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Continuous-Flow Potentiometric Determination of α-Amylase Activity in Serum and Urine

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An automated saccharogenic potentiometric method for serum or urinary α-amylase is described. Amylase is allowed to act on a buffered at pH 6.9 starch solution under controlled continuous-flow conditions and the reducing sugars produced are left to react with periodate. The consumption of periodate is monitored continuously with a newly constructed periodate-sensitive flow-through electrode. The endogenous reducing substances of serum or urine are measured with the same system by incubation of the sample with the same starch solution, at pH 4.7, in the presence of sodium fluoride as an amylase inhibitor. The difference in the reducing power (as glucose) is used to calculate the amylase activity of the samples. Values obtained with this assay correlate fairly well with those obtained with an amyloclastic method (r = 0.90–0.96). © 1985 Academic Press, Inc.

INTRODUCTION

The determination of α-amylase activity in serum and urine is useful as an aid in the diagnosis of acute pancreatitis (*1*). Various methods have been described for the determination of α-amylase in biological fluids. Most of them are based on the hydrolysis of starch by α-amylase and monitoring the disappearance of starch by use of the starch–iodine reaction (amyloclastic) or the amount of reducing sugars formed (saccharogenic) (*7*). Recently, several dye-labeled substrates have been prepared and applied to the determination of α-amylase in biological fluids (*6*, *9*, *10*). These methods give widely varying results when compared with the saccharogenic methods (*8*).

In this paper, the first method for the determination of α-amylase activity in biological fluids by use of ion-selective electrodes is described. Amylase present in serum or urine is allowed to act on a buffered, at pH 6.9, starch solution, at 40°C, for 30 min under continuous-flow conditions. After incubation, periodate is added to the reaction stream and the reducing sugars produced are left to react with periodate for 20 min. The consumption of periodate is monitored with a periodate-sensitive flow-through electrode. The endogenous reducing substances of serum or urine are measured with the same system by incubation of the sample with the same buffered starch solution, at pH 4.7, in the presence of sodium fluoride as an amylase inhibitor. The difference in the reducing power (as glucose) is used to calculate the amylase activity of the unknowns. Comparison of the method with an amyloclastic method gave fairly satisfactory results.

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MATERIALS AND METHODS

Apparatus

The body of an Orion 92 electrode equipped with Teflon membranes (Millipore LCWPO 1300, in quadruplicate) was used. The flow-through channel was constructed by embedding the lower cup of the electrode in a plastic material and drilling holes of appropriate diameters for the inlet and outlet of the solution, as described in detail elsewhere (*4*). The periodate electrode was assembled according to the manufacturer's instructions with the liquid ion-exchanger and the internal reference solution (sodium periodate 0.01 *M*–sodium chloride 0.1 *M*) being injected into the appropriate ports of the electrode body (for the preparation of the liquid ion-exchanger see below). The reference electrode used was a saturated calomel electrode (Corning). All potentiometric measurements were carried out with a Corning Model 12 Research pH/mV meter and recorded on a Sargent–Welch Model XKR potentiometric recorder. The automated analysis system consisted of a sampler (A40 Sampler II, Hook and Tucker, U.K.) and a proportioning pump (pump III, Technicon). A schematic diagram of the automated analysis system is shown in Fig. 1.

Reagents

All solutions were prepared with deionized distilled water from reagent-grade materials.

Buffer-substrate solution. Dissolve 69.0 g of sodium dihydrogen phosphate 1-hydrate and 6.0 g of sodium chloride in about 700 ml of water. Adjust the pH to 6.9 by adding saturated sodium hydroxide solution. Heat to boiling. Weigh 10.0 g of soluble starch (Merck) and mix with 100 ml of cold water. Add at constant stirring 200 ml of the hot buffer solution and pour the resulting solution back to the remaining hot buffer solution, at constant stirring. Boil for 3 min, cool, and adjust the final volume to 1 liter with water (*8*). Add 30 drops of Tween 80 surfactant before use.

