Serum Amylase Determination by Somogyi’s Amyloclastic Method with Use of a Photometric End-Point

Arnold E. Reif and Donald C. Nabseth
With the technical assistance of Lora M. McFety

Somogyi’s amyloclastic amylase assay has been modified by substitution of a spectrophotometric end point for visual observation, thereby increasing precision without changing the Somogyi assay units.

A method of estimating starch concentration from the intensity of the starch-iodine color reaction is described; absorbance is directly proportional to starch concentration.

Somogyi’s finding that corn starch is the substrate showing least variation in ability to be degraded by amylase has been confirmed; a rapid method of standardizing the substrate is described.

High amylase activities obtained using the amyloclastic method with sera containing lysed red cells are artifacts resulting from the interference of hemoglobin with the starch-iodine color reaction; the extent of the interference has been delineated, and means of avoiding it are described.

Somogyi recently expressed doubt that the precision of his widely used amyloclastic method could be enhanced by substituting an optical instrument for visual observance of the end point of the reaction (1). In the present paper, details of a precise method using a spectrophotometer—already described briefly (2)—are given. The method has been used to investigate further Somogyi’s finding that amyloclastic activity varies widely with the type of starch used (3), and to study the interference of lysed red cells with the amylase assay.

From the Biochemistry Section, Department of Surgery, Tufts University School of Medicine and the First (Tufts) Surgical Service, Boston City Hospital, Boston, Mass.

The investigation reported in this article was supported by Grants A-3743 and CY-4469 from the National Institutes of Health, U.S.P.H.S.

Received for publication Feb. 14, 1961.
Materials

Iodine Reagent

Somogyi's stock iodine solution (1) is made by dissolving 10 gm. of KI in 20 ml. distilled water. This solution is used to dissolve 126.9 mg. of I₂ completely and is then diluted to 500 ml. with water. A scrupulously clean flask must be used, since iodine will precipitate out on any trace of impurity that provides condensation nuclei. This stock solution is stable for 4 weeks in the refrigerator (3°).

The iodine reagent is made by diluting 1 part stock solution with 2 parts distilled water and adjusting the pH to 10.0–10.2 using 0.1N NaOH (approximately 0.4 ml./100 ml. of reagent). The iodine reagent is kept at room temperature, remains precisely stable for 1 day only, and should not be kept beyond 3 days (see below).

Standard Substrate Solution

The substrate solution is that described by Somogyi (1), made by a more rapid procedure. This substrate solution is available commercially (Table 1).

The buffer described by Somogyi (1) is used only to prepare the standard substrate solution. It is made by boiling 2.25 gm. of KH₂PO₄, 4.5 gm. of Na₂HPO₄·7H₂O, 2.5 gm. of NaCl, 2.0 gm. of NaF, and 0.3 gm. of propyl parasept (n-propyl p-hydroxybenzoate, Eastman Kodak Co., No. 2992) in 1000 ml. of distilled water, until the molten globules of propyl parasept have dissolved. The buffer is allowed to stand 3 days until excess propyl parasept has settled out, and then the supernatant is decanted.

Then 10.0 gm. of corn starch is placed in a 600-ml. beaker and suspended completely in 50 ml. of buffer, which is added at 55° while the mixture is being stirred mechanically. Next 450 ml. of briskly boiling buffer is decanted into the beaker, with vigorous stirring. Mechanical stirring is continued for 1 min., and then the starch suspension is poured into an Erlenmeyer flask and left to stand covered with an inverted beaker at room temperature, as described by Somogyi (1). After 2 days, the suspension is centrifuged at high speed (1 hour at 2500 g), preferably under refrigeration (10°). The clear supernatant is decanted and can be kept as stock starch solution at room temperature for 1–2 days.

The most accurate way to prepare a substrate solution of 75 mg. of starch per 100 ml. from the stock solution is by determination of starch content, using acid hydrolysis and subsequent dilution with buffer, as
described by Somogyi (1). With any one batch of corn starch powder, this procedure need only be performed once, but then in duplicate. At that time, the optical densities of the two substrate solutions are determined spectrophotometrically, as described in the next paragraph, and averaged; thereafter, substrate solutions are prepared by dilution of fresh stock solutions with buffer to give the same average optical density.

