Automated Assay of Activities of Enzymes Involving the Diphosphopyridine Nucleotide \( \rightarrow \) Reduced Diphosphopyridine Nucleotide Reaction*†

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A continuous flow instrumental system of analysis, first described by Skeggs (I), was previously applied to the determination of phosphohexose isomerase activity and resulted in a method that was approximately eightfold as rapid as the manual procedure (2). The present paper is concerned with the application of this system of analysis to the determination of enzyme activities that utilize the diphosphopyridine nucleotide \( \rightarrow \) reduced diphosphopyridine nucleotide reaction; assays of lactic acid dehydrogenase, alcohol dehydrogenase, and glutamic oxaloacetic transaminase have been used to illustrate the method of application.

EXPERIMENTAL PROCEDURE

Materials—DPN (90 to 100% pure), a hydrated disodium salt of DPNH (90 to 95% pure), and malic dehydrogenase were purchased from the Sigma Chemical Company, \( \text{L} \)-aspartic acid and \( \text{L} \)-ketoglutaric acid from the California Corporation for Biochemical Research, sodium pyruvate from the General Biochemical Company, and human serum albumin from Cutter Laboratories. Yeast alcohol dehydrogenase, twice crystallized, and rabbit muscle lactate dehydrogenase, also twice crystallized, were obtained from the Worthington Biochemical Corporation.

Equipment—The instrument employed for the continuous flow instrumental system of analysis was the AutoAnalyzer (Technicon Instruments Corporation). A special colorimeter, made available to us by the Technicon Instruments Corporation, was equipped with blue sensitive phototubes and interference filters. The filter permitted the passage of light primarily at 680 \( \mu \text{m} \) and to a somewhat lesser extent at 340 \( \mu \text{m} \); the latter band had a half width of 17 \( \mu \text{m} \). A Corning No. 5840 blocking filter was used to cut off the primary band at 680 \( \mu \text{m} \). The cuvette had a 1-cm light path. It was consistently found that the absorbancies of various concentrations of DPNH, read in the automation colorimeter, were 75.3 ± 2.8% of those observed in the Beckman model DU spectrophotometer. This difference is probably due to the use of light from a monochromator in the Beckman spectrophotometer and light from a series of filters in the colorimeter of the automation system. When the cell compartment of the Beckman DU spectrophotometer was modified to accept the automation constant flow cuvette, identical absorbancies were obtained with the automation system and with the Beckman spectrophotometer used in the usual manner. A series of 18 comparative measurements on various concentrations of solutions of DPNH in the Beckman spectrophotometer and in the automation colorimeter yielded a molar absorbancy index, \( \alpha_{\text{m}} \), of 4.68 ± 0.13 \( \times 10^3 \text{ cm}^2 \text{ mole}^{-1} \) for the latter colorimeter. This value has been used in calculations of the automation data reported in this paper.

RESULTS

General Procedure for Automated Enzyme Determinations—The Autoanalyzer has a constant speed turntable with places for as many as 40 plastic cups. Through a system of plastic tubing, a constant flow pump aspirates the enzyme solutions from these cups at predetermined intervals of 40, 60, or 120 seconds, dilutes these solutions if necessary, adds the appropriate reagents, and segments the mixture with air to regulate the rate of flow and to clean the system between specimens. Beyond the pump, the reactants are passed through plastic and glass tubing, the latter being used in the mixing coils and incubation baths. In the last stage, the reaction mixture passes into a constant flow cuvette in the colorimeter described above. For the various enzyme determinations, different arrangements and bores of plastic tubing are employed.

Determination of Lactic Acid Dehydrogenase Activity—A glass-plastic system of tubing was developed that delivered the reagents at average rates yielding a reaction mixture comparable in composition to that employed in the manual procedure (3). The flow diagram is shown in Fig. 1. The reactants were aspirated as follows: the enzyme from the sample plate through a plastic tube, \( A \), at the rate of 0.32 ml per minute; air through tube \( B \) at the rate of 0.80 ml per minute, and 0.067 m sodium phosphate buffer, \( \text{pH} \, 7.4 \), through tube \( C \) at the rate of 2.50 ml per minute. The enzyme, buffer, and air were joined at \( D \), thoroughly mixed in coil \( E \), and passed to a glass reservoir, \( F \), where an aliquot of the total fluid mixture was drawn off through tube \( G \) at the rate of 0.80 ml per minute. This aliquot, which yielded a concentration of enzyme preparation similar to that...
Dilutions of crystalline lactic acid dehydrogenase or tissue homogenates were prepared in 0.067 M sodium phosphate buffer, pH 7.4, containing 0.15% human serum albumin. Serum was usually placed in the sample cups without dilution, except when high enzyme activities were anticipated, and then appropriate dilutions were made with 0.85% NaCl.

