Fructose-1,6-diphosphate Aldolase

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Aldolase catalyses the reaction[1]:

(1) Fructose-1,6-diphosphate $\rightleftharpoons$ dihydroxyacetone phosphate + D-glyceraldehyde-3-phosphate

It also catalyses the reaction between dihydroxyacetone and the following aldehydes: acetaldehyde, D-glyceraldehyde, L-glyceraldehyde[2], glycolaldehyde and glycolaldehyde-2-phosphate[3], to give the corresponding phosphorylated derivatives, methyltetrose phosphate, D-fructose-1-phosphate, L-sorbose-1-phosphate, D-xylulose-1-phosphate and D-xylulose-1,5-diphosphate, respectively. The crystalline enzyme obtained from bovine liver catalyses the cleavage of D-fructose-1-phosphate and the synthesis of erythrulose phosphate from dihydroxyacetone phosphate at about the same rate[4], which contradicts the postulated existence of special enzymes for these reactions[5–7].

Crystalline aldolase has been prepared from rat[8] and rabbit[9,10] muscle and also from yeast[11]. In contrast to the enzyme from mammalian tissue the enzymes from yeast[8], *Clostridium perfringens*[12] and *Aspergillus niger*[13] require divalent metal ions.

Skeletal muscle has the highest concentration of aldolase (Table 1). In this tissue the enzyme accounts for *ca.* 10% of the soluble protein.

Table 1. Aldolase activity in rat tissues[14]
(µl. fructose-1,6-diphosphate/g. fresh weight/hr. at 38° C)

Tissue	Activity	Tissue	Activity
Skeletal muscle	74800	Parotid	3800
Brain	15800	Stomach	3700
Heart muscle	15600	Bladder	3200
Liver	12100	Placenta	3000
Bone marrow	9500	Testicle	2900
Adrenal	8600	Lung	2800
Kidney	7800	Uterus	2100
Spleen	4800	Erythrocytes	900
Thyroid	4800	Pancreas	500
Thymus	4700	Adipose tissue	400
Prostate	4400	Serum	60

The activity of the enzyme in serum is comparatively low; particularly low values are found in human serum (Table 2).

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Table 2. Aldolase activity in the sera of different species[15] (μl. fructose-1,6-diphosphate/ml. serum/hr. at 37° C)

Species	Activity	
	Mean	Range
Man	5.4	3.8—8.0
Dog	15	12—22
Horse	22	10—46
Chicken	35	17—70
Ox	40	24—58
Sheep	40	30—65
Guinea pig	42	17—65
Rat	44	28—65
Pig	47	33—61
Rabbit	54	48—68
Mouse	100	65—120

Four methods are available for the assay of enzyme activity by measuring the hydrolysis of fructose-1,6-diphosphate:

1. determination of the alkali labile triose phosphate formed[1];

2. colorimetric estimation of the triose phosphate formed by the method originally described for lactate[16, 17];

3. measurement of activity by the spectrophotometric method of *Warburg*[8, 10];

4. another colorimetric method in which the dinitrophenylhydrazones of the free trioses are determined[14, 19, 20].

The last two methods are described here.

A. Colorimetric Determination with 2,4-Dinitrophenylhydrazine

Principle

The triose phosphates (dihydroxyacetone phosphate and D-glyceraldehyde phosphate) formed from fructose-1,6-diphosphate by the action of aldolase are trapped with hydrazine. After deproteinization with trichloroacetic acid they are hydrolysed by NaOH. The free trioses are treated with 2,4-dinitrophenylhydrazine, yielding a mixture of methylglyoxal-2,4-dinitrophenylosazone and pyruvic acid-2,4-dinitrophenylhydrazone[18]. Both these compounds dissolve in alkali forming a red dye with an absorption maximum between 535 and 540 mμ.

The following description[19, 20] is a modification of the method of *Sibley* and *Lehninger*[14].