A more rapid method that is adequate for many purposes is to dilute an aliquot of stock solution with the buffer to give an optical density of 1.11 at 700 m\(\mu\). This is measured with the Beckman D.U. spectrophotometer 5 min. after addition of 0.40 ml. of starch solution to 5.0 ml. of iodine reagent containing 0.1 ml. of water. Cuvets of 1.00 cm. light path are used; the blank cuvet contains 5.0 ml. of iodine reagent and 0.5 ml. of water. If the Coleman, Jr., photometer is used, this concentration corresponds to an optical density of 0.90 at 700 m\(\mu\), 5 min. after addition of 0.2 ml. of starch solution to 5.0 ml. of iodine reagent containing 0.3 ml. of water, when read against the same blank in type “A” cuvets.

The substrate solution is placed in an Erlenmeyer flask fitted with a two-holed stopper, one hole holding a Pyrex syphon tube, the other a short Pyrex air inlet tube that is bent 120° (3). The substrate solution is sterilized by heating to boiling and continuing boiling gently for approximately 1 min.; meanwhile suction is used to fill and flush the syphon tube, and a loose cotton plug is inserted in the air inlet tube. A solid Pyrex rod is dipped in absolute ethanol and used to stopper the syphon below the usual rubber tube and pinch-clamp. The same sterilizing procedure is repeated 1 week later. Whenever substrate is required, a few milliliters are discarded to eliminate traces of ethanol; after taking sufficient substrate for use on 1 day, the Pyrex rod is again dipped in ethanol and replaced. The stability of the substrate solution is followed by pipetting 0.4-ml. aliquots into 5.0 ml. of fresh iodine reagent containing 0.1 ml. of water. It is discarded when its optical density falls to a point at which the consequent increase in apparent amylase activity becomes undesirable (Table 1 and Fig. 5).

Procedure

A 5.0-ml. quantity of iodine reagent is pipetted into a series of test tubes, or, if the Coleman, Jr., photometer is being used, into Coleman cuvets. Then 4.0 ml. of standard substrate solution is pipetted into a test tube approximately 15 mm. I.D., and the tube (or several tubes, if
several tests are to be run) is placed in a water bath at 37°. After 10 min. have elapsed, during which temperature equilibration occurs, 1.0 ml. of plasma or enzyme dilution is added from a blow-out pipet, a stopwatch is started, and the tube is mixed and replaced in the bath. At any desired time interval (2 min. is appropriate for taking the first sample), a 0.5-ml. sample is withdrawn from the reaction tube, pipetted into a tube containing iodine reagent, mixed, and the precise time of addition recorded. The number of aliquots of the reaction mixture tested depends on the rate of disappearance of the starch, which the operator quickly learns to judge from the color of the first tube; further samples are taken to fall just before and just after the apparent end point, at which the red-brown color of erythrodextrin is seen with barely a perceptible tint of purple (1, 3); generally, a total of three to five samples suffices. The absorbance of three starch-iodine solutions straddling the end point is read immediately at 700 m\(\mu\) against a blank containing 5.0 ml. of iodine reagent and 0.5 ml. of water. If the end point is earlier than 2 min., a 1 to 10 dilution of enzyme is made with distilled water, and the test is repeated.

A curve of absorbance against time of reaction is constructed. The end point of the reaction is read off this graph as the time at which the absorbance of the reaction mixture has dropped to 15 per cent of the zero reaction tube, which contains 5.0 ml. of iodine reagent, 0.1 ml. of water, and 0.4 ml. of standard substrate solution; this absorbance is 0.167 if the Beckman D.U. spectrophotometer is used and 0.27 if the Coleman, Jr., instrument is used.

Enzyme activity in Somogyi units is calculated from the equation:

\[
\text{Amylase activity} = \frac{2000}{\text{Time} \times \text{volume}}
\]

where time is expressed in minutes and volume in milliliters of undiluted enzyme solution. For instance, if 1.0 ml. of a 1 to 10 dilution of serum gave an end point at 4 min. 45 sec., time would be indicated as 4.75, and volume as 0.1 ml.; the amylase activity would be 4210 Somogyi units.