The reaction mixture was incubated for a precisely measured interval of about 6 minutes. The incubation time was determined each day by measuring the transit time of a colored solution from point L to the cuvette in the colorimeter (Fig. 1). During this passage the enzyme reaction mixture was in coil M for about 30 seconds and in the plastic tube joining the incubation bath and constant-flow cuvette for about 10 seconds. The effects of these short intervals at slightly lowered temperatures on the reaction velocity at 37.5° were found to be negligible.

A graphic record of a series of assays is shown in Fig. 2. Tubes A, B, C, D, and E (Fig. 1) were allowed to aspirate distilled water. When the water reached the cuvette in the colorimeter, a reading near zero absorbancy was transcribed on a moving chart in the recorder. A precise setting to zero absorbancy was made by an adjustment knob on the colorimeter, and a water base-line was recorded (part I of Fig. 2). Tube A was then permitted to aspirate 0.067 M sodium phosphate buffer, pH 7.4, whereas the remaining tubes were inserted into the appropriate reagents as previously described. When this mixture reached the cuvette, it began to displace the distilled water. The absorbancy of these reactants increased and was manifested maximally in about 90 seconds at an absorbancy value of 0.360. This baseline is shown in part II of Fig. 2.

The activities of various enzyme samples in the cups on the turntable were then determined as follows. Tube A was threaded through the crook on the turntable which was set so that a sample from each particular cup was aspirated for precisely 60 seconds. At the end of this interval the crook tilted back, removing the tube from the sample. Air was then aspirated for 30 seconds, a period that permitted the segregation of samples and the cleaning of the system. During this alternating aspiration of samples and air, the extent of oxidation of DPNH in each reactant mixture was manifested as the minimal point of absorbancy in each deflection (part III of Fig. 2). The recording needle did not always return completely to the base-line between successive samples because 30 seconds was not sufficient time for the mixture of reagents without enzyme to clear out the reactant mixture completely from the light path in the cuvette. It can be shown by calculation that this slight residue has no effect on the determination of the succeeding sample. In addition, a series of specimens was sampled at 3-minute intervals with wash-out intervals of 2 minutes, so that the deflections returned to the base-line. The results were the same as those obtained with the usual sampling of specimen at 1½-minute intervals and wash-out periods of 30 seconds.

It was necessary to correct the preceding readings by the readings due to the absorbancy unrelated to the oxidation of DPNH. To determine these “blank” values, tubes A and K (Fig. 1) were permitted to aspirate the buffer. This yielded the base-line without substrate (part IV, Fig. 2), which had a slightly lower absorbancy than the base-line with substrate (part II, Fig. 2). While tube K continued to aspirate buffer, tube A was placed again in the crook on the sample plate, and the latter was allowed to rotate as before. A series of “blank” deflections was thus obtained (part V, Fig. 2). Because of the relatively
high protein content, slight "blank" deflections occurred in the determination of the lactic dehydrogenase activity of samples of sera, but no deflections were obtained in analyses of solutions of crystalline enzyme or of tissue homogenates.

The lactic dehydrogenase activity obtained by the automated procedure was expressed in μmoles of DPNH converted per ml of reaction mixture per minute:

$$ \frac{0.0167}{F} \times \frac{\Delta A}{i} \times \frac{1}{4.08} $$

where ΔA is the sum of the decrease in absorbancy when the enzyme was acting on the substrate and of the increase in absorbancy when the substrate was omitted from the reaction mixture in the blank assay; and i is the reaction time in minutes, determined as described previously. The number 0.0167 represents the concentration of enzyme preparation, in milliliters per ml of reaction mixture, that was employed in the manual method (3). F is a factor that takes into consideration the fact that tubes A, C, G, I, and K, whether because of deviations from the manufacturer's specifications or because of stretching, did not always have the exact internal diameters described earlier in this paper. The value of F was

$$ F = \frac{A_d}{A_d + G_d} \times G_d $$

where the subscript d represents the flow in ml per minute through tubes A, C, G, I, and K as shown in Fig. 1. The flows were determined by aspirating distilled water through each tube from individual graduated cylinders for 10 minutes.

The application of the automated procedure to the determination of lactic dehydrogenase activity is illustrated in the upper part of Table I. A commercial preparation of twice crystallized rabbit muscle lactic dehydrogenase, containing 25 mg of protein per ml, was diluted with 0.15% purified human serum albumin in 0.067 M phosphate buffer, pH 7.4. These concentrations, ranging from 1.25 to 2.50 μg of protein per ml and diluted further in the course of the automated procedure, were such that their action during the incubation period of 5.23 minutes lay within the zero order portion of the reaction. F, the correction factor, as determined by Equation 2, was 0.0294. The last column of Table I shows the agreement between the specific activities at these various concentrations. The average value was 111 μmoles per mg of enzyme per minute. As may be seen from the lower part of Table I, this value is in good agreement with the average, 115 μmoles per mg of enzyme per minute, obtained in a series of determinations by the manual method (3).

