Aldolase

II. Spectrophotometric Determination Using an Ultraviolet Procedure

Patrick V. C. Pinto, Alex Kaplan, and Paul A. Van Dreal*

Two commercial kits for the measurement of serum aldolase activity by a spectrophotometric (UV) method were evaluated. Neither was satisfactory in their present forms, since aldolase activity was not rate-limiting in one, and side reactions utilizing NADH occurred in the other.

A study was made of the optimum concentrations of substrate, NADH, buffer, and coupled enzymes (triosephosphate isomerase and α-glycerophosphate dehydrogenase) in the procedure utilized by one kit, and a method based upon these findings is proposed. The key feature of the proposed method is an increase in the amount of the auxiliary enzymes, GDH/TIM, so that aldolase becomes the rate-limiting enzyme. The proposed method also differs from the kit procedure in the concentrations of substrate and NADH, in the choice of buffer, volume of reaction mixture, and in the order of adding solutions to the reaction mixture.

WHILE THE MEASUREMENT of serum aldolase (fructose-1,6-diphosphate:D-glyceraldehyde-3-phosphate lyase [EC 4.1.2.13]) activity may be performed by a colorimetric procedure (*1*), it can be done more rapidly and conveniently by spectrophotometric procedures that couple the aldolase reaction with that of a dehydrogenase acting upon one of the triosephosphates formed after splitting FDP.† The latter reaction is accompanied by changes in NADH concentration which are measured spectrophotometrically at 340 nm.

Commercial kits are available which utilize different enzyme-coupled

From the Department of Biochemistry, University of Washington, Seattle, Wash 98105.

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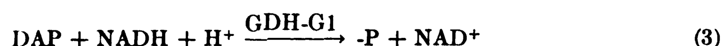
*Senior Fellow supported by Training Grant in Clinical Chemistry 5T1 GM 776 from the National Institute of the General Medical Sciences. Present address: Department of Laboratory Medicine, University of Oklahoma Medical Center, Oklahoma City, Okla 73104.

†The following abbreviations are taken from Bergmeyer, *Methods of Enzymatic Analysis*, Academic Press, New York, 1965: ALD, aldolase (EC 4.1.2.13); DAP, dihydroxyacetonephosphate; FDP, fructose-1,6-diphosphate; GAP, glyceraldehydephosphate; GAPDH, glyceraldehydephosphate dehydrogenase (EC 1.2.1.12); GDH, glycerol-1-phosphate dehydrogenase (EC 1.1.1.8); α-GP, glycerol-1-phosphate; PGA, 3-phosphoglyceric acid; TIM, triosephosphate isomerase (EC 5.3.1.1).

reactions. The Boehringer kit, based upon the methods of Beisenherz *et al.* (*2*) and Racker (*3*) utilizes the following reactions for the determination of aldolase activity:

$$\text{FDP} \xleftrightarrow{\text{Ald}} \text{GAP} + \text{DAP} \tag{1}$$

$$\text{GAP} \xleftrightarrow{\text{TIM}} \text{DAP} \tag{2}$$



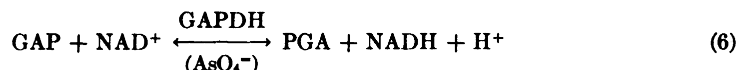
$$\text{DAP} + \text{NADH} + \text{H}^+ \xrightarrow{\text{GDH-G1}} \text{-P} + \text{NAD}^+ \tag{3}$$

The disappearance of NADH is proportional to aldolase activity if this enzyme is made the rate-limiting factor in the above reactions, by providing an excess of TIM and GDH. Two moles of NADH are oxidized per mole of FDP hydrolyzed. The equilibrium in Reaction 2 is far to the side of DAP, while Reaction 3 proceeds to completion.

