a preparation with a salivary/pancreatic inhibitor activity ratio of ~100. This can be achieved without the gel filtration step of the purification procedure (9).

We recommend that the inhibitor solution be stored in tris(hydroxymethyl)aminomethane-HCl (10 mmol/liter, pH 8.0) containing 10 mmol of NaCl per liter, at 4 °C.

Serum Specimens

We obtained control sera from 141 healthy volunteers (72 men and 69 women), 18 to 50 years old. The samples from patients included 11 cases (nine males and two females) of pancreatic exocrine insufficiency (five adults with a previous history of pancreatic disease, and six children with cystic fibrosis), 15 mumps sera (Virus Reference Laboratory, Dublin), and 12 sera from cases of acute pancreatitis. Serum samples were stored at −20 °C before analysis.

Results

Human pancreatic amylase at values greater than 250 U/liter hydrolyzes the insoluble blue starch at a faster rate than the salivary enzyme, when standardized against a saccharogenic (13) method (Figure 1). Unknown samples of high amylase activity were prediluted so that the final extinction value did not exceed 0.4, since the curves diverge above this figure.

Previous studies have shown the much greater (100-fold) specificity of the inhibitor towards salivary than towards pancreatic amylase with both insoluble and soluble starch substrates (9). The effect of a constant amount (0.5 μg) of inhibitor on various concentrations of S and P amylase with blue starch substrate is shown in Figure 3. The degree of inhibition of the salivary enzyme was 90% while there was only 20% inhibition of the pancreatic enzyme over the range of enzyme concentrations shown. Assumption a above is therefore valid.

It might be argued that the 20% inhibition of pancreatic amylase could be due to inhibition of one of the pancreatic isoenzymes. This possibility was investigated by separation of three pancreatic isoenzymes by ion-exchange chromatography on DE52-cellulose (Figure 4) and testing each peak against the inhibitor. Each of the isoenzymes was inhibited 20%, similar to the unfractonated enzyme. A standard curve (Figure 2) was then established by analyzing mixtures of purified S and P amylase over a wide range of P/S ratios (0.1–8). The calculation of values for P and S has been outlined above. The precision of the analyses of pancreatic and salivary-type amylases in serum was determined by within-run and day-to-day replicate determinations of three serum samples of different enzyme levels. The "normal" sample was pooled normal serum. The "high S" sample was pooled normal serum to which salivary amylase had been added to give the values shown (Table 1). Similarly the "high P + S" sample was pooled normal serum to which pancreatic and salivary amylase were added. The precision data (Table 1) attest to the reproducibility of the method.

Analytical recovery of salivary and pancreatic amylase from serum was studied by adding known amounts of each enzyme to amylase-free serum to give enzyme activities in the range 50 to 350 U/liter. The results were

Table 1. Precision of Pancreatic- and Salivary-Type Amylase Assay of Three Pooled Sera

<table>
<thead>
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<th></th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>CV%</th>
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<tr>
<td><strong>Day-to-day precision</strong></td>
<td></td>
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</tr>
<tr>
<td>Normal amylase</td>
<td>18</td>
<td>84.7</td>
<td>3.53</td>
<td>4.16</td>
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<tr>
<td>High S serum</td>
<td>19</td>
<td>82.2</td>
<td>4.06</td>
<td>4.94</td>
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<tr>
<td>High P + S serum</td>
<td>17</td>
<td>312.4</td>
<td>17.43</td>
<td>5.58</td>
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<tr>
<td><strong>Within-day precision</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal amylase</td>
<td>15</td>
<td>86.3</td>
<td>3.43</td>
<td>3.98</td>
</tr>
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<td>High S serum</td>
<td>14</td>
<td>80.4</td>
<td>3.41</td>
<td>4.24</td>
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<tr>
<td>High P + S serum</td>
<td>17</td>
<td>304.5</td>
<td>10.74</td>
<td>3.53</td>
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Purified S amylase was added to an aliquot of pooled normal serum to give the high S sample. Purified P and S amylases were added to pooled normal serum to give the high P + S sample. Each pool was held at −20 °C in aliquots before analysis.

