Aldolase
I. Colorimetric Determination

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The Sigma and Boehringer reagent kits for measuring aldolase activity by a colorimetric method were examined critically. The original Sibley and Lehninger (1) procedure was more satisfactory than the modifications made by the kit manufacturers. Serum aldolase activity measured by the Sigma procedure and our modification of the Sibley-Lehninger method agreed within a standard deviation of ± 2 IU; values obtained with the Boehringer kit were about 50% low.

The enzyme aldolase (fructose-1,6-diphosphate D-glyceraldehyde-3-phosphate lyase [EC 4.1.2.13]), splits fructose 1,6-diphosphate (FDP) into two triosephosphates: glyceraldehyde phosphate (GAP) and dihydroxyacetone phosphate (DAP). Aldolase activity may be measured in a number of ways, but the most commonly used ones are: (1) colorimetric, by the method of Sibley and Lehninger (1) after trapping the triosephosphates with hydrazine, followed by conversion to their colored dinitrophenylhydrazones:

\[ \text{FDP} \xrightarrow{\text{aldolase}} \text{GAP} + \text{DAP} \quad \text{trapped by hydrazine} \]

\[ \text{GAP} + \text{DAP} + \text{NaOH} + 2,4\text{-dinitrophenyldiazine} \xrightarrow{\text{---}} \text{hydrazones} \quad \text{(color)} \]

and (2) spectrophotometric, by coupling the aldolase reaction with that of a dehydrogenase acting upon one of the triosephosphates and measuring concomitant changes in NADH concentration. Several different enzyme systems may be utilized in this manner.

Commercial kits are available for the determination of aldolase activity by both colorimetric and spectrophotometric methods. Since different values in international units for aldolase activity measure-
ments on the same serums were obtained using the different kits, a
critical evaluation was made.
The results obtained with the colorimetric procedures are described
below; the spectrophotometric methods are discussed in a subsequent
publication (2). It was found that improvements could be made in the
sigma kit procedure, and that changes in the procedure and concentra-
tions of reagents were necessary for the Boehringer kit.

Materials and Methods

Reagents

1. **FDP solution, 0.05 M** Dissolve 0.5 g FDP sodium salt* in 20 ml
distilled water. For solutions of lower concentration, dilute as required
with distilled water.

2. **Collidine buffer, 0.1 M, pH25° 7.4 and 8.6** Prepare a stock
collidine solution by dissolving 4.84 g (2.8 ml) collidine† in water and
make up to 100 ml volume. A 25-ml portion of this stock solution is
brought to pH25° 7.4 (glass electrode) by the addition of 0.1 N HCl, and
diluted to 100 ml with distilled water. In like manner a 25-ml aliquot
of the stock solution is adjusted to pH25° 8.6 with 0.1 N HCl and diluted
to 100 ml with distilled water. Dilute as required for other concen-
trations.

3. **Tris buffer, 0.1 M, pH25° 7.4 and 8.6** Prepare a stock solution
by dissolving 12.1 g Tris* in distilled water and make up to 100 ml.
Aliquots of 10 ml are brought to pH 7.4 or pH 8.6 by the addition of
0.1 N HCl, and diluted to 100 ml. Dilute as required for other concen-
trations.

4. **Crystalline aldolase solution** Dilute the stock suspension of
rabbit muscle aldolase (from either Sigma* or Worthington†) with
distilled water to give a final concentration of 10–200 Sibley-Lehninger
units per milliliter.

5. **2,4-Dinitrophenylhydrazine solution, 0.1% (w/v)** Grind 250 mg
of 2,4-dinitrophenylhydrazine in a mortar with small portions of 2 N
HCl; filter into a 250-ml volumetric flask and make up to volume with
the acid.

6. **Trichloracetic acid, 10% (w/v)** Dissolve 10 g of trichloracetic
acid in distilled water and make up to 100 ml.

7. **Sodium hydroxide solution, 0.75 N** Prepare by approximate
dilution from a saturated solution of NaOH. Check the concentration
by titration against standard HCl and adjust to 0.75 N.