Optimum Conditions for Measurements

With collidine buffer the aldolase activity of serum, blood haemolysates and muscle homogenates has a broad pH optimum between 7 and 8, while with veronal buffer it is between 8.5 and 9. Phosphate and borate inhibit the enzyme; the latter reacts with the *cis*-hydroxyl groups of the substrate[21] (Fig. 1). In the presence of cyanide, crystalline aldolase from bovine liver has a pH optimum between 9.1 and 9.4 (glycylglycine-NaOH buffer) with fructose-1,6-diphosphate and between 8.1 and 8.4 with fructose-1-phosphate. However, it should be noted that the pH optimum measured in the pre-

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[18] *W. S. Beck*, J. biol. Chemistry *212*, 847 [1955].
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[21] *F. H. Bruns*, Biochem. Z. *325*, 429 [1954].

sence of ketone and aldehyde trapping agents is influenced by the dependence of the trapping reaction on pH. With fructose-1,6-diphosphate concentrations above 0.005 M the serum enzyme is saturated with substrate ($K_M = 0.75 \times 10^{-3}$ M). Even with greatly increased serum aldolase activity, as is found in certain pathological conditions, the rate of hydrolysis under the given assay conditions is directly proportional to the time and enzyme concentration (amount of serum). For animal sera and homogenates of liver, kidney, *etc.* this is only true after suitable dilution. Maximum rates are obtained with serum at 46°C. At 37°C the serum enzyme is not denatured, while crystalline muscle aldolase is partially inactivated above 30°C[17].

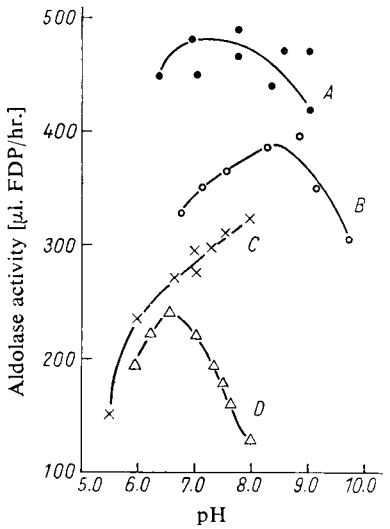

Fig. 1. pH-Activity curves for aldolase (human haemolysate 1 : 10). Final concentration of the buffer: 0.033 M; A) collidine buffer; B) veronal buffer; C) phosphate buffer; D) borate buffer.

Reagents*)

1. Fructose-1,6-diphosphate, FDP
   dibarium salt (*ca.* 65% FDP-Ba_2); commercial preparation, see p. 1014.
2. Sodium sulphate, anhydrous
3. Hydrochloric acid, A. R., 2 N
4. Sodium hydroxide, A. R., 1 N
5. Collidine (2,4,6-trimethylpyridine)
   the liquid should be colourless; if not, it must be distilled; b. p. 171°C, sp. gr. 0.917.
6. Hydrazine sulphate
7. 2,4-Dinitrophenylhydrazine
8. Trichloroacetic acid

Preparation of Solutions

I. Fructose-1,6-diphosphate (*ca.* 0.1 M FDP):
In a centrifuge tube suspend 3 g. fructose-1,6-diphosphate (dibarium salt) in *ca.* 20 ml. distilled water and slowly add 2 N HCl dropwise until complete solution is obtained. Dissolve 2 g. sodium sulphate in 5 ml. distilled water and add to the FDP solution. Stir and centrifuge off the precipitate of $BaSO_4$. Adjust the clear, usually yellowish

*) Complete reagent kits are commercially available (see p. 1036).

supernatant to pH 7.4 by the dropwise addition of 1 N NaOH (pH-meter). Dilute the solution to 30 ml. with distilled water. This solution is approximately 0.1 M with respect to FDP.

II. Collidine buffer (0.1 M; pH 7.4):
Dissolve 4.84 g. (= 5.28 ml.) collidine in distilled water and make up to 100 ml. To 25 ml. of this stock solution add 45 ml. 0.1 N HCl and dilute to 100 ml. with distilled water. If necessary, adjust to pH 7.4 with dilute HCl or NaOH.

III. Hydrazine (0.56 M):
Dissolve 9.08 g. hydrazine sulphate in *ca.* 70 ml. distilled water and add *ca.* 5 g. solid NaOH. Add 10 N NaOH until pH 7.4 is obtained (pH-meter) and dilute to 100 ml. with distilled water.