The Calbiochem Calsul procedure, based upon the method of Warburg and Christian (*4*), utilizes the following reactions:

$$\text{FDP} \xleftrightarrow{\text{Ald}} \text{GAP} + \text{DAP} \tag{4}$$

$$\text{GAP} \xleftrightarrow{\text{TIM}} \text{DAP} \tag{5}$$



$$\text{GAP} + \text{NAD}^+ \xleftrightarrow[(\text{AsO}_4^-)]{\text{GAPDH}} \text{PGA} + \text{NADH} + \text{H}^+ \tag{6}$$

GAP is oxidized by GAPDH in the presence of NAD and arsenate to 3-phosphoglyceric acid; the increase in absorbance of the NADH is measured at 340 nm. Baranowski and Niederland (*5*) and Taylor (*6*) have pointed out that the Warburg and Christian method (*4*), however, is subject to large errors if interfering enzymes, particularly GDH, may be present. They also recommend that TIM should be excluded because it favors so strongly the conversion of GAP to DAP. Since GDH and TIM are present in both serum (*7, 8*) and the Calbiochem reagent mixture (GDH as a slight contaminant of GAPDH), no attempt was made to investigate the Calbiochem procedure for optimum rate conditions.

Reagents and Materials

1. Tris and collidine buffer solutions, 0.1 M, $pH_{25°}$ *7.4* Prepare as for colorimetric procedure (*1*).

2. FDP solution, 0.022 M Dissolve 112 mg FDP sodium salt in 10 ml distilled water. Store in freezer in small aliquots (0.9 ml is sufficient for four determinations). Discard any excess FDP after thawing and using.

3. NADH, 1.5 mM Dissolve 100 mg of NADH* in 10 ml of the appropriate buffer. Store in freezer in small aliquots (0.9 ml is sufficient for four determinations). Discard any excess NADH after thawing and using. An NADH inhibitor may form if the solution is thawed and frozen repeatedly (*9*). The use of a control serum high in aldolase activity is recommended as a check against possible development of an inhibitor.

4. GDH/TIM, 10 mg/ml Obtained as an ammonium sulfate suspension from Boehringer or Calbiochem.

5. TIM suspension Use as obtained from Calbiochem (ammonium sulfate suspension).

6. GAPDH suspension Use as obtained from Calbiochem (ammonium sulfate suspension).

7. Crystalline rabbit muscle aldolase solution Prepare as for colorimetric procedure (*1*).

8. Boehringer Kit TC-D 15974

9. Aldolase Calsuls, Calbiochem

Spectrophotometer: Changes in absorbance were measured in a Beckman DU spectrophotometer equipped with the Gilford 2000 attachments for recording, scale expansion, positioning, and temperature control.

Results and Discussion

After measuring the aldolase activity in a series of serums by the use of the Boehringer and Calbiochem kits, it was apparent that the values obtained by the latter procedure were consistently lower than those obtained by the Boehringer system. This led to a thorough investigation of the various parameters involved in the aldolase assay, in the attempt to find optimum conditions for measurement. A satisfactory spectrophotometric method was obtained by modifying the Boehringer procedure, which compared favorably with the colorimetric method (*1*).

Reagents and solutions similar to those used in the Boehringer kit were prepared in our laboratory. Several changes in procedure were instituted as the testing period started. These were:

1. Changing the final volume from 2.76 to 3.0 ml.
2. Adding the substrate last to the mixture after an equilibration period of 5 min. in order to eliminate the effect of dehydrogenases acting upon endogenous substrates in the presence of added NADH.
3. Substituting Tris buffer for collidine in order to avoid the unpleasantness of the latter material.

*Boehringer Mannheim Corp., New York, NY; Calbiochem, Los Angeles, Calif; and Sigma Chemical Co., St. Louis, Mo.

4. Following the reaction rate on the Gilford attachment for a period of 6 min.

Parameters Investigated

Buffer System

Four different serum pools and two solutions of purified crystalline aldolase were taken as the test material. Aldolase activity was measured in four buffer mixtures consisting of 0.035 M collidine at $pH_{25°}$ 7.4 and 8.6, and 0.035 M Tris at $pH_{25°}$ 7.4 and 8.6. With all samples, the aldolase activity was independent of the buffer type or pH. The ionic strength could be varied from 0.0035 to 0.05 M without affecting aldolase activity. While the buffer employed by Boehringer (0.05 M collidine) is satisfactory, it is more pleasant to use 0.035 M Tris.