*Sigma Chemical Co., St. Louis, Mo.
†Matheson, Coleman, and Bell, Cincinnati, Ohio.
‡Worthington Chemical Corporation, Freehold, N.J.
8. Dihydroxyacetone, 1.5 mM  Dissolve 27.0 mg of dihydroxyacetone* (DHA) in 200 ml of Tris buffer and use for the preparation of the standard curve. The Sigma calibration solution No. 750-11 may be used with their directions.

9. Sigma kit No. 750
10. Boehringer kit† TC-O 15961

Procedure
The procedure for the determination of serum aldolase activity was carried out according to the directions of the respective manufacturers supplying the kits. The various steps in the procedures and concentrations of reagents are summarized in Table 1. The original Sibley-Lehninger (1) procedure is also listed for comparison.

Results and Discussion
The lack of agreement found in the different procedures made necessary a study of the various parameters involved. Since there are gross differences between the Sigma and Boehringer methods with respect to buffer system, substrate concentration, serum volume, time of incubation, and time for hydrazine formation, optimum conditions for the measurement of aldolase activity were investigated.

Buffer Systems
Four different serum pools and two solutions of purified (crystalline) aldolase were assayed for their enzyme activities by the Sigma procedure. The aldolase activities were measured using the Sigma buffer of 0.005 M Tris at pH 8.6 and 7.4. The relative aldolase activities at pH 7.4 and 8.6 of the four serum pools and of crystalline aldolase solutions are shown in Fig. 1, with the aldolase activity obtained at pH 7.4 being taken as 100. The type of buffer did not influence the enzymatic activity; Tris and collidine gave identical results at the same pH. The activity of the serum aldolase was the same whether measured at pH 7.4 or 8.6. The broad pH optimum of serum aldolase is well known (1).

The pH of the buffer, however, affected the activity of the crystalline aldolase solutions. The activities obtained at pH 8.6 were only 65% of the activities obtained at pH 7.4. The lower pH optimum for crystalline aldolase solutions has been reported already by Dounce and Beyer (3) and probably is due to the presence of hydrazine in the mixture and the relatively low concentration of protein in the crystalline aldolase

*Calbiochem, Los Angeles, Calif.
†Boehringer Mannheim Corporation, New York, NY.
solutions. It cannot be ascribed to a modification in the aldolase structure during isolation, because the serum enzyme and the aldolase concentrates from muscle have the same pH optimum in the absence of hydrazine when measured by a UV procedure.

Since neither the buffer type (Tris or collidine) nor the buffer pH (7.4 or 8.6) affected the aldolase activity obtained with serum samples,

<table>
<thead>
<tr>
<th>Table 1. Comparison of Various Colorimetric Procedures</th>
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<tr>
<td><strong>I. Reaction mixture:</strong></td>
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<td>pH</td>
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<td>Concentration (M)*</td>
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<tr>
<td>Substrate concentration (M)*</td>
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<tr>
<td>Trapping hydrazine agents (M)*</td>
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<td>Iodoacetate</td>
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<td>Serum volume (ml)</td>
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<td>TOTAL REACTION VOLUME</td>
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<tr>
<td>Temperature</td>
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<tr>
<td>Time (min.)</td>
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<tr>
<td>Volume 10% TCA (ml)</td>
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<td><strong>III. Color development:</strong></td>
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<td>Volume 0.75 N NaOH (ml)</td>
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<tr>
<td>Hydrolysis at room temperature (min)</td>
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<tr>
<td>Volume dinitrophenylationhydrazine (ml)</td>
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<td>Chromogen formation at 37° (min.)</td>
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<td>Color development at room temp. (min.)</td>
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<tr>
<td>10</td>
</tr>
<tr>
<td>540</td>
</tr>
</tbody>
</table>

* Refers to the final concentration in the reaction mixture.

*Aldolase activity at pH 7.5 token as 100%
either one may be used for the assay of serum aldolase. Tris buffer, pH 7.4, is recommended, however, because it is more convenient to use, does not have the unpleasant odor of collidine, and makes it possible to assay crystalline aldolase samples without having to change buffer systems.