According to Somogyi (3), the amylase activity of normal human serum ranges "from 80 to 150 units, with occasional extensions of moderate degrees beyond either limit." Boyd, Boyd, and Byrne (10) have studied serum amylase values of hospital patients recently, using Somogyi’s original method (3), and these workers do not attach clinical significance to values below 300 Somogyi units.
If sera are cloudy owing to high lipid content or are slightly hemolyzed,* absorbance at 700 m\(\mu\) is read against a serum blank containing 5.0 ml. of iodine reagent, 0.4 ml. of water, and 0.1 ml. of serum, instead of against a water blank. If the sera are hemolyzed at a level above 0.5 gm. of hemoglobin per 100 ml. of serum, the present assay gives high amylase results which are artifactitious. Such sera are therefore diluted to this level for the assay; if this is impracticable, iodine solution 7 (see Table 2) can be used in conjunction with soluble starch (Lintner), which is dissolved in Somogyi's buffer (see above), boiled for 20 min., and standardized against the standard substrate solution to give the same reaction time with a given enzyme solution.

Once familiarity with the technic has been attained, two assays can be run simultaneously (1). All the results reported below were obtained using a water-bath thermostated to \(\pm 0.1^\circ\). Unless otherwise specified, dog serum was used as the source of enzyme.

### Experimental Results

#### Assay Method

The wavelength-absorption curve of intact corn starch solution, following pipetting of an aliquot into 5.0 ml. of iodine reagent, shows a flat maximum at 624 m\(\mu\) (curve A in Fig. 1). The corresponding curve for an equivalent sample of corn starch solution taken at the end of the amylase assay, when it has been broken down to "erythrodextrin" (1, 3), has a maximum at 547 m\(\mu\) (curve B in Fig. 1). The wavelength for the absorbance readings was chosen to be 700 m\(\mu\), to give a relatively high absorbance with intact starch and a relatively low absorbance with "erythrodextrin."

The absorbance at 700 m\(\mu\) of starch solutions added to the iodine reagent increased by 3–4 per cent within the first 5 min., and thereafter only very gradually (Fig. 2). In preparing standardized starch solutions, the absorbance was read precisely 5 min. after the starch and iodine solutions were mixed. In running the amylase assay, absorbances were read during or immediately following each assay, without

---

*To determine the approximate hemoglobin concentration, 0.2 ml. of plasma is pipetted into 4.8 ml. of 0.1N HCl and the optical density is read 10 min. later at 520 m\(\mu\) against a water blank. Then hemoglobin concentration in grams per 100 ml. of serum equals optical density times 3.39 if read in 1.00 cm. cuvets in the Beckman D.U. spectrophotometer; times 2.17, if read in "A" cuvets in the Coleman, Jr., instrument. With the use of the more precise potassium ferriyanide-cyanide solution (containing 1 gm. of NaHCO\(3\), 50 mg. of KCN, and 200 mg. of K\(_3\)Fe(CN)\(_6\) in 1 L. of water) instead of 0.1N HCl, the respective factors are 3.64 and 2.55.
observing a standardized time of reading. Maximum accuracy could be achieved by waiting 10 min. before reading the absorbances, but this method accentuated interference by hemoglobin when hemolyzed sera were used.

![Fig. 1. Wavelength-absorption curve of corn starch-iodine reagent mixtures. Curve A indicates starch before addition of amylase; Curve B, degraded starch ("erythrodextrin") at end point of amylase assay.](image)

Direct proportionality between the absorbance of the starch-iodine mixture and the amount of starch added was maintained precisely up to an absorbance of 1.3 as determined with the Beckman D.U., or 2.0 as determined with the Coleman, Jr., spectrophotometer. In these experiments, which proved conformity to Beer's law, all tubes stood more than 5 min. after mixing before their absorbances were determined. The absorbance obtained was insensitive to change in pH of the iodine reagent between pH 7 and 10.