It should be noted that serum aldolase and solutions of crystalline aldolase had the same broad pH optimum in the spectrophotometric method. In contrast to this, higher aldolase activity had been found at pH 7.4 by colorimetric methods (*1*) for solutions of crystalline aldolase, even though pH 8.6 was equally satisfactory for measuring aldolase of serum. The presence of hydrazine, a strong buffer, in the colorimetric method and the relatively low concentration of protein that might bind hydrazine in solutions of crystalline aldolase may account for this difference in behavior.

Substrate Concentration

The same six test solutions used above were employed in the search for optimum substrate concentration. The aldolase activity was measured in five final substrate concentrations that varied from 0.35 to 6 mM. The aldolase activity of the test solutions varied from 10 to 60 mU. As shown in Fig. 1, an FDP concentration of 0.7 mM is the lowest con-

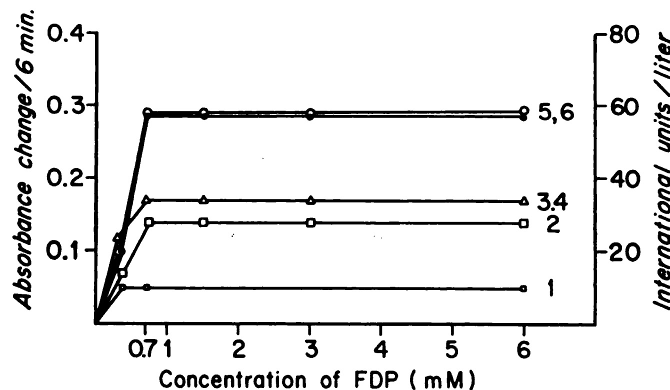


Fig. 1. Effect on six test solutions of varying FDP concentration on aldolase activity.

centration that permits optimum aldolase activity. A doubling of the minimal substrate concentration to 1.5 mM would provide a substrate concentration that would be more than adequate to take care of the usual pathologic specimens. Thus, this substrate concentration would permit measurement of a serum activity of 125 mU when incubated for 6 min., with a net absorbance change of approximately 0.6. A serum of approximately 400 mU, or 13 times the upper limit of normal, could be assayed by measuring the net absorbance change in 2 min. and projecting the slope for the 6-min. period.

Boehringer employs an FDP concentration of 2.7 mM, which is far more concentrated than necessary. The cost per analysis is reduced when the lower concentration of substrate is utilized.

NADH Concentration

The optimum concentration of NADH, another costly material, was investigated in a similar fashion. Results are shown in Fig. 2, where the absorbance change in 6 min. is plotted against NADH concentration, which varied from 0.025 to 0.2 mM in the assay mixture. The lowest NADH concentration for maximum activity was 0.075 mM. An NADH concentration of 0.10 mM would permit the measurement of a serum aldolase activity of approximately 130 mU in a 6-min. period or of 400 mU in a 2-min. period. An NADH concentration of 0.1 mM is recommended because it is adequate, economical, and convenient, and because the absorbance of the solution at zero time does not exceed 1.2. Higher NADH concentrations give absorbance readings that are too high to measure accurately.

The Boehringer kit contains 0.35 mM NADH, which is completely unsatisfactory for measurement of absorbance at 340 nm because it is so high. Their method is geared to measurement of absorbance at 366

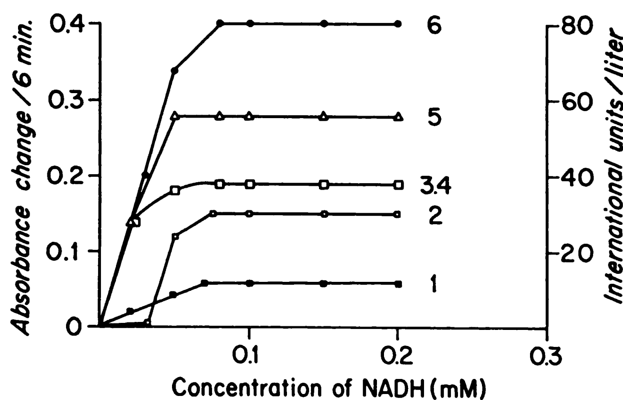


Fig. 2. Effect on six test solutions of varying NADH concentration on aldolase activity.

nm, where the molar extinction coefficient of NADH is about 0.5 of that at 340 nm. Even so, the absorbance is too high to read accurately at zero time, and it is wasteful of a costly ingredient.