**Trapping Agents**

Hydrazine has been employed as the trapping agent for the triosephosphates by most workers. Both Boehringer and Sigma employ the same trapping agent and at approximately the same molar concentration. The hydrazine concentration is not critical because it can be reduced from 0.056 to 0.004 M without affecting the trapping capacity. It should be noted that hydrazine is a very strong buffer. Its incorporation at 0.053-0.056 M may well be the reason that gross differences in the ionic strength of Tris or collidine do not affect the aldolase activity.

Iodoacetate has been employed only by Boehringer. The rationale for its use is to inhibit a side reaction, the oxidation of one of the triosephosphates (GAP) by glyceraldehyde dehydrogenase* (GAPDH). In our hands, an iodoacetate concentration of 0.0002 M had no effect upon the aldolase activity of various serum samples. It can be omitted from the reaction mixture without affecting the accuracy of the method since its presence is superfluous.

**Substrate Concentration**

Four serum samples and two crystalline aldolase solutions were measured for aldolase activity according to the Sigma procedure, but using substrate concentrations varying from 0.001 to 0.01 M, in the reaction mixture. As shown in Fig. 2, maximal enzyme activity could be obtained with a substrate concentration of 0.0025 M or higher. It is evident that both Sigma and Boehringer are using adequate amounts of substrate, but the concentration of substrate in the Boehringer procedure could be reduced 60% without affecting the aldolase assay.

**Incubation Conditions**

Both Sigma and Boehringer employ an incubation temperature of 37°, and both stop the reaction by adding an amount of 10% TCA equivalent to the total reaction volume. The period of incubation, however, is only 30 min. in the Sigma procedure but 60 min. in the Boehringer procedure. Our data covering an incubation period ranging

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*O-Glyceraldehyde-3-phosphate:NAD oxidoreductase (phosphorylating); EC 1.2.1.12.
from 10 to 30 min. indicate linearity over this period even with serum volumes of 1.0 ml. This is in agreement with the work of Sibley and Lehninger (1), who reported that the reaction was not linear over 60 min., but was linear to at least 30 min. Even though Bruns (4) reported that the aldolase reaction was linear for 60 min., inspection of his data reveals that the reaction rate decreases somewhat after 40 min. For this reason, a 30-min. incubation time is recommended, and the reaction time in the Boehringer procedure should be reduced.

**Serum Volume**

A serum sample of 0.2 ml is adequate for the routine determination of aldolase activity, since the absorbance obtained with normal serum and an incubation time of 30 min. will be up to 0.05 absorbance units. Since many of the determinations of aldolase activity are performed on children, the 0.2 ml of serum employed in the Sigma procedure is preferable to the 1.0-ml volume recommended by Boehringer.

**Chromogen Formation**

In order to form the chromogen complex of dinitrophenylhydrazine with the trioses after decomposition of the triose-hydrazine complex with alkali, Sibley and Lehninger (1) warmed the mixture with an acid solution of dinitrophenylhydrazine for 10 min. at 37°. This technic was retained by Boehringer, but Sigma increased the time for chromogen formation to 60 min., based upon the report of Beck (5) that this increases sensitivity and accuracy.
Sibley and Lehninger (1) assumed that hydrazine traps the triosephosphates, GAP and DAP, in a 1:1 ratio as soon as they are formed from the splitting of FDP by aldolase. Working with purified triosephosphates, Beck (5) showed that the reaction rates for the formation of their chromogen complexes with dinitrophenylhydrazine are not equal, when the reaction proceeds for 10 min. at 37°. The molar extinction coefficient (E_m) of DAP is 1.8 times that of GAP; the E_m's were equal when the reaction time was extended to 60 min. Under the latter condition, a pure triose could be used as a standard for the reaction in place of the cumbersome alkali-labile phosphorus technic.

To check this point, a series of serum specimens and crystalline aldolase solutions were assayed, employing a color development time of 10 min. in one series, and 60 min. in another. In a similar fashion, purified solutions of glyceraldehyde (GALD) and dihydroxyacetone (DHA) were reacted with dinitrophenylhydrazine for 10 and 60 min., respectively. The results are summarized in Table 2.