On addition of serum dilutions to starch solution, a rapid decrease was obtained in the absorbance of samples pipetted subsequently into the iodine reagent (Fig. 3). The end point of the assay was set at an absorbance of 15 per cent of its initial level. This end point was a trace earlier than that obtained in parallel experiments with Somogyi's visual method (3), but it had the advantage that the curves were still steep (Fig. 3), resulting in a sharp end point. The measured color reaction does not follow first-order kinetics, since plots of the logarithm of absorbance against time gave S-shaped curves for data like those shown in Fig. 3.
Fig. 2. Change in absorbance following addition of corn starch solutions to iodine reagent at room temperature. Curve A indicates starch before addition of amylase; Curve B, partially degraded starch; Curve C, degraded starch ("erythrodextrin") at end point of amylase assay.

Fig. 3. Change in absorbance following addition of 1.0 ml. of various dilutions of dog serum (Curve A, undiluted; Curve B, 1 to 3.3 dilution; Curve C, 1 to 8 dilution) to 4.0 ml. of standardized starch solution held at 37.0°. At times indicated, 0.5-ml. aliquots of reaction mixture were pipetted into 5.0 ml. of iodine reagent standing at room temperature.
A plot of amylase activity against volume of plasma assayed gave a curve that deviated significantly from direct proportionality only at amylase levels above 5000 (Fig. 4). Similar curves were obtained with human plasma and with dilutions of peritoneal fluid from dogs with acute experimental pancreatitis.

![Fig. 4. Relationship between volume of dog plasma contained in 1 ml. of solution assayed and amylase activity of the solution, when determined at 37.0°.](image)

To determine the variation in amylase activity caused by using starch concentrations other than the standard one used in this method, the experiment recorded in Fig. 5 was done. There was a progressively sharper increase in apparent amylase activity with decrease in starch concentration. This confirms the belief that low amylase activities can be determined most sensitively at low substrate concentrations, as noted by Somogyi (1).

The reproducibility of the present method was checked by running six identical assays. With the end point at 5.1 min., the standard deviation was 2.4 sec., or 0.8 per cent. Somogyi himself mentioned that the reproducibility of his method is within 30 sec. if care is taken, and that 1 min. represents a degree of precision that is easily attainable. The average variance of assays run by Boyd et al. (4) using Somogyi’s method was 1.45 min.

Since Somogyi ran his assay at 40° (1), while the present assay was run at 37.0°, the ratio of activities of the serum enzyme at these two
temperatures was determined. It averaged 1.133, and there was no significant variation, regardless of whether it was determined with an enzyme concentration giving an end point in 4, 12, or 29 min. This ratio of activities raised the value of Somogyi’s constant $K$ from 1800 to 2040 for assays run at $37^\circ$; since Somogyi’s original method is not precise to 2 per cent (see above), adoption of the round figure of 2000 for the present assay is sufficient to preserve identity of results.

Exhaustive experiments were run on the stability of iodine stock solution, iodine reagent, standard substrate solution, and serum enzyme. Soluble starch content of substrate solutions was measured in terms of a drop in starch-iodine color intensity at 700 m$\mu$. Deterioration of stock iodine solution of $pH$ 6.7 when held at $3^\circ$ was less than 0.2 per cent after 2 weeks’ storage, and less than 2 per cent after 2 months’ storage. The iodine reagent of $pH$ 10.0–10.2 deteriorated less than 1 per cent after 1 day at room temperature, and less than 3 per cent after 3 days. Since iodine concentration can affect the starch-iodine color in the presence of hemoglobin in a manner not evident in its absence (see Table 3), the iodine reagent was not kept beyond 3 days.

Surprisingly, triplicate repeat experiments with Somogyi’s corn
starch substrate buffered with propyl parasept (1) showed a drop of 6–12 per cent in absorbance readings in the first 4 days after preparation, and occasionally a 50 per cent drop in 7–15 days. This drop evidently was the result of failure to achieve sterility by use of propyl parasept as preservative (11). However, when sterile precautions (3) as well as propyl parasept buffer (1) were used, there was no deterioration within 35 days (sample 1 in Table 1). The commercial substrate preparation, which was prepared in the same way, also proved to be stable (samples 5, 6, and 7 in Table 1). Omission of propyl parasept from Somogyi’s medium but inclusion of two sterilizations resulted in a slight or questionable deterioration (sample 2), and a similar result was obtained with Somogyi’s original substrate (sample 3). Addition of penicillin and streptomycin failed to halt deterioration of a nonsterile substrate (sample 4).