Coupled Enzymes (GDH/TIM)

In order to employ a coupled enzyme system for the measurement of aldolase, it is essential that the coupled enzymes are present in excess so that the activity of aldolase becomes the rate-limiting factor. The Boehringer system uses two auxiliary enzymes, TIM and GDH, as shown in Reactions 2 and 3 above. Both enzymes are obtainable in a single solution at a concentration of 10 mg protein per milliliter.

The optimal amount of GDH/TIM solution to add to the aldolase assay system was estimated by employing varying amounts of the enzyme mixture when measuring aldolase activity of the six test solutions (four serum pools and two solutions of crystalline aldolase). Three different concentrations of GDH/TIM were tested: 0.02 mg per tube (the amount used in Boehringer's method), 0.04 mg per tube, and 0.10 mg per tube. The relative activity of the three mixtures are plotted as bar graphs in Fig. 3, with the activity of the 0.02-mg mixture taken as 100. The doubling of the GDH/TIM concentration to 0.04 mg resulted in a 30% increase in activity, while raising the concentration to 0.10 mg yielded an aldolase activity that was 40% greater than for the Boehringer concentration.

It is obvious that the GDH and/or TIM was not present in excess in the Boehringer procedure. To test this further, 0.02 mg of TIM (obtained from Calbiochem) was added to the 0.02 mg of GDH/TIM

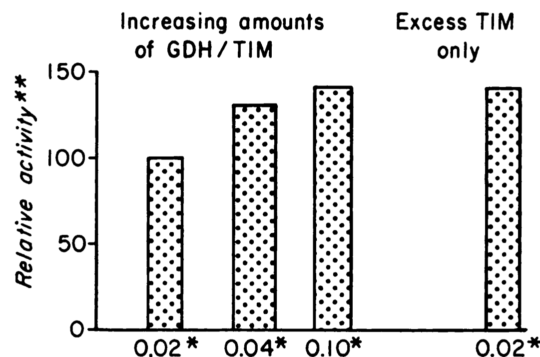


Fig. 3. Effect of GDH/TIM concentration on aldolase activity.

mixture for assaying the test solutions. As shown in Fig. 3, the aldolase activity with this supplemental TIM was as great as with the 0.1 mg GDH/TIM mixture. This indicates that TIM was rate-limiting in the Boehringer mixture that we tested, and that with excess GDH/TIM (0.1 mg) or with 0.02 mg TIM added to the 0.02 mg GDH/TIM mixture, aldolase activity will be increased by 30 or 40%.

Iodoacetate as Inhibitor of Side Reactions

The Boehringer kit contains iodoacetate ostensibly to inhibit a possible side reaction, the oxidation of GAP by NAD and GAPDH. Slater (*10*) has shown that this side reaction in muscle extracts containing aldolase and all of the other enzymes is so slow that it cannot be measured, and he concluded that no inhibitor is necessary. We have measured aldolase activity in serum with and without the presence of iodoacetate and confirm Slater's conclusion that iodoacetate is superfluous.

Sample Volume

Both Boehringer and the proposed procedure employ 0.2 ml of serum. With serums of high and low activity, the sample volume could be lowered to 0.01 ml or raised to 0.5 ml, respectively, without adversely affecting the measurement of aldolase activity.

Order of Adding Reagents

In the Boehringer procedure, the reagents are preincubated and the test is started immediately upon the addition of serum. This may give erroneous values for aldolase activity, because there may be variable amounts of endogenous substrates and dehydrogenases in serum that utilize NADH and cause a change in absorbance that cannot be attributed to aldolase.

To eliminate the effect of extraneous serum enzymes utilizing NADH, in our proposed procedure the serum is preincubated with all reagents, except the substrate for aldolase (FDP), until the absorbance is constant. Then FDP is added and the test started.

Proposed Procedure

1. To 1.0 ml Tris buffer, 0.1 M and pH 7.4, add 0.2 ml 1.5 mM NADH, 10 μl of GDH/TIM solution (10 mg/ml), 0.2 ml serum, and 1.4 ml distilled water.

