A Micromethod for the Determination of Plasma Amylase

Roger W. Marsters, Thomas D. Kinney, and Ku Yin Lin

A micromethod has been developed for the determination of plasma amylase levels. The advantages of this method are that it requires very small quantities of blood and that it is accurate and reproducible. It therefore is useful for determining the serum amylase of infants and lends itself to experimental work on small animals such as rats, where there is a relatively high level of amylase in the blood.

Briefly, the method consists of incubating 0.02 ml. of plasma or serum in an excess of a buffered starch substrate for exactly 15 minutes, precipitating proteins with dry picric acid, filtering, developing color with 15% sodium carbonate, and reading in a photoelectric colorimeter. Picric acid has the advantage that it not only stops enzyme action and precipitates the proteins, but with the addition of sodium carbonate the amount of reducing power developed can be easily determined. Furthermore, the color developed with heat and alkali is very stable and can be conveniently read any time within 2-3 hours following its development.

REAGENTS

0.75 PER CENT SOLUBLE STARCH

Suspend 7.5 gm. soluble starch (Merck according to Lintner) in about 60 ml. of cold water and then add very slowly with stirring, about 600 ml. of boiling water. Boil this solution with continuous

From the Department of Pathology, Western Reserve University School of Medicine at Cleveland Metropolitan General Hospital (formerly Cleveland City Hospital) Cleveland, Ohio.

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stirring for exactly 1 minute, remove from the heat, add preservatives consisting of 1.350 gm. Methyl Parasept* and 0.270 gm. Propyl Parasept, allow to cool, and make up to 1 liter. Store at 5° and make up fresh each month. This solution does not need to be heated before use; merely remove from refrigerator, shake, and use.

0.25 M SODIUM CHLORIDE

Dissolve 14.61 gm. of NaCl and make up to 1 liter of solution.

0.2 M PHOSPHATE BUFFER

Dissolve 7.62 gm. of anhydrous potassium dihydrogen phosphate in about 600 ml. of water and add 20.45 gm. of disodium hydrogen phosphate. When dissolved, make up to a volume of 1 liter. Check pH and adjust if necessary to 7.2.

GLUCOSE STANDARD

Carefully weigh 200 mg. anhydrous glucose and make up to 1 liter with saturated picric acid.

15% SODIUM CARBONATE

Dissolve 150 gm. anhydrous sodium carbonate and make up to 1 liter of solution.

METHOD

Set up 16 x 125-mm. test tubes labeled A, B, and C for each determination and add to each tube the following: 2 ml. 0.75% starch, 2 ml. 0.2 M phosphate buffer, 1 ml. 0.25 M NaCl. Mix the contents of each tube and place the entire rack in a water bath at 40° for approximately 5 minutes' preheating.

Prepare three 0.02 ml. pipettes for each serum as detailed under TECHNIC FOR COLLECTING BLOOD SPECIMEN. Store in moist, covered trays. Set the timer for 15 minutes and immediately pipet 0.02 ml. of each serum into Tubes A and B for each determination. This will provide duplicate tubes for the activity determination.

After exactly 15 minutes, remove the rack and add dry powdered picric acid to Tubes A, B, and C. Go through the series, shaking each tube several times to hasten the saturation of the solution with picric acid. Make sure that at least 0.5 gm. of dry picric acid has been

*Heyden Chemical Corporation, 342 Madison Avenue, New York 17, N. Y.
added to each tube and that some solid picric acid remains in the bottom of each tube to guarantee saturation.

Add 0.02 ml. of each serum to the "C" series and snap each tube again several times to complete precipitation of the protein. Filter each tube through Whatman No. 2 paper into another series of large test tubes. Transfer 1 ml. of the filtrate into 13 × 100-mm. tubes previously graduated at 5.0-ml. volume. Add 1 ml. of 15% sodium carbonate solution; mix and place in a boiling water bath for 20 minutes. At the same time, a blank composed of 1 ml. saturated picric acid and also a 1 ml. glucose standard (0.200 gm./l.) in saturated picric acid are run with each group of unknowns.

Remove the entire rack of tubes and allow to cool for several minutes; make up to volume with distilled water. Read in a Klett photo-electric colorimeter with filter No. 54 (green) with the instrument set at "O" with the picric acid blank.

Results are calculated by multiplying the difference (average of A and B less C) by the K value (from the glucose standard reading) by the dilution factor as follows:

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\frac{100}{0.02} \times 5.02 \times K = \text{units per 100 ml. serum}
\]

Normal range for humans is 120-260 units per 100 ml., and for rats, 8,000 to 13,000 units per 100 ml.