IV. 2,4-Dinitrophenylhydrazine (0.1% w/v):
Grind 250 mg. 2,4-dinitrophenylhydrazine in a mortar with small portions of 2 N HCl, filter and dilute to 250 ml. with 2 N HCl.

V. Trichloroacetic acid (10% w/v):
Dissolve 10 g. trichloroacetic acid in distilled water and make up to 100 ml.

VI. Sodium hydroxide (0.75 N):
Dissolve 3 g. (dry!) NaOH in distilled water and make up to 100 ml. Alternatively dilute 75 ml. 1 N NaOH to 100 ml. with distilled water.

VII. Collidine-hydrazine buffer:
Mix 100 parts collidine buffer (solution II) with 25 parts hydrazine solution (III) and 50 parts distilled water.

Stability of the solutions

Store all solutions, with the exception of IV and VI, in a refrigerator at 0 to 4° C. The collidine buffer will become acid with time due to evaporation of the collidine and therefore, if necessary, it must be re-adjusted to pH 7.4 with dilute NaOH.

Buffer, substrate and hydrazine solutions should be renewed after 2 to 3 weeks. Bacterial contamination can be prevented by the addition of a few drops of chloroform or toluene.

Procedure

Use only fresh serum free from haemolysis.

Colorimetric measurements

Wavelength: 540 mμ (absorption maximum of the colour) or an adjacent wavelength, *e.g.* Zeiss filter S 53 E or 546 mμ Eppendorf photometer; light path: 1 cm.; final volume: 11 ml.; temperature: 37° C. Measure against the control tube.

Pipette successively into test tubes:

	Experimental	*Control*
serum	1.00 ml.	1.00 ml.
collidine-hydrazine buffer (solution VII)	1.75 ml.	1.75 ml.
FDP solution (I)	0.25 ml.	—
Incubate for 60 min. at 37° C (water bath). Add		
trichloroacetic acid	3.00 ml.	3.00 ml.
FDP solution (I)	—	0.25 ml.

Filter and mix

1.00 ml. filtrate with
0.75 ml. NaOH (solution VI).

Allow to stand for 10 min. at room temperature. The alkali labile triose phosphate is hydrolysed. Then add

1.00 ml. 2,4-dinitrophenylhydrazine solution (IV)

mix and incubate for 10 min. at 37°C in a water bath. Add

8.25 ml. NaOH (solution VI)

and mix. Between 3 and 15 min. measure the brown-red colour against the control with a colorimeter. After 15 min. the colour decreases. It is about 90% of its original value after 30 min. and about 50% after 2 hours.

Glyceraldehyde and dihydroxyacetone give different colour intensities. Hydrazine traps the two trioses to the same extent, so that the formation of a new equilibrium by the triosephosphate isomerase of the serum is avoided.

Standard curve

Pure triose phosphate preparations are difficult to obtain. However, the method can be easily standardized by the determination of the alkali labile triose phosphate: the amount of inorganic phosphate formed on incubation of a portion of the trichloroacetic acid filtrate (1.0 ml.) with 1.0 ml. 1 N NaOH is determined. The NaOH must be free from SiO_2 and should be freshly prepared from NaOH pellets. If the amount of alkali labile phosphate is plotted against the measured optical densities, a straight line is obtained[19].

Calculations

The aldolase activity is expressed as the amount of fructose-1,6-diphosphate (FDP) cleaved under the above conditions by 1.0 ml. serum. This amount is then given in µl. FDP[14, 19].

1 µmole FDP = 340 µg. FDP = 22.4 µl. FDP
1 µl. FDP = 15.2 µg. FDP = 0.045 µmoles FDP
1 µmole FDP = 2 µmoles triose phosphate = 62 µg. orthophosphate
1 µl. FDP = 2.77 µg. inorganic phosphate.

According to *Bruns*[19], if the measured optical densities are multiplied by 37, the aldolase activity in µl. FDP/ml. serum/hr. at 37°C is obtained.