2. Mix and equilibrate at 37° for at least 5 min. Check whether absorbance at 340 nm is constant.

3. Add 0.2 ml of 1.5 mM FDP, start the timing, and follow the reaction at 37° for 6 min. in the Gilford attachment to the Beckman DU.

4. Calculation:

$$\text{Aldolase activity (mU)} = \Delta A \times \frac{3.0}{6.2} \times \frac{1}{2} \times \frac{1000}{0.2} \times \frac{1}{6} = \Delta A \times 200$$

where ΔA is the change in absorbance in 6 min. in 3 ml reaction mixture; 6.2 is the millimolar extinction coefficient of NADH; 2 moles of NADH are oxidized per mole of FDP split.

A comparison of the Boehringer and proposed procedure is shown in Table 1. Changes have been made in the concentrations of substrate, NADH, and in the strength of the coupled enzyme systems. Tris buffer has been substituted for collidine, and the volume of the reaction mixture has been increased from 2.76 to 3.0 ml.

Temperature Correction and Factors

There is considerable confusion in the literature concerning the correction factors to be employed for converting the units of aldolase activity from one temperature to another. Thus, Calbiochem performs the Calsul assay at 37° and reports it at 37°; for the "Supercalsul," they suggest 30° but give temperature correction factors so that the units may be expressed at any temperature from 25° to 37°. Boehringer carries out the aldolase reaction at 37° but converts activity to mU at 25°. Their literature states that the temperature correction is +12% for every degree rise in temperature. We have calculated the temperature correction for 25°, 30°, and 37° from the data of Bruns and Bergmeyer (*11*). Table 2 indicates the relative correction factors employed by these authors (*11*), Boehringer, Calbiochem, and the present study. The correction factors employed by Calbiochem differ con-

Table 1. Comparison of Boehringer and Proposed (This Study) UV Procedures for the Determination of Aldolase Activity

	Boehringer	*Proposed*
Buffer	Collidine	Tris
$pH_{25°}$	7.4	7.4
Concentration (M)*	0.05	0.035
Substrate: FDP (mM)*	2.7	1.5
Cofactor: NADH (mM)*	0.36	0.10
Coupling system: GDH/TIM (mg)	0.02	0.1
Temperature	37°	37°
Sample volume (ml)	0.2	0.2
Total volume (ml)	2.76	3.00
Component added last	Serum	Substrate
Equilibration period (min.)	5	5
Wavelength (nm)	340/366	340
Incubation time (min.)	20	6

* Final concentration in reaction mixture.

siderably from the others, particularly at 37°, and may explain partially the lack of agreement in aldolase activity measurements.

Evaluation of Aldolase Methods

The aldolase activity of 12 serums was measured by three methods: (1) by the Calbiochem procedure using their Calsuls; (2) by the Boehringer procedure, using their kit and directions; and (3) by the procedure proposed above. The results are presented in Table 3.

The results obtained with the Calbiochem Calsul were significantly lower than either of those of the Boehringer or the proposed method and were considered unsatisfactory. As mentioned above, this may be ascribed to the TIM added and to the presence of GDH in serum. No attempt was made to modify the Calbiochem procedure.

Values obtained by the proposed method consistently were 1.0–1.5 times as high as those found with the Boehringer system, with a mean factor of 1.3. This is consistent with our finding that the Boehringer system was deficient in TIM. When the amount of GDH/TIM used by

Table 2. Temperature Conversion Factors for Serum Aldolase Activity

Method	*Conversion factor at temperature**		
	25°	*30°*	*37°*
Bruns & Bergmeyer (*11*)	1.0	1.54	2.60
Boehringer	1.0	1.60	2.44
Calbiochem	1.0	1.80	4.56
This study	1.0	1.73	2.65

* Activity at 25° taken as unity.