To prepare a buffer-substrate solution for the determination of the reducing power of the sample, proceed as above with two changes: (a) add 6.0 g of sodium

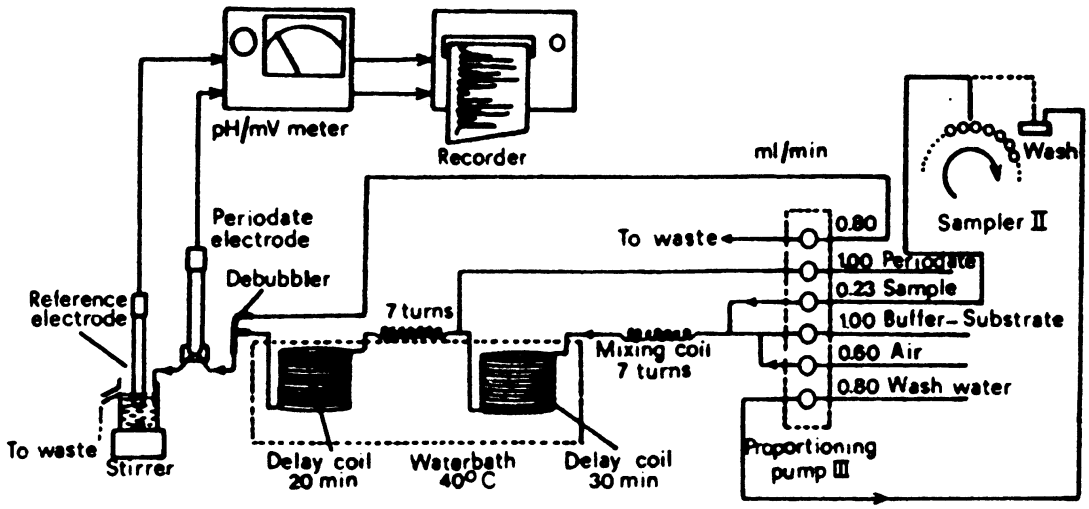

FIG. 1. The flow diagram of the automated analysis system.

fluoride instead of sodium chloride and (b) eliminate the addition of sodium hydroxide solution for pH adjustment. This solution has a final pH of 4.7.

Sodium metaperiodate ($NaIO_4$) working solution, 0.100 M. Dissolve 21.4 g of sodium metaperiodate in water and dilute to 1 liter. This solution should be kept in an amber-glass bottle.

Glucose stock solution, 0.100 M. Dissolve 18.0 g of pure anhydrous glucose in water and dilute to 1 liter. Working standards, 10^{-2}, 3×10^{-2}, and 5×10^{-2} *M* are prepared by dilution. All glucose solutions should be refrigerated.

Tetrapentylammonium bromide solution (TPABr), 0.100 M. Dissolve 1.89 g of TPABr (Eastman Organic Chemicals Co.) in water and dilute to 50 ml.

Procedures

(*a*) *Preparation of the periodate liquid ion-exchanger.* Mix 20.0 ml of equimolar (0.1 *M*) aqueous solutions of tetrapentylammonium bromide and sodium metaperiodate in a separatory funnel, to precipitate tetrapentylammonium periodate. The water-insoluble salt is extracted with 20.0 ml of 2-nitrotoluene. The organic phase is passed through a filter paper containing anhydrous sodium sulfate to remove traces of water. The filtrate is used for the electrode construction.

(*b*) *Measurements.* The optimum conditions for the analysis are indicated in Fig. 1. Load the serum or urine (diluted 1 + 1 or 1 + 2 with water) samples in the 2-ml plastic sample cups and set the system to operate at 30 samples per hour with a sample:wash ratio of 1:1. Include three standard solutions of glucose (0.010, 0.030, 0.050 *M*) which are sufficient to establish the calibration curve. Repeat the analysis by simply replacing the buffer-substrate solution of pH 6.9 with that of pH 4.7. Measure the peak height, $\Delta E = E_{peak} - E_{baseline}$, corresponding to each sample in both cases.

Calculations

For this assay, one unit of activity (expressed per liter of specimen) is defined arbitrarily as the amount of enzyme that liberates reducing sugars equivalent to 1 m*M* of glucose under the experimental conditions outlined. Measure the peak height, ΔE, for each glucose standard, from the recorded trace, and calculate A = [1-antilog($-\Delta E/S$)], where S is the slope of the electrode. (The slope was found equal to 46 mV. The slope should be determined experimentally for every periodate electrode.) Plot A vs m*M* of glucose standards (units/liter) to find the calibration curve (see Fig. 3). For each serum or urine sample, measure the peak heights (from the recorder trace) ΔE_1 and ΔE_2, at pH 6.9 or 4.7, respectively. Calculate B = [1-antilog($-\Delta E_1/S$)] and C = [1-antilog($-\Delta E_2/S$)]. Use the difference $B - C$ to find the activity of amylase from the calibration curve.

