Dihydroxyacetone Phosphate, Fructose-1,6-diphosphate and D-Glyceraldehyde-3-phosphate

Determination with Glycerol-1-phosphate Dehydrogenase, Aldolase and Triosephosphate Isomerase

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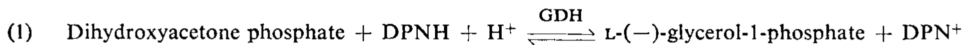
As the same indicator reaction can be used for the spectrophotometric determination of dihydroxyacetone phosphate, fructose diphosphate and glyceraldehyde phosphate[1-4], the description of the method is similar for all three substrates.

Principle

Dihydroxyacetone phosphate

Glycerol-1-phosphate dehydrogenase (GDH) catalyses the reduction of dihydroxyacetone phosphate (DAP) with reduced diphosphopyridine nucleotide (DPNH):



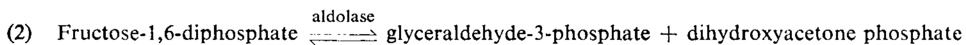
(1) Dihydroxyacetone phosphate + DPNH + H^+ $\overset{GDH}{\rightleftharpoons}$ L-(−)-glycerol-1-phosphate + DPN^+

The equilibrium constant of this reaction K_c is 1.78×10^{11} [moles/l.]$^{-1}$ at pH 0 and 25°C[5,6]; even at pH 7 ($K_c' = 1.78 \times 10^4$ [moles/l.]$^{-1}$) the equilibrium lies far to the right, so that dihydroxyacetone phosphate reacts quantitatively.

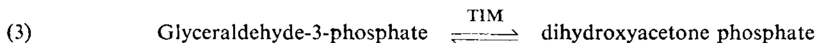
With a measurable excess of DPNH and sufficient enzyme the reaction proceeds rapidly. The decrease in optical density at 340 or 334 (also 366) mμ due to the oxidation of DPNH is a measure of the reaction.

Fructose-1,6-diphosphate

Aldolase cleaves fructose-1,6-diphosphate (FDP) to give glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate (DAP) (equation 2). GAP is isomerized to DAP by triosephosphate isomerase (TIM) (equation 3) and the DAP is reduced by DPNH and GDH (equation 1).

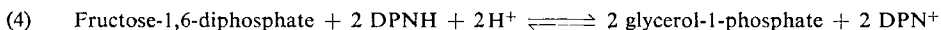


(2) Fructose-1,6-diphosphate $\overset{aldolase}{\rightleftharpoons}$ glyceraldehyde-3-phosphate + dihydroxyacetone phosphate



(3) Glyceraldehyde-3-phosphate $\overset{TIM}{\rightleftharpoons}$ dihydroxyacetone phosphate

The equilibrium of reaction (2) lies to the left[7] ($K_c = 6.8 \times 10^{-5}$ [moles/l.] at pH 7 and 20°C), while the equilibrium constant of reaction (3) K_c is 22 at pH 7 and 20°C[7]. Coupling reactions (1)−(3) gives the balance equation (4), whose equilibrium lies to the right ($K_c = 4.75 \times 10^4$ [moles/l.]$^{-1}$ at pH 7 and 20°C).



(4) Fructose-1,6-diphosphate + 2 DPNH + 2H^+ $\rightleftharpoons$ 2 glycerol-1-phosphate + 2 DPN^+

For each mole of FDP 2 moles of DPNH are oxidized. The inclusion of the triosephosphate isomerase reaction (3) therefore increases the sensitivity of the assay.

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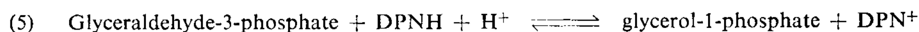
1) *Th. Bücher* et al., unpublished.
2) *E. Racker*, J. biol. Chemistry *167*, 843 [1947].
3) *E. C. Slater*, Biochem. J. *53*, 157 [1953].
4) *W. Thorn* et al., Pflügers Arch. ges. Physiol. Menschen Tiere *261*, 334 [1955].
5) *H. L. Young* and *N. Pace*, Arch. Biochim. Biophysics *75*, 125 [1958].
6) *H. J. Hohorst*, Ph. D.-Thesis, Universität Marburg, 1960.
7) *O. Meyerhof* in *J. B. Sumner* and *K. Myrbäck:* The Enzymes. Academic Press, New York 1951, Vol. II, p. 162.