The amylase activity of a sample of dog serum that was stored frozen at $-10^\circ$, determined on the day of preparation of the substrate and 21 days later, is recorded in the last two columns of Table 1. The activity obtained using the latest commercial batch of substrate tested

Table 1. Deterioration of Corn Starch Substrate Solutions with Time

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Description</th>
<th>Absorbancies of starch solutions*</th>
<th>Apparent amylase activity (Somogyi units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 0</td>
<td>Day 7</td>
</tr>
<tr>
<td>1</td>
<td>Standard Somogyi (1) substrate (this study)</td>
<td>1.101</td>
<td>1.090</td>
</tr>
<tr>
<td>2</td>
<td>Like 1, but propyl parasept omitted</td>
<td>1.175</td>
<td>1.158</td>
</tr>
<tr>
<td>3</td>
<td>Original Somogyi (3) substrate</td>
<td>1.137</td>
<td>1.064</td>
</tr>
<tr>
<td>4</td>
<td>Like 3, but 1000 U. penicillin G and 1000 µg streptomycin per ml. added.</td>
<td>1.077</td>
<td>1.051</td>
</tr>
<tr>
<td>5</td>
<td>Somogyi (1) substrate, batch 30 B†</td>
<td></td>
<td>0.803</td>
</tr>
<tr>
<td>6</td>
<td>Like 5, batch 80 B†</td>
<td></td>
<td>0.959</td>
</tr>
<tr>
<td>7</td>
<td>Like 5, another sample batch 80 B†</td>
<td></td>
<td>0.958</td>
</tr>
</tbody>
</table>

*0.4 ml. of starch solution added to 5.0 ml. of iodine reagent containing 0.1 ml. of water. Read 5 min. later in a Beckman D.U. spectrophotometer at 700 mµ, using cuvets of 1-cm. light path. Each value is the mean of triplicate determinations.
†Sigma Chemical Company, St. Louis 18, Mo.
(samples 6 and 7) compared well with that obtained using our substrate (sample 1). The lower amylase activity obtained with sample 2 as compared to sample 1 was not explained, and this difference vanished when both samples were made up fresh a second time. While propyl parasept did not inhibit amylase activity, 1% benzoic acid, which was used as a preservative by Caraway (5), did inhibit if substituted for propyl parasept in preparation of Somogyi's substrate. It caused 20 per cent inhibition when the end point was at 17 min., increasing to 50 per cent inhibition at an end point of 4 min. Benzoic acid added to the assay system but not used in the buffer in preparation of the substrate was almost inactive as an inhibitor.

The effect of storage on amylase activity was tested with three non-sterile heparinized human plasmas. Storage for 0, 1, 3, and 8 days at 3° resulted in mean activities of 100, 101.6, 96.9, and 93.7 per cent, respectively. After storage for 8 days at −10°, the mean activity was 95.5 per cent.

**Standardization of Substrate**

Somogyi maintained that use of corn starch as substrate was essential in his amyloclastic method, since different samples of soluble starch obtained from the same source gave amylase activities varying by as much as 100 per cent (1). In the present study, different starches were standardized both on the basis of the glucose content of their acid hydrolysates as described by Somogyi (1, 3), and on the basis of absorbance of starch solutions with iodine reagent. The results (Table 2) confirm the findings of Somogyi.

A better index for standardizing the substrate was starch content rather than absorbance (Table 2). Thus, the standard deviation of the apparent amylase activity of four corn starch lots was 6.9 per cent for solutions of constant starch content but 16.7 per cent for solutions of constant optical density. Nevertheless, the degree of reproducibility obtained by standardizing corn starch solutions on the basis of absorbance is sufficient for clinical use and for many research purposes and has the advantage of convenience. Such solutions should be adjusted (see Materials) to an absorbance reading of 1.11, which is the mean absorbance obtained with four solutions each containing 75 mg. of starch per 100 ml., each solution having been prepared from different batches of corn starch (Table 1).