RESULTS AND DISCUSSION

Electrode

The Orion's liquid-membrane perchlorate ion-selective electrode has already been used as a periodate sensor in various analytical applications (*5*). This electrode exhibits poor response (drift) and short lifetimes when immersed in con-

centrate periodate solutions probably because of the slow oxidation of the Fe(II) contained in the Orion's liquid ion-exchanger. The electrode proposed in this study is stable, free of drift and its lifetime is two months or more, when used in dilute or concentrate periodate solutions. This is due to the stability of the liquid ion-exchanger and the mechanical resistance of the Teflon membranes. The electrode potential is independent of pH in the range 4–7, where metaperiodate species are the main ions in solution. The constructed periodate electrode exhibits linear response for periodate at concentrations of 5×10^{-5}–10^{-1} M in accordance with equation

$$E = E' - S \log \alpha_{IO_4^-}, \quad (1)$$

where S is found experimentally, from a plot of E vs log $\alpha_{IO_4^-}$, to be 46 mV. The electrode selectivity over a number of common anions (j), such as acetate, bicarbonate, halogens, sulfate, nitrate, and benzoate is good ($K^{pot}_{IO_4^- - j} < 0.01$ in all cases).

Reaction Conditions

The pH of incubation chosen (6.9) lies in the optimal pH ranges of α-amylase activity (6.9–7.2) and electrode response (*4–7*). Chloride activates amylase and

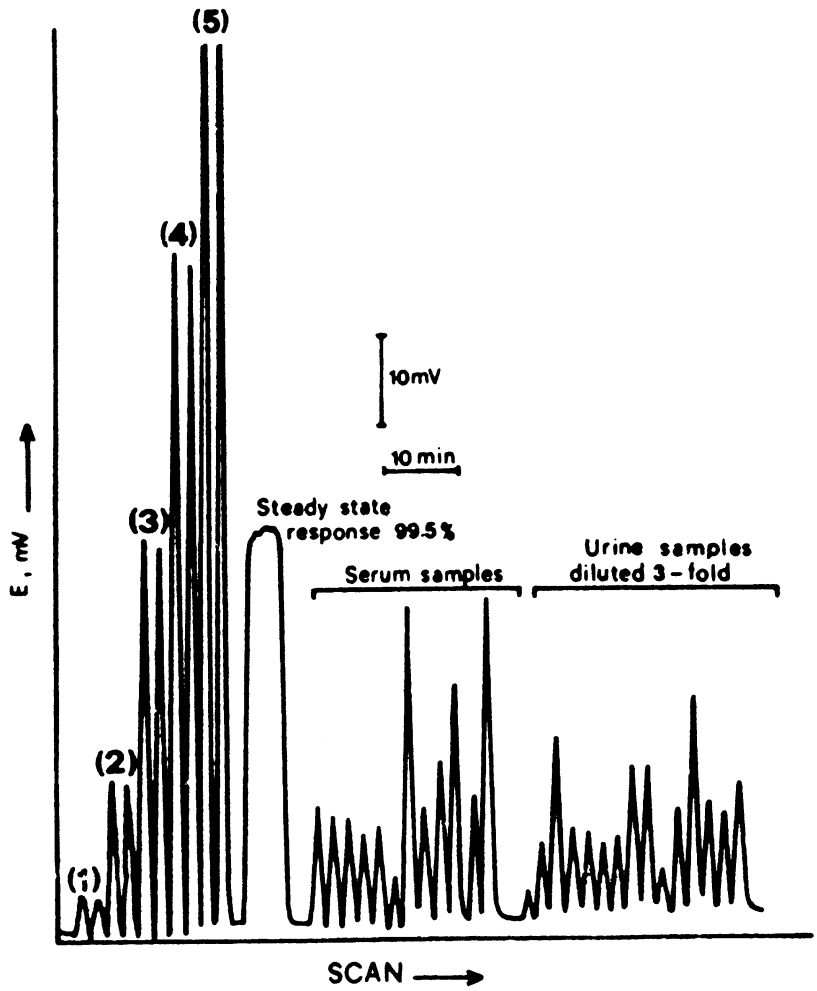

FIG. 2. Recorder tracings for glucose standards and serum or urine samples (*M*): (1), 0.010, (2) 0.030, (3) 0.050, (4) 0.060, (5) 0.070.