**Determination of Activity of Glutamic Oxaloacetic Transaminase**—This determination was based on the interaction of aspartate with α-ketoglutarate, and the resulting reduction of the formed oxaloacetate by DPNH in the presence of malic dehydrogenase (4). The oxidation of DPNH was measured spectrophotometrically at 340 μM. The flow diagram is shown in Fig. 3. Dilutions of enzyme preparations were made in 0.067 M sodium phosphate buffer, pH 7.4, containing 0.15% human serum albumin; all other reactants were also dissolved in the buffer and were adjusted to pH 7.4. These were aspirated as follows: enzyme specimen from the sample plate through tube A at the rate of 0.32 ml per minute; air through tube B at 0.80 ml per minute; a mixture of 0.057 M sodium m-aspartate containing 140 mg of DPNH per liter through tube C at 2.0 ml per minute; malic dehydrogenase, 370 Sigma units per ml through tube F at 0.60 ml per minute; 0.056 M sodium α-ketoglutarate through tube H at 0.42 ml per minute. As shown in Fig. 3, the enzyme, aspartate, and DPNH were mixed in coil E, were combined with malic dehydrogenase aspirated through tube F, and mixed again in coil G. D represents the juncture of the contents of tubes A, B, and C which are then mixed in coil J and the point at which α-keto glutarate is introduced into and mixed with the other components to start the reaction.

**Table I**

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Lactic dehydrogenase in μmoles/min/mg enzyme</th>
<th>Change in absorbancy, ΔA</th>
<th>ΔA/4μM</th>
<th>Specific activity</th>
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<tr>
<td>Specimen</td>
<td>Reaction mixture</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Automated</td>
<td>1.25 0.0318 0.0174 0.00371 110</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.67 0.0425 0.0228 0.00476 112</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.00 0.0508 0.0266 0.00567 112</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.50 0.0635 0.0392 0.00644 102</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Manual</td>
<td>0.125 0.0208 0.0140 0.00225 108</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>0.167 0.0278 0.0204 0.00328 118</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.200 0.0333 0.0242 0.00389 117</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>0.250 0.0410 0.0290 0.00447 119</td>
<td></td>
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</tr>
</tbody>
</table>

**Fig. 3.** Flow diagram for determination of glutamic oxaloacetic acid transaminase. The aspiration tubes are designated as follows: A, sample of enzyme; B, air; C, sodium m-aspartate and DPNH; F, malic dehydrogenase; H, sodium α-ketoglutarate. D represents the juncture of the contents of tubes A, B, and C which are then mixed in coil E, combined with malic dehydrogenase aspirated through tube F, and mixed again in coil G. I represents the beginning of coil J and the point at which α-ketoglutarate is introduced into and mixed with the other components to start the reaction.
Comparison of automated and manual determinations of glutamic oxaloacetic transaminase activity of human serum and mouse liver homogenates

The incubation period was 15.2 minutes for the human serum and 14.7 minutes for the mouse liver homogenates. The factor F, determined by Equation 2, was 0.130 for serum and 0.128 for the tissues.

### Table II

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Automated µmoles/ml/min</th>
<th>Manual µmoles/ml/min</th>
<th>Average µmoles/ml/min</th>
<th>Average deviation %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human serum</td>
<td>1</td>
<td>0.0228</td>
<td>0.0237</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.0036</td>
<td>0.0037</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.0051</td>
<td>0.0051</td>
<td>0.0</td>
</tr>
<tr>
<td>Mouse liver</td>
<td>1</td>
<td>69.0</td>
<td>71.4</td>
<td>70.2</td>
</tr>
<tr>
<td></td>
<td>2</td>
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<td></td>
<td>3</td>
<td>76.9</td>
<td>82.9</td>
<td>79.9</td>
</tr>
</tbody>
</table>

* The reaction velocity has been calculated per g of tissue instead of per ml of reaction mixture.

Fig. 4. Applicability of automated enzyme assays to purification of glutamic oxaloacetic transaminase from pig heart. Enzyme activities of successive 1-ml aliquots eluted from a hydroxylapatite column in a later stage of purification are recorded as transmittancies. The rise in transmittancy at the left-hand part of the record was due merely to the removal of reagents preparatory to setting up another sample plate containing the remaining specimens.