To determine whether the method of preparing the corn starch solution affected its apparent amylase activity, three different samples of
Table 2. Variation of Amylase Activity with Type of Starch Substrates*

<table>
<thead>
<tr>
<th>Type of starch</th>
<th>Brand and packer or manufacturer</th>
<th>Apparent amylase activity at constant starch concentration† of 75 mg./100 ml. (%)</th>
<th>Absorbance of starch of 75 mg. starch/100 ml.</th>
<th>Apparent amylase activity at constant absorbance of 1.11 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>“Carver,” M. Stoll</td>
<td>100.0</td>
<td>1.049</td>
<td>96.5</td>
</tr>
<tr>
<td>Corn</td>
<td>“Swansdown,” S. S. Pierce</td>
<td>104.4</td>
<td>1.044</td>
<td>96.8</td>
</tr>
<tr>
<td>Corn</td>
<td>“Argo,” Corn Products</td>
<td>112.1</td>
<td>1.314</td>
<td>132.2</td>
</tr>
<tr>
<td>Corn</td>
<td>“Cream,” A. E. Staley Mfg.</td>
<td>115.0</td>
<td>1.020</td>
<td>108.1</td>
</tr>
<tr>
<td>Potato</td>
<td>Baker Chemical</td>
<td>88.8</td>
<td>0.448</td>
<td>34.9</td>
</tr>
<tr>
<td>Soluble</td>
<td>Mallineckrodt</td>
<td>63.6</td>
<td>0.402</td>
<td>24.1</td>
</tr>
<tr>
<td>Soluble</td>
<td>Merck</td>
<td>130.4</td>
<td>0.473</td>
<td>72.8</td>
</tr>
</tbody>
</table>

*Mean results for two experiments are given. In each experiment, a single enzyme solution of constant amylase content was used to determine apparent amylase activity with different substrates.

†The apparent amylase activity obtained with 75 mg. corn starch “Carver”/100 ml. was designated 100% activity in each experiment. Starch concentration was determined from glucose content of acid hydrolysate (1).

‡0.4 ml. starch solution added to 5.0 ml. iodine reagent containing 0.1 ml. water.

The same corn starch were prepared and standardized to the same absorbance as described under Materials; the apparent amylase activity of an enzyme solution showed a maximum difference of 3.3 per cent when determined with the use of these substrate solutions.

The effect of deviating from the procedure described under Materials by using less or more heat during solubilization of the starch suspension initially at 55° was investigated as follows. Instead of adding boiling buffer to the starch suspension, a delay of 20 sec. and 1 min., respectively, was made between removal of the boiling buffer from the Bunsen burner and addition to the suspension. When the activity of an enzyme solution was determined with these substrates following standardization to an absorbance reading of 1.11 (see Materials), the 20-sec. delay had no effect, while the 1-min. delay increased the apparent amylase activity by less than 2 per cent above that obtained with the use of standard substrate solution. If the initial starch suspension was lumpy, as when starch powder was added to the buffer at 55° instead of the reverse procedure, the same 1-min. delay increased the apparent amylase activity by 35 per cent, suggesting that only shorter-chain molecules were being solubilized at the lower temperature caused by the delay. The effect of more heat was investigated by boiling the starch suspension for 1 min. following addition of the boiling buffer, then standardizing the absorbance to 1.11, as above. With this sub-
strate, the apparent amylase activity of an enzyme solution was 42 per cent lower than with the standard substrate solution, suggesting that boiling had solubiлизed starch molecules with longer chain lengths. It must be stressed that the above effects apply only to the solution of starch from a suspension. Once solution had been effected and any insoluble starch removed by centrifugation, refluxing the starch solution for 30 min. had no effect on starch content, nor on apparent amylase activity as determined with a given enzyme solution.

The action of amylase on different starches was tested further by running parallel amylase assays at three enzyme concentrations, each differing by a factor of two. Solutions of corn starch, soluble starch (Lintner), and a commercially standardized starch solution (Sigma Chemical Co., No. 700-1) were compared in this way. While there were differences in absolute activity as indicated in Table 2, the ratio of activities at any enzyme concentration was constant within 2 per cent. These results indicate that relative values obtained with soluble starch (Lintner) are as reliable as relative values obtained with corn starch.