FIG. 3. Calibration curve for the analysis of amylase in serum or urine.

a concentration of at least approximately 0.01 *M* is required for optimal activity (*7*). A starch concentration of 10 g/liter in the buffer-substrate solution was chosen to ensure saturation of the enzyme. Although optimal temperature for α-amylase lies in the range of 50–55°C, an incubation temperature of 40°C was chosen because the electrode response was unstable when serum samples were measured at 45–50°C. The selection of a 30-min incubation time has been widely employed in saccharogenic methods. The hydrolysis of starch and the reaction of reducing sugars with periodate are carried out in the same water bath (40°C). It was decided to separate the hydrolysis and oxidation steps because it was found that periodate affects markedly the activity of amylase. Starch alone reacts to a small extent with periodate; this reaction does not affect the analysis since the electrode signal after the reaction of starch with periodate is taken as baseline.

Calibration Curve

The relation between periodate electrode potential, *E*, and periodate activity is logarithmic in nature (Eq. (1)). Therefore, there is not a linear relationship between potential change, ΔE (peak height) and glucose concentration of the

TABLE 1
Precision of the Method for Serum and Urine Samples

Sample	Activity (U/liter)	SD (U/liter)	*CV*	*N*
Serum	23.2	0.25	1.1	10
Serum	36.5	0.24	0.7	6
Urine	43.8	0.32	0.7	7
Urine	51.1	0.10	0.2	7

standards. A linear calibration curve is taken by plotting [1-antilog($-\Delta E/S$)] vs concentration of glucose, as described in detail elsewhere (*3*). Figure 2 shows a typical recording for a series of amylase determinations carried out by the proposed procedure. A typical calibration curve is shown in Fig. 3.

Precision

The within-run precision of the method for serum and urine is shown in Table 1. It must be mentioned that due to the logarithmic nature of the calibration curve a precision (*CV*) of about 1% for peak heights of about 40 mV is reduced to 0.3%, when translated to activity units.

Comparison

Serum and urine samples were analyzed by the proposed method and by an established amyloclastic procedure (*2*). The results are presented graphically in Figs. 4 and 5. There is fairly good correlation between the two methods. Using the regression equation of the correlation plots amylase units found by the present method can easily be transformed to standard amylase units of the amyloclastic method.

The proposed method for the determination of α-amylase activity in serum and urine is a new saccharogenic procedure which utilizes periodate for the deter-