Fig. 4. Separation of pancreatic amylase isoenzymes on DE52-cellulose column (13 cm × 1 cm). Buffer was tris(hydroxymethyl)aminomethane (10 mmol/liter, pH 9.0) containing 10 mmol of NaCl and 3 mmol of CaCl₂ per liter. After application of 2 ml (33 U) of pancreatic amylase, column was washed with four column volumes of eluting buffer, followed by a gradient with respect to increase in concentration of NaCl (0–0.2 mol/liter). 2.5-ml fractions were collected.
in good agreement with expected values for both enzymes. The recovery (%) for salivary amylase ranged from 96.8 to 98%, while that for pancreatic amylase was from 93.4 to 101.2%

Possible interference by bilirubin was investigated. Bilirubin was added to pooled serum in increasing amounts to give a final concentration in the range 50–500 mg/liter. No significant effect on the action of the inhibitor was observed, when bilirubin was included in the blank.

Pancreatic and salivary-type amylases in sera from males and females are shown in Table 2. The mean pancreatic amylase value in females, 99.5 ± 29.8 (SD), was significantly higher than that found in males (82.4 ± 29.9) when compared by Student’s t-test (P < 0.0005). Salivary activities were not statistically different. Histograms of serum pancreatic and salivary-type amylase activity in males and females are shown in Figure 5.

Differential serum amylase values for patients with pancreatic insufficiency are shown in Table 3. These included six patients with confirmed cystic fibrosis and five adults with a history of pancreatic exocrine insufficiency. In all cases the total serum amylase was within normal limits, i.e., within 2 SD’s of the controls. When the values were compared with those of the control group via the Student’s t-test the difference obtained was not statistically significant (0.05 < P < 0.1) (Table 3). The mean (U/liter) of serum pancreatic amylase levels, 25.9 ± 16.1 (SD), was significantly lower than the figure of 82.4 ± 29.9 for the 72 male controls (P < 0.0005). In addition, the mean for salivary amylase activity in pancreatic insufficiency patients was significantly higher than for the controls—80 ± 35.4 (SD) as compared with 49.6 ± 30.4 (P < 0.0025).

When the differential amylase assay was applied to mumps sera and sera from patients with acute pancreatitis, the results were in agreement with the clinical condition. In 15 mumps sera with total amylase activities in the range 255–1270 U/liter, the hyperamylasemia in all cases was due to salivary amylase. Pancreatic enzyme activities were within the normal range (Table 4). In 12 sera from pancreatitis patients with total amylase values in the range 360–9750 U/liter, the increase was in all cases due to the pancreatic component. Accurate analysis of salivary amylase can not be done if there is more than an eight-fold excess of pancreatic to salivary enzyme. The reason for this is apparent from Figure 2. At P/S values greater than 8, the enzyme uninhibited exceeds 73%, which approaches the figure of 80% obtained for pure pancreatic enzyme as the P/S value increases. Because the CV at high amylase activities can be up to 6% (Table 1), analysis of salivary amylase is not accurate when P/S exceeds 8. In practice this means that the lower limit of measurement of salivary amylase in the presence of 900 U of pancreatic amylase per liter is 112 U/liter. This is within 2 SD’s of the mean salivary level for controls. At higher pancreatic amylase concentrations, as was obtained in nine of the 12 cases of

| Table 2. Pancreatic- and Salivary-Type Amylase Activity in Serum of Normal Subjects |
|---------------------------------|-------------|-------------|-------------|
| No. analyses                    | Total amylase | Pancreatic | Salivary |
| Females                         | 69           | 151.7 ± 47.7 | 99.5 ± 29.8 | 52.4 ± 31.7 |
| Males                           | 72           | 131.9 ± 49.8 | 82.4 ± 29.9 | 49.6 ± 30.4 |
| Significance*                   |              | P < 0.01    | P < 0.0005 | P > 0.3   |

* t-test

<table>
<thead>
<tr>
<th>Table 3. Differential Serum Amylase Activities in Patients with Pancreatic Insufficiency, as Compared with Values for Men Controls</th>
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<td>No. analyses</td>
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<td>Controls</td>
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<td>Pancreatic insufficiency</td>
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<td>Significance*</td>
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* t-test

Fig. 5. Distribution of (a) serum pancreatic amylase and (b) serum salivary amylase in a population of normal adults
acute pancreatitis, the limit of detection of salivary amylase would be outside 2 SD’s of the mean control value. For example, in the presence of 9000 U of pancreatic amylase per liter, the limit of accurate detection of salivary amylase would be 1125 U/liter.

Discussion

This paper describes the application of an inhibitor that has a much greater specificity for human S amylase than for human P amylase (9) in the assay of pancreatic and salivary-type amylases in human serum. The fact that the inhibitor behaved similarly towards each of the three pancreatic isoenzymes separated by DE52-cellulose ion-exchange chromatography fortunately rules out any interference due to alteration in the concentration of any of the individual isoenzymes in serum which may occur in some pathological conditions (14–16).