**Limits of Starch-Iodine Reaction**

In investigating the interference of lysed red cells with the amylase assay, the effect of altering the concentrations of the reactants in the starch-iodine reaction was investigated. The interaction between iodine, potassium iodide, starch, serum components, and hemoglobin, and its dependence on pH, proved to be complicated. Limits of the starch-iodine reaction were found beyond which the reaction broke down, either as the result of formation of a precipitate or from decoloration of the starch-iodine color.

Table 3 presents results obtained in a typical qualitative experiment. Addition of lysed red cells of known hemoglobin concentration caused decoloration of the starch-iodine color. A green rather than a blue starch-iodine color was caused by the intense yellow background of the iodine solution, superimposed onto the blue starch-iodine color. The higher the concentration of $I_2$ at constant KI concentration, the higher the permissible hemoglobin concentration (last column, Table 3). However, the higher the $I_2$ concentration, the more rapid the formation of a precipitate. When the concentration of KI was raised at constant $I_2$ concentration, the capacity to withstand interference by hemoglobin was raised and the rate of formation of a precipitate was retarded (Table 3). In every case, raising the pH of the iodine solution slowed the rate of formation of a precipitate, but it also reduced
Table 3. Breakdown of Starch-Iodine Color Reaction: Dependence of Precipitate Formation and Decoloration on Concentration of KI, I₂, Hemoglobin, and on pH at Constant Starch Concentration*

<table>
<thead>
<tr>
<th>Starch-iodine reaction</th>
<th>Precipitate formation</th>
<th>Iodine solution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>KI (gm./100 ml.)</td>
</tr>
<tr>
<td>1-A</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>1-B</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>2-A</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>2-B</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>3-A</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>3-B</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>6-A</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>6-B</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>15</td>
</tr>
</tbody>
</table>

*Each tube contained 5 ml. iodine reagent, hemoglobin concentrations expressed as if contained in plasma used in the present amylase assay, 0.5 ml. soluble starch (Lintner) boiled for 20 minutes, and distilled water to 6.3 ml. total volume.

The level of hemoglobin that was tolerated without interference with the starch-iodine color (Table 3). Iodine solution 7 gave no precipitate with soluble starch (Lintner) and could tolerate a high level of hemoglobin without detriment to the starch-iodine reaction. When its absorbance was read at 650 mμ following addition of starch solution, it performed as well in conforming to Beer's law as the iodine reagent finally chosen for the present amylase assay. However, it formed a precipitate rapidly if corn starch solution was used rather than soluble starch (Lintner). In general, corn starch performed in the same manner as soluble starch (Table 3) but formed a precipitate more rapidly.

The iodine reagent finally chosen (see Materials) corresponds closely to a 1 to 3 dilution of iodine solution 2-B in Table 2. At pH 10, no visible precipitate formed with corn starch solutions until 5–15 min. after addition of corn starch, and up to 1 hour later the precipitate was so minute as to escape all but the most careful inspection. However, in common with most starch-iodine complexes seen in this investigation, complete precipitation occurred within 2–3 days, leaving a colorless supernatant. The presence of minute particles 1 hour after starch and iodine reagents were mixed had no effect on the absorbance at 700 mμ,
nor did it necessitate mixing. Precipitate formation was considerably more rapid with Somogyi's iodine reagent (1), which is almost identical with iodine solution 2-A (Table 3).

The high values given by Somogyi's method (1) with sera containing high degrees of hemolysis are explained by the results shown in Fig. 6. Curves Aₕ and Aₖ demonstrate that the starch-iodine color disappears progressively as lysed red cells are added. The important curves, however, are Bₕ and Bₖ and Cₕ and Cₖ. They demonstrate that when the starch is partially broken down, the presence of far lower amounts of lysed red cells will decolorize the starch-iodine color, giving falsely high amylase values. There is also a decrease of starch-iodine color with time, so that the time curves for points on Curve C at higher percentages of hemolysis do not resemble those in Fig. 2. Solutions of bovine hemoglobin (Wilson & Co., Inc., Chicago) caused disappearance of the starch-iodine color at concentrations similar to those of human hemoglobin (Fig. 6).