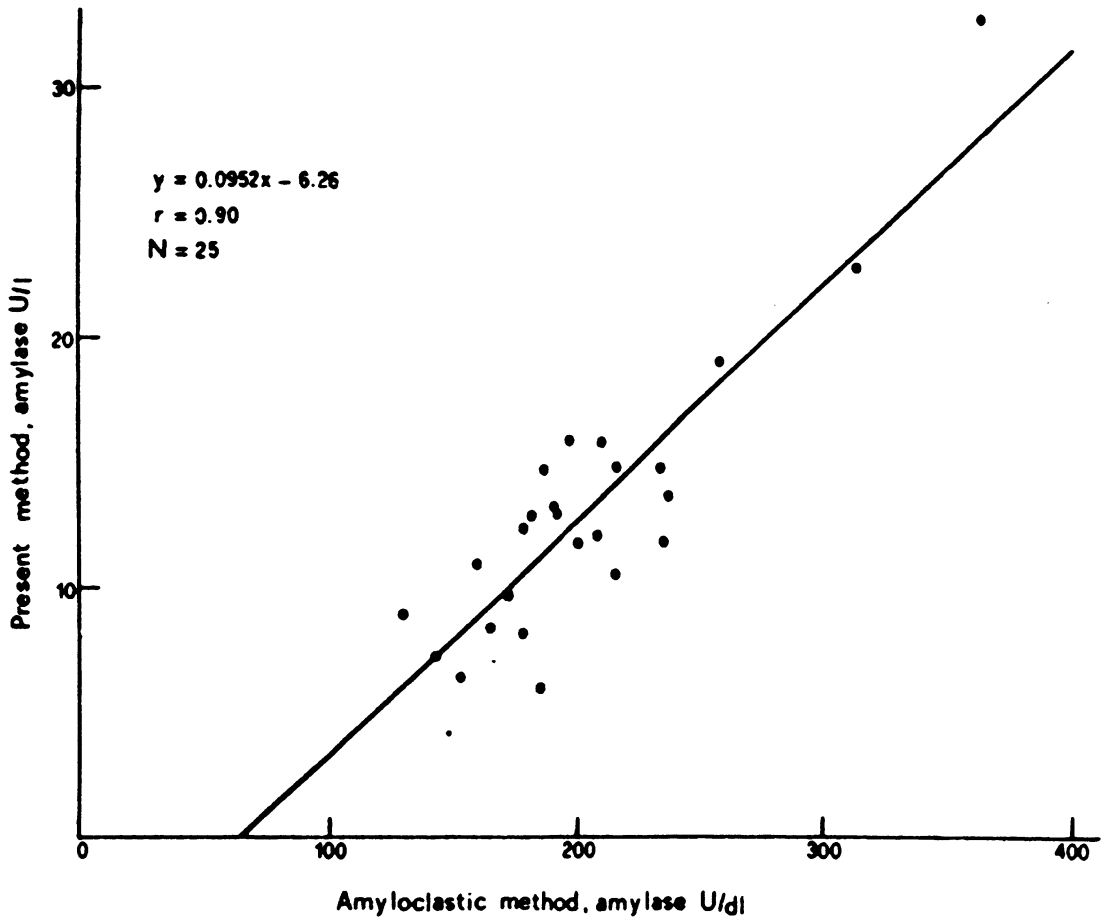

FIG. 4. Correlation of amylase levels in serum determined by the proposed automated method and an amyloclastic method.

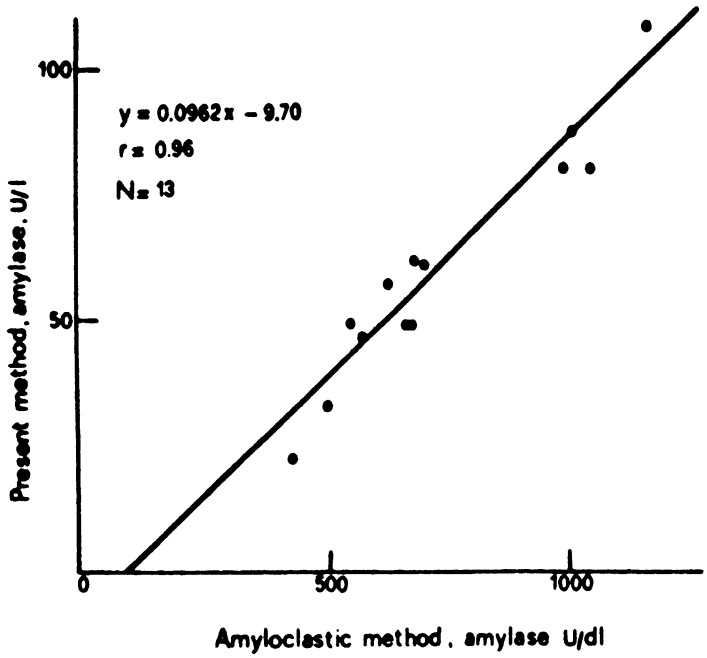

FIG. 5. Correlation of amylase levels in urine determined by the proposed automated method and an amyloclastic method.

mination of the endogenous reducing substances of the sample and those produced when amylase hydrolyses starch. Periodate activity is monitored continuously with a newly constructed periodate-sensitive liquid-membrane flow-through electrode. The method is automated and no sample pretreatment is required. The advantages of the proposed method for amylase are high precision and sensitivity. In contrast to the widely used amyloclastic procedures which exhibit precisions for serum of about ± 10% in the normal range and about ± 5% in the higher activity levels, the proposed procedure exhibits precision of better than 1%. This is due to the automation, the stability, and the logarithmic nature of the electrode response.

Samples in the pathological range can be detected more easily with the proposed procedure in comparison with photometric procedures, because of the logarithmic nature of the electrode response. For example, a serum sample in the upper normal range with activity of 180 U/dl as measured by the amyloclastic procedure (*2*) will decrease the absorbance of the blank from 0.40 to 0.31 absorbance units, while serum samples of 360 and 540 U/dl will exhibit absorbance values of 0.22 and 0.13 absorbance units, respectively. The same serum samples, when measured by the proposed potentiometric method, will exhibit ΔE values (peak heights) of 4.8, 13.9, and 32.2 mV, respectively, that is the measured peak heights increase exponentially when the amylase activity increases linearly.

In addition, the potentiometric procedure offers other competitive advantages over photometric procedures such as indifference to optical interferences, turbidity, etc.

The main limitation of the proposed procedure is the need for two measurements for the same sample, which reduce the number of samples analyzed and increase the sample consumption by a factor of 2. This is a general limitation of all saccharogenic procedures.

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