By comparing the absorbance obtained after 60 min. of color development with that after 10 min. (A_60:A_10), a ratio of 1.2 was found for DHA compared to 1.6 for GALD (Table 2). This confirms that the absorbance of the chromogenic complex of GALD increases much more with time than that of DHA. When a solution of crystalline aldolase was incubated with FDP, the ratio of A_60:A_10 was 1.4, the average

Table 2. Effect of Time on the Chromogen Formation with 2,4-Dinitrophenylhydrazine in the Aldolase Assay

<table>
<thead>
<tr>
<th>Sample</th>
<th>Absorbance at 640 nm at 2 color development times*</th>
<th>Activity:</th>
<th>Activity:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 min.</td>
<td>60 min.</td>
<td>A_60:A_10</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
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<tr>
<td>A</td>
<td>0.56</td>
<td>0.68</td>
<td>1.20</td>
</tr>
<tr>
<td>B</td>
<td>0.50</td>
<td>0.60</td>
<td>1.20</td>
</tr>
<tr>
<td>C</td>
<td>0.36</td>
<td>0.43</td>
<td>1.20</td>
</tr>
<tr>
<td>D</td>
<td>0.16</td>
<td>0.19</td>
<td>1.20</td>
</tr>
<tr>
<td>Crystalline aldolase solutions</td>
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</tr>
<tr>
<td>A</td>
<td>0.15</td>
<td>0.21</td>
<td>1.40</td>
</tr>
<tr>
<td>B</td>
<td>0.085</td>
<td>0.12</td>
<td>1.40</td>
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<tr>
<td>GALD‡</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.25</td>
<td>0.40</td>
<td>1.60</td>
</tr>
<tr>
<td>B</td>
<td>0.48</td>
<td>0.97</td>
<td>1.65</td>
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<td>DHA§</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
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<td>1.22</td>
</tr>
<tr>
<td>B</td>
<td>0.80</td>
<td>0.96</td>
<td>1.20</td>
</tr>
<tr>
<td>Sigma calibration solution</td>
<td>0.60</td>
<td>0.75</td>
<td>1.25</td>
</tr>
</tbody>
</table>

* The 10 min. and 60 min. refer to the periods of color development with 2,4-dinitrophenylhydrazine at 37°.
† Based on Sigma calibration solution for 10 and 60 min., respectively, of color development.
‡ Aqueous solution of GALD, prepared from GALD powder, obtained from Sigma.
§ Aqueous solution of DHA, from DHA powder, obtained from Sigma.
value that one would expect when DAP and GAP are produced and fixed in equimolar quantities.

In contrast to the above, incubation of a series of serum specimens with FDP gave a ratio of $A_{260}:A_{10} = 1.2$, the value of the ratio for pure DHA. Since serum is known to contain triosephosphate isomerase* (TIM) (6), which brings about an equilibrium ratio of DAP:GAP of 95:5, it is not surprising that the $A_{260}:A_{10}$ ratio reflects the preponderance of DAP. Evidently, the hydrazine does not prevent the conversion of GAP to DAP by TIM.

In view of the above, the original 10-min. period of color development proposed by Sibley and Lehninger (1) is more appropriate than the 60-min. period proposed by Beck (5) and adopted by Sigma. In addition, the shorter time period is more convenient.

**Recommended Procedure**

As a result of the above studies, the recommended procedure may be summarized as follows: It is the same as the Sigma procedure except that (1) Tris buffer pH 7.4 is substituted for the pH 8.6 buffer; (2) the time for chromogen formation is reduced to 10 min. It is more closely related to the original Sibley-Lehninger procedure than to Sigma’s and is carried out as follows:

1. To tubes labeled Test and Blank add the following:
   - Tris buffer, 0.05 M, pH 7.4 1.4 ml
   - Hydrazine sulfate, 0.056 M 0.2 ml
   - Serum 0.2 ml

2. Equilibrate both tubes in a 37° water bath for 5 min. and add 0.2 ml substrate (0.05 M FDP) to the tube marked Test and mix. Start timing when substrate is added.

3. Incubate Test and Blank tubes for exactly 30 min. To each add 2.0 ml of 10% TCA at the end of the incubation period. Then add 0.2 ml FDP substrate to the tube labeled Blank and mix.

