Reaction Rate Measurement of Multiple Enzyme Samples by Continuous Flow Analysis

Harold H. Brown and Mary R. Ebner

A simple technic to adapt the advantages of continuous flow analysis to the kinetic assay of multiple enzyme samples is described. It will permit adequate standardization by primary or secondary standards run through the entire analysis of those procedures that do not exhibit spontaneous changes in light absorption or fluorescence with time. The adaptation of the Kind-King method for alkaline phosphatase (1) to this technic is given to demonstrate the superiority of such a system over single-point enzyme assays.

The assay of enzymatic activity by a single determination of a product formed or substrate consumed at a fixed time after mixing the substrate and sample is inherently poor methodology. The application of such methodology to continuous flow analysis, when the flow is maintained by the squeezing action of rollers on flexible tubing, variegates the single fixed time and makes standardization a problem.

The increasing use of commercially available enzyme preparations (which are not standards in the chemical sense, regardless of the designations applied to them by the manufacturers) as the only solution to this standardization problem commits an even more serious breach of chemically acceptable methodology.

The use of the AutoAnalyzer to study the kinetics of enzyme-substrate complexes and the effects of such parameters as pH and substrate concentration has already been described (2-4).

We have developed a simple means of utilizing the advantages of continuous flow analysis for measuring by reaction rates the enzymatic activity of a series of samples. We were particularly concerned with those reactions that cannot be incubated in a cuvet and followed spectrophotometrically or fluorometrically. Our primary goal, however, was to achieve a system that could be adequately standardized.

To illustrate the simplicity of this system we present as an example the Kind and King modification of the King and Armstrong procedure.
for alkaline phosphatase (1), although any procedure suitable for continuous flow analysis should be adaptable.

Methods and Materials

Equipment

Standard AutoAnalyzer components were used, with the exception of the modifications of the sample plate described below. A 5-cm. deep water bath to fit on the Sampler II was constructed of sheet copper (Fig. 1). The heating element of a laboratory immersion heater (Blue M Electric Co., Model TH-2004, 7-in. diameter) was rebent to fit the water bath.

The riser portion of the sample plate was cut flush with the underside of the sample plate, and a 9-cm. diameter disc was cut from the center of the base of the sample plate. The base and the ring that holds the sample cups were joined together by strap metal at such a height that the sample cups were immersed to an adequate depth in the bath (Fig.

Fig. 1. Constant temperature bath assembled and positioned on Sampler II.
1). The microswitch arm of Sampler II must be bent so it will be in the ON position when the water bath is in place.

Figure 2 shows the flow diagram for alkaline phosphatase.

Reagents

1. Carbonate buffer, 0.05 M, pH 10.2 Dissolve 3.13 gm. anhydrous sodium carbonate and 1.68 gm. sodium bicarbonate in water and dilute to 1 L. The pH is adjusted to 10.2.

2. Buffered substrate, 0.005 M phenyl phosphate Dissolve 1.09 gm. pure disodium phenyl phosphate in 1 L. of the carbonate buffer (Reagent 1).

3. Stock phenol standard, 50 mg./100 ml. A stock phenol standard was made and standardized by the usual iodine-thiosulfate procedure. The concentration was adjusted to 50 mg./100 ml. by diluting with 0.1 N HCl.

4. Working standards These were diluted in buffered substrate
as indicated in Table 1. The working concentrations are twice the actual concentrations because we used only half the standard amount of serum (see Procedure).

Five and 10 mg./100 ml. standards were made by diluting 5-ml. aliquots of the 20-mg. standard with 15 and 5 ml., respectively, of the buffered substrate.

The following reagents were described by Marsh et al. (5) for use with their automated procedure.

5. 4-Aminoantipyrine Dissolve 1.0 gm. in water and dilute to 1 L.
6. Potassium ferricyanide Dissolve 5.0 gm. in water and dilute to 1 L.

Procedure

One major advantage of this system is its flexibility. The choice of procedure depends on the number of samples and standards to be analyzed and the frequency with which they are to be repeated. Two examples are presented.

The water bath is assembled and allowed to come to temperature. The pump is started with all lines placed in their respective reagent bottles. The sampler reservoir is filled with the blank solution. The standards are placed on the sample plate and the sampler started; 0.2 ml. of the first unknown serum is mixed with 8 ml. of the buffered substrate and 0.2 ml. distilled water and placed on the sample plate following a cup containing the blank solution. The remaining serums are treated in an identical manner. The time of mixing is not critical, but two factors must be controlled: (1) the mixture must be in the water bath long enough to attain the set temperature before sampling; and (2) if the activity is elevated, the zero-time reading may be too high if the sample and substrate were mixed too long before sampling, and thus must be adjusted for.

The unknowns are sampled for zero-time activity followed by a standard and blank. The series of unknowns is repeated at whatever time interval was chosen, and for as many repeat determinations as desired.