As pointed out earlier (9), the order of addition of the reactants is vitally important to the proper working of the method. Enzyme must be pre-incubated with inhibitor for 20–25 min for maximum inhibition of S amylase to be achieved, before the substrate is added. This is because the starch substrate can itself combine with inhibitor. When maximum binding of enzyme and inhibitor is obtained after the 25 min pre-incubation period, the ability of the starch to combine with the inhibitor is vastly decreased (9). The extent to which the substrate displaces the inhibitor from the enzyme–inhibitor complex and the effect of the combination of substrate with any free inhibitor present after the pre-incubation period will be studied separately by special kinetic treatment.

The reason for the greater specificity of the inhibitor for S amylase than for P amylase is also under study. Until recently, human salivary and pancreatic amylases have been regarded as being closely alike, and their immunologic properties caused McGeachin (17) to regard them as structurally identical. While recent work (18, 19) has shown that rat pancreatic and parotid amylase can be differentiated immunologically, the actual measurement by immunological methods of one enzyme in the presence of the other in man has still to be achieved.

Our observations of P- and S-type amylase patterns in hyper- or hypo-functioning states of pancreas or salivary glands would seem to make it possible to specify the origin of the amylase that appears in human serum. However, while the pancreas and salivary glands are believed to be the main sources of the amylases that are present in serum and urine (15, 20), the possible contribution of additional amylases, from fallopian tube and liver as postulated by Janowitz and Dreiling (21) cannot be ruled out. Their chromatographic similarity to the salivary and pancreatic enzymes, respectively, has been demonstrated (20). The higher serum amylase activity found in women relative to men (Table 2) is in agreement with the findings of others (22). We have demonstrated this increased amylase activity to be of the pancreatic type, but its tissue source remains to be established. Increased serum amylase has also been found in subjects who are taking oral contraceptives (22) and in pregnancy (22–24). Serum amylase activity in the woman may be under hormonal influences. Messer and Dean (18) claim, from immunochemical studies, that serum and liver amylase of the rat are identical, suggesting that the liver is the main source of serum amylase. Takeuchi et al. (19) have shown by electrophoretic methods that amylase in rat liver is a complex of parotid-type amylase and glycogen. The very low a-amylase activity found in liver homogenate and its almost complete absence in bile suggest that in man little a-amylase is produced by the liver (25).

Quantitation of P-type amylase in serum by this inhibitor appears to measure pancreatic-type amylase directly. A limitation of the method is the inability to measure salivary enzyme activity accurately at P/S values greater than 8. This is because (a) the inhibitor is not absolutely specific and (b) there is a CV of 6% inherent in the method. As pointed out above, this means that in cases of acute pancreatitis with pancreatic amylase values >1000 U/liter and a P/S ratio >8, the method could give falsely high values for salivary amylase. At values for pancreatic amylase <1000 U/liter, the problem does not arise.

Even though the method measures S-type amylase indirectly (since it is calculated by difference, T-P) the results obtained for the mumps sera were in agreement with the clinical condition. Increased serum amylase activity was in all cases due to the salivary fraction. The higher salivary amylase activity found in some cystic fibrosis sera, relative to normals, is in agreement with the findings of Skude (25). S-type hyperamylasemia has also been reported in some cases of lung and pancreatic cancer (8, 26).

The changes in salivary and pancreatic-type amylases obtained in the pathological conditions studied are in agreement with the findings of other authors who used electrophoretic (5, 14–16, 25, 27) or chromatographic (8, 20) methods. The P/S ratio of 1.8 obtained for the control sera is higher than that (~1.0) obtained by others (5, 8, 25, 27). This may be due to the overestimation of salivary enzyme by some forms of electrophoresis, because the pancreatic isoenzyme A2 tends to overlap with the salivary isoenzymes A1 and A2 (28). It is also possible that the pancreatic enzyme is underestimated by electrophoretic methods because of its greater lability (3).

This preliminary report demonstrates the application of an inhibitor to the assay of salivary and pancreatic-type amylases in human serum. Further studies will be
necessary to prove its general application, but it does appear to hold great promise. It seems to be ideally suited for laboratory investigation of pancreatic insufficiency and chronic pancreatitis and may even in some situations obviate the need for duodenal intubations.

The application of the method to other disorders that can produce abnormally high serum amylase activity—intestinal obstruction or inflammation, common-duct stones, hepatitis, and facial or abdominal trauma—should help to identify the tissue source of amylase. These studies, together with investigation of renal clearance of P- and S-type amylases, will be reported separately.

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References