4. Centrifuge; transfer 1.0 ml of the clear supernatant fluid to tubes marked Test and Blank, respectively.

5. To each tube add 1.0 ml of 0.75 N NaOH, mix, and let stand for 10 min. at room temperature. It must not stand longer than 20 min.

6. To each tube add 1.0 ml of dinitrophenylhydrazine solution and mix. Place in a 37° water bath for exactly 10 min.

7. Remove tubes, develop color by the addition of 7.0 ml of 0.75 N NaOH; mix, and after 5 min. at room temperature read the absorbance at 540 nm.

*α-Glyceraldehyde-3-phosphate ketol-isomerase (EC 5.3.1.1).
8. Calculate aldolase activity by reference to a calibration curve prepared thus:
   a. Add the respective volumes of 1.5 mM DHA and Tris buffer listed in Table 3 to each of 6 Erlenmeyer flasks (50 ml).
   b. To each flask, add the following, mixing after each addition:
      - Hydrazine solution 0.1 ml
      - TCA solution 1.0 ml
      - NaOH, 0.75 N 2.0 ml
      - Color reagent 2.0 ml
   c. Place all flasks in a water bath at 37° and incubate for exactly 10 min.
   d. At end of the incubation period, remove flasks from water bath and add 14 ml of 0.75 N NaOH solution. Mix well and let stand at room temperature (see e below for length of time).
   e. Read the absorbance of these solutions in the spectrophotometer at 540 nm against the blank. Complete readings within 15 min. after addition of alkali.
   f. Plot a calibration curve of the absorbance against aldolase units (Table 3) and use this curve for reading aldolase activity of the samples (Step 7 of the procedure). When the light path is 10 mm, enzyme activity may be calculated from the absorbance \( A \) according to Bruns (4) as follows:
      \[ A \times 225 = \text{S-L units} \]
      or
      \[ A \times 167 = \text{mU}^* \]

The aldolase activities in 8 serums were measured using the Sigma kit, the Boehringer kit, and our proposed method. The results obtained with the Boehringer kit are listed in two columns in Table 4. In Column A, the activity values were obtained using our standard curve and correcting for dilution differences, while in Column B, the values

\*International units = \( \mu \)moles/ml/min.; Sibley-Lehninger units = \( \text{mU} \times 1.34 \).

**Table 3. ALDOLASE CALIBRATION CURVE**

<table>
<thead>
<tr>
<th>Flask No.</th>
<th>Vol. 1.5 mM DHA (ml)</th>
<th>Vol. Tris buffer (ml)</th>
<th>mU*</th>
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<td>0</td>
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<tr>
<td>2</td>
<td>0.1</td>
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</tr>
<tr>
<td>6</td>
<td>0.5</td>
<td>0.4</td>
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\*International units = \( \mu \)moles/ml/min.; Sibley-Lehninger units = \( \text{mU} \times 1.34 \).
Table 4. Comparison of Serum Aldolase Activities by Three Colorimetric Procedures

<table>
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<th>Serum No.</th>
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<th>Column B†</th>
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<td>9.7</td>
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<td>22.5‡</td>
<td>22.5‡</td>
<td>33.3</td>
<td>40.9</td>
</tr>
</tbody>
</table>

* Values obtained using our graph and appropriate corrections for dilution differences.
† The milliunits obtained by using the Boehringer table and their factor of 2.65 to convert units from 25° to 37°.
‡ Converted S-L units to mU by multiplying by 0.75.
§ Used 0.2 ml of serum instead of the recommended 1.0 ml.

obtained from the Boehringer table were multiplied by a factor of 2.65 to convert units from a temperature of 25° to 37°. Although the activities in Column A agreed with those in Column B, the aldolase values obtained with the Boehringer kit were approximately 50% of those measured by the Sigma or the proposed method. There was much closer agreement between the aldolase activities measured by the Sigma procedure and the proposed method. In 6 of 8 serums, the Sigma values were 9–14% lower than in the proposed method; one was the same, while the other was 32% higher. The standard deviation was ± 2.0 units. The discrepancies would largely disappear if the time for hydrazine formation in the Sigma procedure would be reduced from 60 to 10 min.

The values obtained with the Boehringer kit were unacceptable: Their incubation time is too long, and too large a volume of serum is employed.

References