The glutamic oxaloacetic transaminase activity obtained by the automated method was expressed in µmoles of DPNH converted per minute per ml of reaction mixture:

\[
\frac{0.167}{F} \times \frac{\Delta A}{t} \times \frac{1}{4.68}
\]

where ∆A, F, and t have the same meanings as previously described for the calculation of lactic dehydrogenase activity. The number 0.167 represents the concentration of enzyme preparation in milliliters per ml of reaction mixture that was employed in the manual method.

The reproducibility of determinations of transaminase activity by the automated method compared well with that by the manual method. Thus the means of the average deviations of a series of duplicate determinations by the two procedures were 1.1% and 1.3%, respectively. Table II shows a comparison of the results of automated and manual determinations of the transaminase activity in human serum and in mouse liver homogenate. The means of the values obtained by the two methods show average deviations ranging from 0.0 to 3.8%.

The way in which automated analyses may facilitate certain types of enzyme purification is shown in the following example. Glutamic oxaloacetic transaminase was prepared from pig heart according to the procedure of Jenkins et al. (5). In one of the later steps in which the enzyme was eluted from the hydroxylapatite column with 0.08 M potassium phosphate buffer, pH 6.8, the fraction collector was adjusted so as to yield successive 1-ml aliquots. These were placed in batches of 30 to 40 specimens on the sample plate (Fig. 2). The enzyme activity began to increase markedly at aliquot 8, reached a peak at aliquots 12 to 15, declined and reached a low level at aliquot 30. Enzyme activities rose in the succeeding aliquots (Fig. 4), and reached another peak between aliquots 41 to 50. Indeed, because of the practicability of the analyses, aliquots 50 to 100 were also analyzed. These showed very little activity except for a slight peak at aliquots 70 to 75.

### Determination of Alcohol Dehydrogenase Activity

This procedure was based on the increase in absorbancy of DPN at 340 mp and follows the manual method described by Racker (6). A flow system was devised which was similar to those shown in Figs. 1 and 2. The reactants were aspirated as follows: 0.32 ml of enzyme solution prepared in 0.067 M phosphate buffer, pH 7.4, containing 0.15% human serum albumin; air at the rate of 0.80 ml per minute; 0.0240 sodium pyrophosphate buffer, pH 8.5, at 2.50 ml per minute. These reactants were joined and mixed with additional air coming through a tube at the rate of 0.80 ml per minute and with substrate, 0.341 M ethanol, coming through a tube at 2.0 ml per minute. The reactants were warmed to 37.5° by passage through an incubation bath for about 30 seconds and were mixed with an aqueous solution of 113 mg of DPN per liter introduced at the rate of 2.0 ml per minute. The final concentrations were: pyrophosphate buffer, 0.009 M; DPN, 0.05 mm; ethanol, 0.1 M. These were essentially the same as in Racker’s procedure (6). The complete reaction mixture was then incubated at 37.5° for a precisely determined interval of about 2 minutes. The extent of the reduction of DPN was recorded, and the velocity per minute was calculated.

The activity of crystalline alcohol dehydrogenase was determined at 1.37 and 2.74 µg per ml of final reaction mixture at 37.5°. The averages of the values obtained by the automated procedure and by the manual procedure, with the Beckman
model DU spectrophotometer, were 3.67 and 3.62 μmoles of DPN changed per minute per mg of enzyme, respectively.

**Speed of Determination**—The preparation of solutions of the reagents for the lactic dehydrogenase determinations takes approximately 1 hour. The automated procedure permits the performance of 40 determinations per hour. While these determinations are being performed during the first hour, the cups in five or six sample plates may be filled with appropriate samples and stored, if necessary, in the refrigerator. Since a buzzer sounds when the last cup is sampled, about 8 minutes before its contents pass through the system and its activity is recorded, there is adequate time for the complete sample plate to be removed and another inserted. As many as 240 determinations can therefore be made per day; except for the first 2 hours, the operator can be engaged in other activities in the laboratory. This rate of performance is about fourfold the rate that may be performed with a manually operated Beckman DU spectrophotometer. A similar differential exists between the automated and manual procedures for the determination of glutamic oxaloacetic transaminase.

**SUMMARY**

Methods for the automated determination of lactic acid dehydrogenase, glutamic oxaloacetic transaminase, and alcohol dehydrogenase activity have been described, and it has been shown that the results are the same, within experimental error, as those obtained by manual methods. The speed of performance of the automated methods has been discussed. The principles of the methods described in this paper are applicable to the automated assay of other enzymes utilizing the diphosphopyridine nucleotide reduced diphosphopyridine = nucleotide or the triphosphopyridine nucleotide = reduced triphosphopyridine nucleotide reaction.

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Automated Assay of Activities of Enzymes Involving the Diphosphopyridine Nucleotide \textsuperscript{-} Reduced Diphosphopyridine Nucleotide Reaction
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