NOTES ON THE METHOD

As with all enzyme activity determinations, it is essential to adhere strictly to an arbitrary set of conditions. In this method a 15-minute incubation period is utilized and the timing should be carefully observed. If many specimens are being tested, then as much time must be taken to add the dry picric acid as was originally required to add samples to the incubation mixture.

The rack of tubes should be removed from the water bath just before the end of the incubation period, prior to adding the dry picric acid. For dispensing the picric acid, a crude syringe can be constructed from a short length of 7-cm. glass tubing with a plunger of glass rod fitted loosely. The tubes should be agitated individually several times to aid solution of the picric acid.

The picric acid used for stopping enzyme action and developing color is air-dried in a large evaporating dish. A thin layer of moist picric acid as it comes from the manufacturer is spread out on the
bottom of the dish, covered with filter paper, and left for a day or two in a warm place, such as on top of a drying cabinet where the temperature will be about 100°.

Not all makes of picric acid are satisfactory and, rarely, certain lots from one manufacturer will cause difficulty because of off-shade color development. For several years we have standardized on Baker Analyzed Reagent which yields clear yellow to orange colors quite consistently.

It is good practice to leave a small amount of solid picric acid on the bottom of both the saturated picric, and glucose standard, reagent bottles so as to compensate for any seasonal variations in room temperature which may occur.

The tubes employed for incubation were 16 × 125 mm., which was short enough to rinse the 0.02-ml. pipets in the incubation mixture. The tubes employed for color development were Pyrex 13 × 100-mm. lipless test tubes, graduated in our laboratory at 5.0 ml. using a diamond point pencil.

The blank value with soluble starch was not far from the blood glucose value. High blood glucose values will decrease the accuracy of the method, since the activity is measured by difference and elevated blood glucose will produce a high blank. The occurrence of such high blanks was rare.

With normal random rat serums, the activity tube (A and B) gave readings five to seven times higher than the blank (C) readings. The use of any starch other than that prepared by the Lintner technic will yield high-blank reducing values.

In rinsing the plasma from the pipets into the incubation mixture, it is important to avoid contamination from saliva. The rubber mouthpieces must be kept clean and dry, and it is worthwhile to connect a saliva trap in the line tubing between the mouth and the pipet containing the sample.

Following color development, the rack of tubes may be immersed in cold water for a few moments, but they should not be chilled because picric acid will crystallize out. It is best to add some water to all tubes immediately after removal from the cooling bath and make to volume when the tubes have reached room temperature.

In boiling the tubes for color development, it is very important to have the water rapidly boiling and deep enough to cover the tubes to a greater depth than the contained solution.

It is convenient to make up sufficient solution for a day’s run
beforehand from accurately measured buffer, starch, and salt solution.

TECHNIC FOR COLLECTING BLOOD SPECIMEN

Experience has shown that collecting blood directly into capillary tubes was not always successful because of clotting, and therefore bleeding is carried out into 5-ml. beakers containing a small quantity of dry anticoagulant such as potassium oxalate or powdered heparin (Hynson, Westcott, and Dunning).

In drawing blood from infants, use the heel. The new blood lancets recommended for hematologic finger pricking are inadequate and a No. 11 Bard-Parker scalpel blade is recommended. With rats, hold the tail upon a large-size rubber stopper and make a 2-3-mm. longitudinal incision near the tip with a scalpel. The rat can be held in a length of mailing tube with wire screen over one end.

It is important to keep the parts being pricked warm, and with rats the tail is conveniently held in front of a 250-w. infrared bulb. From the beaker transfer the blood to glass capillary tubes—of the type recommended by Natelson (1)—by tipping the beaker and allowing the blood to run into the capillary. It is not necessary to use suction to fill the tubes, as this introduces air bubbles.

After filling the capillary tube, seal the large end over a microburner, allow to cool, and then centrifuge. Discard the column of red cells later by breaking the tube after nicking with an ampoule file. Fill three 0.02-ml. Sahli hemoglobin pipets by touching the pipet tip to the capillary and allowing each to fill to the mark. Place the pipets in a moist, covered tray for immediate determination of enzyme activity. With infants, the use of 0.02 ml. serum yields very low activities and a sample size of at least 0.3 ml. is recommended.