B. Determination with Enzymatic Auxiliary and Indicator Reactions (Spectrophotometric Assay)[10]

Principle

To determine the activity of aldolase, the amount of dihydroxyacetone phosphate (DAP) and D-glyceraldehyde-3-phosphate (GAP) formed from fructose-1,6-diphosphate per unit time is measured. The two triose phosphates are interconverted by triosephosphate isomerase (TIM):

(2) $$\text{DAP} \overset{\text{TIM}}{\rightleftharpoons} \text{GAP}$$

The equilibrium lies 96% to the left.

The DAP is reduced with glycerol-1-phosphate dehydrogenase (GDH) and reduced diphosphopyridine nucleotide (DPNH) in the following indicator reaction which is quantitative:

(3) $$\text{DAP} + \text{DPNH} + \text{H}^+ \longrightarrow \text{L-glycerol-1-phosphate} + \text{DPN}^+$$

With an excess of TIM and GDH, reaction (1), p. 724, is rate limiting, and DAP and GAP are completely converted; for each mole of FDP 2 moles of DPNH are oxidized. The decrease of optical density per min. at 340 or 366 mμ is the measure of the activity.

Optimum Conditions for Measurements

See p. 725.

Reagents *)

1. Collidine (2,4,6-trimethylpyridine)
   The liquid should be colourless; if not, it must be distilled; b. p. 171°C, sp. gr. 0.917.
2. Hydrochloric acid, A. R., *ca.* 5 N
3. Mono-iodoacetic acid, sodium salt
4. Fructose-1,6-diphosphate
   sodium salt, FDP-Na_3H or crystalline cyclohexylammonium salt, FDP-$(C_6H_{13}N)_3$. Commercial preparation, see p. 1014.
5. Reduced diphosphopyridine nucleotide, DPNH
   disodium salt, DPNH-Na_2. Commercial preparation, see p. 1011.
6. Sodium hydrogen carbonate, 1% (w/v)
7. L-Glycerol-1-phosphate dehydrogenase, GDH **)
   crystalline suspension in 2.0 M ammonium sulphate solution; commercial preparation, see p. 981.
8. Triosephosphate isomerase, TIM **)
   crystalline suspension in 2.8 M ammonium sulphate solution; commercial preparation, see p. 998.

Purity of the enzyme preparations

The GDH and TIM preparations should contain <0.01% aldolase, relative to their specific activity. The specific activity of the GDH should be at least 30 units+)/mg. and that of the TIM at least 1000 units+)/mg.

Preparation of Solutions

I. Buffer-iodoacetate-FDP solution (0.056 M collidine buffer, pH 7.4; 3×10^{-4} M iodoacetate; *ca.* 2×10^{-3} M FDP):
   Dissolve 0.679 g. (= 0.74 ml.) collidine, 6.2 mg. Na iodoacetate and 127.5 mg. FDP-$(C_6H_{13}N)_3$ or 100 mg. FDP-Na_3H in *ca.* 90 ml. distilled water, adjust to pH 7.4 with *ca.* 0.6 ml. 5 N HCl (glass electrode) and dilute to 100 ml. with distilled water.

II. Reduced diphosphopyridine nucleotide (*ca.* 1.5×10^{-2} M β-DPNH):
   Dissolve 25 mg. DPNH-Na_2 in 2 ml. 1% $NaHCO_3$ solution.

III. Glycerol-1-phosphate dehydrogenase-triosephosphate isomerase, GDH-TIM (*ca.* 1.8 mg. GDH/ml.; *ca.* 0.2 mg. TIM/ml.):
   Dilute the commercially available crystalline suspensions with 2.4 M ammonium sulphate solution and mix; pH *ca.* 6. Protein ratio GDH:TIM *ca.* 10:1; activity ratio *ca.* 1:3.

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*) Complete reagent kits are available commercially, see p. 1036.
**) GDH and TIM are available commercially as a mixed crystalline suspension (C. F. Boehringer & Soehne, GmbH, Mannheim, Germany), see p. 999.
+) According to *E. Racker et al.*: μmole substrate/min., see p. 32, 33.

Stability of the solutions

Store all solutions at 0 to 4° C. The mixed enzyme suspension keeps for longer than a year; the DPNH solution for at least a week. Solution I becomes acid with time due to the evaporation of collidine and, if necessary, should be re-adjusted to pH 7.4 with dilute NaOH.