Table 1. Preparation of Working Standards

<table>
<thead>
<tr>
<th>Buffered substrate (ml.)</th>
<th>Stock phenol standard (ml.)</th>
<th>H2O (ml.)</th>
<th>Phenol equiv. conc. (mg./100 ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0</td>
<td>5.0</td>
<td>0*</td>
</tr>
<tr>
<td>20</td>
<td>0.2</td>
<td>0.8</td>
<td>20.0</td>
</tr>
<tr>
<td>10</td>
<td>0.2</td>
<td>0.3</td>
<td>40.0</td>
</tr>
<tr>
<td>10</td>
<td>0.3</td>
<td>0.2</td>
<td>60.0</td>
</tr>
<tr>
<td>10</td>
<td>0.4</td>
<td>0.1</td>
<td>80.0</td>
</tr>
</tbody>
</table>

* Blank.
Figure 3 shows the recording of an analysis of a series of standards and eight unknowns followed by a standard and blank. The unknown mixtures are resampled at 15-min. intervals after the zero-time determination. The milligrams of phenol liberated in each sample is determined from the standard curve. The zero-time value is subtracted from the 15-min. value, and represents King-Armstrong units (K-A U.) of activity.

Figure 4 shows another series of the same unknowns (the preceding standards were omitted from the reproduction). This time, however, the sequence is repeated at 10, 20, and 30 min. after the zero time. The milligrams of phenol are determined from the standard curve, the blank value subtracted, and the results plotted against time (Fig. 5). The linearity of the reaction with time is obvious. Although the graph does not extend far enough to show it, even the activity of Sample 6 was constant to the 30-min. point. King-Armstrong units may be obtained from the graph by taking the 15-min. value. The results of the 15-min. incubation from Fig. 3 are plotted as x's for comparison.

To illustrate further the inherent flexibility of this technic; if 12
unknowns were to be assayed, the time sequence could be 0, 14, and 28 min. (to allow for a standard and a blank). The values would be plotted vs. time as in Fig. 5 and the 15-min. activity (K-A U.) taken from the graph.

![Graph](image)

**Fig. 4.** Recording of same unknown samples as in Fig. 3, but at a different sampling sequence. Samples 7 and 8 are duplicates, and are moved back 1 min. during last sequence. St, standard; Bl, blank.

We continue to use King-Armstrong units, although the equipment with its adjustable temperature control on the incubating bath is adaptable to computing international units.

When we tried the classic ratio of 0.1 ml. serum to 2 ml. buffered substrate, the useful range of assay was limited, especially when the incubation time was continued for 30 min. We therefore used only half the usual amount of serum and compensated by doubling the values of our working standards.

**Discussion**

In addition to the method described in the procedure we have also experimented with another assay for alkaline phosphatase and with glutamic oxalacetic transaminase. We believe the following points
should apply to most other enzyme analyses that are adaptable to this technic.

The minor amount of "noise" at the blank position and at zero-time activity of the samples is caused by the small air bubbles that enter the system while the sample-aspirating probe travels from the reservoir to the sample cup and vice versa, producing a momentary change in concentration and pH of the remaining reagents.

When the sampler is running at 60 per hour, it acts as a convenient timer. The operator must remain attentive, however, to reset the sample plate for each new period of sampling. It is also necessary to fill any empty spaces in the sequence with cups containing the blank solution. With these precautions the sampler and synchronously running recorder provide close control of incubation time of each sample.

A good automatic dilutor is convenient for measuring the standards or samples and buffered substrate because they do not have to be at incubator temperature when mixed: only when they are aspirated for zero-time activity.

---

**Fig. 5.** Plot of results from recordings of Fig. 4. Numbers at lines: sample numbers. Sample 6 linear to 30-min. Phenol (mg.) at 15 min. are K-A U. Results from Fig. 3 plotted as $x$. 
One notorious problem in multiple sample analysis by continuous flow has been the interference to a normal sample or one of low concentration by a preceding high one. Samples 7 and 8 of Fig. 3 and 4 are actually duplicates of the same serum that were included to demonstrate the degree of such interference; the 20-min. activity of Sample 6 did not obscure Sample 7 but did contribute slightly to it (Fig. 4). One method of rescuing Sample 7 is demonstrated at the 30-min. incubation time: the blank cup was placed in Sample 7's position and Sample 7 and all following cups were moved back one position; their "third" time is then 31 min. An alternative would be to remove Sample 6 and replace it with the blank; this would have the advantage of not altering the timing of the following samples.

There is another way to save a sample that is in danger of invalidation from preceding high activity if it is apparent on the zero-time sequence. The high sample can be replaced by a blank, and the samples run through an additional time sequence. The zero-time activity is taken as the second run, rather than the first.

If at zero time an unknown shows such great activity that its second sequence value may be above the highest standard, it could be inserted at an earlier time to save setting it up again.

This technic provides a simple method of establishing the reaction rate slope for the routine colorimetric assay of enzymes. It also promises a means of measuring some enzymes (e.g., glutamic oxalacetic transaminase) kinetically without coupling the desired reaction to other complex and unstable biochemicals in order to produce changes in U.V. absorbance or fluorometric emission. We are continuing to work in this area.

Finally, this technic permits adequate standardization of enzyme reactions by primary or secondary standards that can be run through the entire procedure. It provides an excellent system for each laboratory to establish values for its own control serums for use on the multichannel instruments that are becoming increasingly popular.

References