DISCUSSION

This technic represents a modification of a method originally described by Myers, Free, and Rosinski (2). As used here, 15 mg. of starch substrate was available in each incubation mixture. Much experience with the method was had with 10 mg. of starch in each incubation tube, but the amount was increased to 15 mg. so as to achieve full linearity between enzyme concentration and reaction rate. With normal levels of amylase in the rat the reduction observed was from 0.2 to 0.25 mg. expressed as glucose. Therefore, usually less than 1-2 per cent of the substrate was consumed during an aver-
age incubation so that during the incubation period, substrate was present in relatively great excess. In the presence of adequate substrate, enzyme concentration will be proportional to rate, so that if the rate is at a maximum, the quantity of endproduct will be proportional to enzyme concentration. Thus, if the starch substrate does undergo some degradation due to bacterial or mold contamination, the apparent activity of any specimen of amylase enzyme will tend to decrease, since the ratio of enzyme to substrate is critical, even in the range of substrate excess.

The use of preservatives is recommended because unprotected starch quickly becomes contaminated and rapidly develops high blanks. The mixed methyl-propyl esters of hydroxybenzoic acids employed for the starch substrate were very effective, as evidenced by visual inspection and bacteriologic studies. When a starch solution
Fig. 2. Relation of boiling time to color development.

Fig. 3. Relation of enzyme concentration to activity.
was made up and divided between two sterile containers, with preservative added to only one, it was found that even after deliberately contaminating both with cultures of various bacteria, the starch with preservative added continued to produce the same blank reading. On the other hand, the unprotected starch yielded high values within a few days. The preserved starch was sterile upon culture after several months’ time, and a few samples of such preserved starch left standing on the shelf for periods up to 12 months were still apparently free of contaminants. It is good practice, however, to occasionally run a starch blank, always to store the substrate at 5°, and to make up fresh starch each month if not used in less time. The preservative does not interfere with the action of either human or rat amylase enzyme.

The unit of activity for this method is in terms of glucose reducing power expressed in milligrams per 100 ml. serum. Since picric acid is more effective than alkaline copper in determining reducing activity of maltose, the glucose equivalent is higher. This is partially compensated by the fifteen minute incubation period so that the actual normal range in units is only slightly higher than for conventional Somogyi amylase units.
Fig. 5. Spectrophotometric absorption curves of picric acid, glucose, rat serum, and starch.

One of the possible variables in this method is the saturation step, where dry picric acid is added to stop enzyme action. In a series of 120 rat serums performed in triplicate, one series was saturated at 20°, the second at 25° and the third at 30°. The mean Klett values for these three groups were 260, 262, and 261. All three determinations were processed in the same manner except for the temperature at which saturation with picric acid was carried out at the end of the incubation period. Apparently the temperature of the tubes following incubation when the picric acid is added is not critical.

In the original method of Myers, Free, and Rosinski "saturated" sodium carbonate was employed to develop color with the picric acid filtrate. The concentration of sodium carbonate in saturated solution was determined gravimetrically and found to be almost exactly 30
per cent. The color developed with a glucose standard in saturated picric acid employing various concentrations of alkali yielded the results presented in Fig. 1. As a result, a strength of 15% sodium carbonate was selected to furnish a final concentration of 7.5% or approximately one-fourth saturated sodium carbonate.

The effect of various boiling times was investigated as shown in Fig. 2 and, as a result, a time of 20 minutes was selected as optimum.

The relationship of the amount of enzyme present to amylase activity was studied by preparing a series of dilutions of a pooled rat serum to yield enzyme concentrations of 10, 20, 30% and up to full strength serum. The activity followed a straight line function as shown in Fig. 3.

The effect of varying the incubation time was studied and the data are presented in Fig. 4. It can be observed that during and following the 15-minute period of incubation, the activity bears almost a direct relationship to time, confirming earlier studies with the original macromethod as applied to duodenal contents (3, 4).

In addition, the spectrophotometric absorption curves of a picric acid blank, starch blank, picric glucose standard, and an unknown were determined on a Coleman Universal spectrophotometer, with 19 × 105-mm. round cuvets. The curves are plotted in Fig. 5 and indicate that the use of the Klett instrument with filter at a maximum transmission of 540 mμ is satisfactory.

**SUMMARY**

A micromethod for the determination of serum amylase has been presented, which is useful in pediatric practice and in experimental work on small animals. The optimum conditions for the procedure have been studied and the data obtained recorded.

**REFERENCES**