Glyceraldehyde-3-phosphate

Glyceraldehyde-3-phosphate can also be determined spectrophotometrically by coupling reactions (1) and (3). The balance equation is:



(5) Glyceraldehyde-3-phosphate + DPNH + H^+ $\rightleftharpoons$ glycerol-1-phosphate + DPN^+

Reagents

1. Potassium carbonate, K_2CO_3, A. R.
2. Methyl orange indicator
3. Perchloric acid, A. R.; sp. gr. 1.67; *ca.* 70% (w/w)
4. Triethanolamine hydrochloride
5. Sodium hydroxide, A. R., 2 N
6. Ethylene-diamine-tetra-acetic acid, EDTA
   disodium salt, $EDTA\text{-}Na_2H_2 \cdot 2H_2O$ (Titriplex III, Trilon B, Versene)
7. Potassium dichromate, $K_2Cr_2O_7$, A. R.
8. Reduced diphosphopyridine nucleotide, DPNH
   disodium salt, $DPNH\text{-}Na_2$; commercial preparation, see p. 1011.
9. Glycerol-1-phosphate dehydrogenase, GDH
   crystalline, from skeletal muscle, suspension in 2.0 M ammonium sulphate solution. Commercial preparation, see p. 981.

Additional for the determination of FDP and GAP:

10. Triosephosphate isomerase, TIM
   crystalline, from skeletal muscle, suspension in 2.8 M ammonium sulphate solution. Commercial preparation, see p. 998.

Additional for the determination of FDP:

11. Aldolase
   crystalline, from skeletal muscle, suspension in 2.0 M ammonium sulphate solution. Commercial preparation, see p. 970.

Purity of the enzyme preparations

The GDH preparation should have a specific activity of at least 2000 units/mg. (according to *Bücher* *)) corresponding to *ca.* 36 units/mg. (according to *Racker* *)). Contamination by lactic dehydrogenase should not exceed 0.05%, by aldolase 0.01% and by phosphoglyceraldehyde dehydrogenase 0.05% (relative to the GDH activity).

The aldolase preparation should have a specific activity of at least 1000 units/mg. (according to *Bücher* *)) or 18 units/mg. (according to *Racker* *)). Contamination by lactic dehydrogenase should not exceed 0.05% and by phosphoglyceraldehyde dehydrogenase 0.01% (relative to the aldolase activity).

The triosephosphate isomerase preparation should have a specific activity of 2×10^5 units/mg. (according to *Bücher* *)) corresponding to *ca.* 3.6×10^3 units/mg. (according to *Racker* *)). Contamination by lactic dehydrogenase should not exceed 0.01% (relative to the TIM activity).

Preparation of Solutions

All solutions should be prepared with fresh, doubly distilled water.

I. Potassium carbonate (*ca.* 5 M):
   Dissolve *ca.* 69 g. K_2CO_3 in distilled water and make up to 100 ml.

*) Definition of units, see p. 33.

II. Methyl orange indicator:
Dissolve 50 mg. methyl orange in distilled water and make up to 100 ml.

III. Perchloric acid (*ca.* 6% w/w):
Dilute 7.7 ml. $HClO_4$ (sp. gr. 1.67) to 150 ml. with distilled water.

IV. Triethanolamine buffer (0.4 M; pH 7.6):
Dissolve 18.6 g. triethanolamine hydrochloride in about 200 ml. distilled water, add 18 ml. 2 N NaOH and 3.7 g. EDTA-$Na_2H_2 \cdot 2H_2O$ and dilute to 250 ml. with distilled water.

V. Reduced diphosphopyridine nucleotide (*ca.* 5×10^{-3} M β-DPNH):
Dissolve 7 mg. DPNH-Na_2 in triethanolamine buffer (solution IV) and make up to 1.5 ml.

VI. Glycerol-1-phosphate dehydrogenase, GDH (*ca.* 1.5 mg. protein/ml.):
Dilute the crystalline suspension containing *ca.* 10 mg. protein/ml. with distilled water.

VII. Triosephosphate isomerase, TIM (*ca.* 100 μg protein/ml.):
Dilute the crystalline suspension containing *ca.* 20 mg. protein/ml. with distilled water *).

VIII. Aldolase (*ca.* 2 mg. protein/ml.):
Dilute the crystalline suspension containing *ca.* 10 mg. protein/ml. with distilled water.

IX. Potassium dichromate (*ca.* 1% w/v, to partially compensate for the optical density):
Dissolve *ca.* 1 g. $K_2Cr_2O_7$ in distilled water and make up to 100 ml.

Stability of the solutions

Store all solutions, stoppered, in a refrigerator at 0—4°C. Under these conditions even the enzyme solutions are stable for several weeks without appreciable loss of activity. The DPNH in triethanolamine buffer is stable for 2—3 weeks.

Procedure

Experimental material

Obtain blood without constriction of the vein and deproteinize. Freeze tissue samples within a fraction of a second and do not allow to thaw until ready to deproteinize [8].