Under the assay conditions described by Somogyi (1), lysed red cells had precisely the same effect as in the present system (Fig. 6), except that interference with the starch-iodine color was more marked, occurring at about 40 per cent lower concentrations of lysed cells.

![Fig. 6. Effect of hemolysis of 1.0 ml. of serum added to 4.0 ml. of starch solution on absorbance of 0.5 ml. of mixture and 5.0 ml. iodine reagent. Curves Aₕ, Bₕ, and Cₕ were read against usual water blank (5.0 ml. of iodine reagent, 0.5 ml. of water); Curves Aₖ, Bₖ, and Cₖ were read against a serum blank (5.0 ml. of iodine reagent, 0.4 ml. of water, and 0.1 ml. of hemolysed serum). Curve A indicates starch before degradation; Curve B, starch partially degraded; Curve C, degraded starch ("erythrodextrin") at end point of amylase assay. In this figure, 100 per cent hemolysis corresponds to a serum hemoglobin concentration of 14 gm./100 ml.](image)
Under the present assay conditions, adding an amount of freshly lysed human red cells equivalent to 4 per cent hemolysis increased the amylase activity of human serum artfactually by 10.6 per cent. The fact that three-times-washed, freshly lysed human red cells contain zero or negligibly low amounts of amylase was determined in the present system and checked by running aliquots of 40% lysed cells in Somogyi's saccharogenic assay system (1), with normal human sera used as positive controls.

**Discussion**

Somogyi's amyloclastic amylase assay is used so widely that a simple photometric adaptation that preserves the basic concepts and assay units of Somogyi's method is a logical development. Amylase assays recently reviewed by Boyd et al (4) and other methods that have appeared subsequently (5-7) have not produced such a method. The present adaptation is almost as simple as Somogyi's visual method, yet considerably more precise.

The present investigation has confirmed Somogyi's finding (1) that if a standard substrate is to be prepared, corn starch and not soluble starch must be used. A simple method of standardizing the substrate solution is described which uses the intensity of the starch-iodine color reaction. However, in preparing standard substrate solutions from different batches of corn starch, determination of starch content by acid hydrolysis (1) gives substrates of more uniform degradability.

Boyd et al. questioned whether the high amylase activity of lysed sera, when tested by Somogyi's amyloclastic method, might not be caused by the high amylase content of lysed red cells (4). The present study has shown that the amylase content of lysed red cells is negligibly small and that the high amylase activity of sera containing lysed red cells is an artifact caused by interference of hemoglobin with the starch-iodine color reaction. Since many of the decoloration phenomena observed in this investigation are reversible, the mechanism of the interference may be competitive adsorption of iodine, either on starch to produce the starch-iodine color complex, or on hemoglobin and presumably also on other normal serum proteins such as albumin, leaving insufficient iodine to fill the available active sites on starch. Close similarities with other comparable biologic systems involving adsorption are obvious (8, 9). Differences in the intensity of the starch-iodine color obtained with the same concentrations of starches of different origin (Table 2) suggest that such starches have different numbers of
active sites capable of adsorbing or reacting with iodine.* In addition, average chain length and consequently rate of degradation by amylase appear to vary in different samples of starch. Present data on the complex interaction between iodine, potassium iodide, serum components, hemoglobin, and starch are couched mainly in qualitative terms (Table 3), leaving a challenge to future investigators to delineate this area more precisely.

References


*A paper by Bailey and Whelan published since submission of this paper has clarified greatly the relationship between the starch-iodine color reaction and the chain length of starch. Data obtained with synthetic starches indicate that at average chain lengths of below 70 glucose units, both intensity and maximum wavelength of the starch-iodine color falls off rapidly with decrease in chain length, and the color disappears entirely below 12 glucose units. The interaction between starch and iodine was interpreted as a specific complexing of iodine along the major axis of the helical spiral of the glucose units that compose the starch molecule; in addition, adsorptive phenomena of a less specific type were thought to be involved (16).