Table 3. Comparison of Serum Aldolase Values by Three Spectrophotometric (UV) Procedures

Serum No.	*Enzyme activity in mU at 37°*			*Ratio (This study: Boehringer)*
	Calbiochem	*Boehringer*	*This study*	
1	7.2	9.6	12.0	1.3
2	4.8	7.2	9.6	1.3
3	4.8	7.2	9.6	1.3
4	9.6	9.6	12.0	1.3
5	3.6	7.2	7.2	1.0
6	6.0	9.6	12.0	1.3
7	9.6	13.2	14.4	1.1
8	6.0	9.6	12.0	1.3
9	11.4	13.2	19.2	1.5
10	6.0	6.6	7.2	1.1
11	14.4	13.2	19.2	1.5
12	9.6	10.8	14.4	1.3
Mean				1.3

Table 4. COMPARISON OF SERUM ALDOLASE ACTIVITIES OBTAINED BY THE PROPOSED PROCEDURE IN TWO LABORATORIES

	Aldolase activity in mU at 37°		
Sample No.	*Our laboratory*	*C.O.H.**	*Difference*
Normals			
1	1.9	1.0	+0.9
2	2.4	1.6	+0.8
3	2.4	2.8	−0.4
4	3.0	2.9	+0.1
5	3.1	2.4	+0.7
6	4.8	5.5	−0.7
7	5.0	3.6	+1.4
8	6.0	4.0	+2.0
9	7.2	5.8	+1.4
10	8.0	8.0	0.0
Abnormals			
1	10.0	8.4	+1.6
2	12.0	11.5	+0.5
3	15.9	12.5	+3.4
4	21.6	21.6	0.0
5	39.0	39.0	0.0
6	42.0	45.0	−3.0
7	48.0	45.0	+3.0
8	55.0	59.0	−4.0
9	66.0	65.0	+1.0
10	81.0	79.0	+2.0
S.D.†			±1.8

* Children's Orthopoedic Hospital, Seattle, Wash.

† Standard deviation of the two series = $\sqrt{\frac{\Sigma (d^2)}{N}}$

Boehringer was increased from 0.02 to 0.10 mg, the activity of aldolase increased by a factor of 1.4 (Fig. 3).

The reproducibility of the proposed procedure by two different hospital laboratories was tested by determining the aldolase activities of 20 serums, half of which were abnormal. These are shown in Table 4.* The results on both normal and abnormal serums show a close correlation between the two laboratories, with a mean difference of about 0.4 mU and a standard deviation of ±1.8.

A comparison was made of serum aldolase activity determined by the colorimetric (*1*) and spectrophotometric methods. Sixteen serums with aldolase activities ranging from 5.0 to 62.5 mU were measured by both methods and are tabulated in Table 5. The difference in aldolase activity between the colorimetric and UV methods ranged from −3.6

*We are indebted to Dr. E. Smith and to Miss Ann Bauer of Children's Orthopedic Hospital for their cooperation in this phase of the investigation.

Table 5. COMPARISON OF SERUM ALDOLASE ACTIVITIES OBTAINED BY RECOMMENDED COLORIMETRIC AND SPECTROPHOTOMETRIC METHODS

	Aldolase activity in mU at 37°		
Serum No.	*Colorimetric*	*Spectrophotometric (UV)*	*Difference* (Color − UV)*
1	7.5	8.0	−0.5
2	7.5	6.4	+1.1
3	8.0	8.0	0.0
4	7.5	6.4	+1.1
5	7.5	6.4	+1.1
6	5.0	4.0	+1.0
7	8.0	8.0	+0.0
8	7.5	6.4	+1.1
9	7.5	8.0	−0.5
10	5.0	5.3	−0.3
11	10.0	9.6	+0.4
12	7.5	6.4	+1.1
13	42.5	40.2	+2.3
14	50.0	53.6	−3.6
15	62.5	60.1	+2.4
16	7.5	8.0	−0.5
MEAN			+0.4

* S.D. = ±1.4 mU.

to 2.3 mU, with a mean of 1.1. The standard deviation was ±1.4 mU. Both methods are satisfactory, but the spectrophotometric one is more convenient to use with a rate-recording instrument, such as the Gilford apparatus.

The following normal ranges ±2 S.D. were obtained: adult males (24), 8.0 ± 4.0 mU; adult females (23), 4.7 ± 3.2 mU; children (20): 8 males and 12 females (3–14 years), 8.0 ± 4.0 mU.

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