Procedure

Use only fresh serum free from haemolysis.

Spectrophotometric measurements

Wavelength: 340 or 366 mμ; light path: 1 cm.; final volume: 3.0 ml.; temperature: 37°C (constant temperature cuvette holder). Measure against air or water. A control cuvette is not necessary.

Pipette successively into the cuvette:

2.74 ml. buffer-iodoacetate-FDP solution (I)
0.05 ml. DPNH solution (II).

Equilibrate for 5—10 min. and then mix in

0.01 ml. GDH-TIM suspension (III).

After *ca.* 1 min. any DAP and GAP contaminating the FDP preparation will have been reduced by the DPNH. Mix in

0.20 ml. serum.

The aldolase reaction now starts. After 1—2 min. measure the optical density E_0 and at the same time start a stopwatch. Read the optical densities E_2, E_4, E_6.....E_{20} at 2 min. intervals for 20 min. The optical density changes per 2 min. should be similar; the reaction is usually linear with time. $E_0-E_{20} = \Delta E/20$ min. is used for the calculations.

If no constant temperature cuvette holder is available or if the 20 min. incubation period is required for other work, equilibrate the reaction mixture without the serum in a test tube in constant temperature water bath (37°C), pour into a cuvette, mix in the serum and measure E_0. Then pour back into the test tube, incubate for 20 min. at 37°C and after pouring into the cuvette again, measure E_{20}.

With very high aldolase activity, ($\Delta E/20$ min.) >0.250 at 366 mμ, dilute the serum 1 : 1 to 1 : 10 with solution (I).

Calculations

According to[19] an aldolase unit is the amount of enzyme which splits 1 μl. FDP = 0.0446 μmoles FDP per hour at 37° C. At 366 mμ $\Delta E = 1.000$ corresponds to a conversion of 0.303 μmoles DPNH/ml. or $(^1/_2)\times 0.303\times 3 = 0.4545$ μmoles FDP in a 3 ml. assay mixture. Therefore if ΔE/hr. = 1.000/hr. this equals

$$\frac{0.4545}{0.0446} = 10.2 \text{ units.}$$

So for measurements at 366 mμ, with 0.2 ml. serum and an incubation time of 20 min.:

$$\frac{(\Delta E/20 \text{ min.}) \times 10.2 \times 6}{0.2\times 20} = (\Delta E/20 \text{ min.})\times 153 = \text{aldolase units/ml. serum}$$

For measurements at 340 mμ it is necessary to divide by 1.89 because of the ratio of the extinction coefficients of DPNH at 340 and 366 mμ (6.22 : 3.3).

$$\frac{(\Delta E/20\,\text{min.}) \times 153}{1.89} = (\Delta E/20\ \text{min.}) \times 81 = \text{aldolase units/ml. serum.}$$

For conversion to other units, see p. 33.

Example

Acute hepatitis; 0.2 ml. serum was analysed and the following optical densities were measured at 366 mμ:

$$\begin{aligned} E_0 &= 0.505 \\ E_{20} &= 0.290 \\ \Delta E/20\ \text{min.} &= 0.215/20\ \text{min.} \\ 0.215 \times 153 &= 32.9\ \text{units/ml. serum.} \end{aligned}$$

Normal Values

The normal values lie between 3 and 8 units. Raised activity (over 20 units) is found particularly in hepatitis[19, 20], progressive muscular dystrophy[22] and myocardial infarction[23]. For a review, see[24] and also p. 703.

Stability of the Enzyme in the Sample

In the presence of large amounts of protein aldolase is relatively thermostable. Blood samples can be allowed to clot at room temperature and the serum obtained by centrifuging. The activity in plasma is identical with that of serum. Leaving serum samples at room temperature for several hours is not harmful. The serum activity remains unchanged on storage at 0 to 4°C for at least 3 to 4 days. Interference with the activity assays by substances present in serum has so far not been observed.

Influence of Therapeutic Agents

Significant increases in serum aldolase activity have been observed after treatment with deoxycorticosterone[25], cortisone and ACTH[26]. The raised serum activity in prostatic carcinoma is decreased by treatment with oestrogens and this has been described as a test for the therapeutic activity[27–29].

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