Deproteinization and extraction

Preliminary remarks: Add perchloric acid to deproteinize the sample. There are two possible methods of extraction: single extraction and calculation of the volume of the extract on the basis of an assumed, mean water content for the sample (see p. 254) or repeated and therefore quantitative extraction of the tissue. The second method is preferable if, apart from dihydroxyacetone phosphate or fructose-1,6-diphosphate, other metabolites, which may be difficult to extract, are to be determined in the same extract. In both cases, it is convenient always to use the same ratio of volume of extract to tissue weight of 8:1.

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*) The commercially available GDH-TIM preparation (Boehringer) can be used instead of solutions VI and VII for the determination of FDP and GAP.

[8] *H. J. Hohorst, F. H. Kreutz* and *Th. Bücher*, Biochem. Z. *332*, 18 [1959].

Use the following amounts of perchloric acid for the single extraction method:

7.2 ml. perchloric acid solution (III) to 1 g. of blood
7.15 ml. perchloric acid solution (III) to 1 ml. of blood
7.25 ml. perchloric acid solution (III) to 1 g. of tissue.

With the repeated extraction method it is sufficient to extract twice and to make up the extract to the corresponding volume to weight ratio of 8:1. In this case the error due to retention of the compound in the precipitate does not exceed 3—4%. This error can usually be ignored, if not, the results must be suitably corrected.

Method: Weigh a centrifuge tube containing a glass rod and 5 ml. perchloric acid solution (III). Add about 1 g. of sample: allow blood to flow directly from the cannula; powder frozen tissue[8] and add. Quickly mix and re-weigh.

Single extraction: From the difference in weight calculate the total volume of perchloric acid required to give a ratio of volume of extract to tissue weight of 8:1 (see above) and add the requisite amount of perchloric acid to the 5 ml. already present. Mix the suspension thoroughly, grind lumps of tissue against the walls of the tube and centrifuge at a minimum of 3000 g for 10 min. Transfer the supernatant to a cooled 10 ml. flask for neutralization.

Repeated extraction: Disintegrate a suspension of the sample in 5 ml. perchloric acid solution (III) with a homogenizer and centrifuge at 3000 g for 10 min. Decant the supernatant, mix the precipitate with 1 ml. perchloric acid solution (III) + 1 ml. doubly distilled water and re-centrifuge. Combine the supernatants, measure the volume and make up to 8 ml./g. with doubly distilled water.

Neutralization: Pipette 0.02 ml. indicator solution (II) into 8 ml. extract and, while stirring vigorously with a magnetic stirrer and cooling in ice, add about 0.1 ml. carbonate solution (I) from a 0.2 ml. capillary pipette. Wait until the CO_2 evolution has nearly ceased and then add more carbonate solution until the mixture is salmon-pink (pH *ca.* 3.5). A total of about 0.18 ml. carbonate solution is required. Allow to stand for 10 min. in an ice bath, decant or pipette off the supernatant from the precipitated perchlorate and use a measured portion for the determination.

Spectrophotometric measurements of DAP and FDP

Preliminary remarks: The calculation of the results is made simpler if the same ratio of total volume to sample volume is used. A control cuvette can usually be omitted.

Method: Wavelength: 340 or 334 mμ; light path: 5 cm.; final volume: 4.52 (DAP) or 4.54 (FDP) ml. Read the experimental cuvette against the blank cuvette.

Pipette the solutions into the cuvettes in the following order *):

Experimental cuvette	*Blank cuvette* (light path 1 cm.)
2.00 ml. buffer (solution IV)	2.00 ml. buffer (solution IV)
2.50 ml. deproteinized tissue extract	
0.01 ml. DPNH solution (V).	

*) This order must be followed even if only fructose diphosphate is to be determined. In other words, the dihydroxyacetone phosphate present in the extract must be reduced first before the FDP assay can be commenced.

To compensate for the absorption of the sample, mix

ca. 0.02 ml. potassium dichromate solution (VIII)

into the blank cuvette. Mix the contents of the cuvettes thoroughly and allow to warm to room temperature. Read the optical density E_1 two or more times at 3 min. intervals. For the determination of dihydroxyacetone phosphate mix into the experimental cuvette

0.01 ml. GDH solution (VI)

with a small glass spatula.

On completion of the reaction (6–9 min. after the addition of the enzyme, depending on the DAP concentration) read the optical density E_2 two or more times at 3 min. intervals. For the determination of fructose diphosphate, mix into the experimental cuvette

0.01 ml. TIM solution (VII) and
0.01 ml. aldolase solution (VIII)

and on completion of the reaction (6–9 min. after the addition of the enzymes) read the optical density E_3 two or more times at 3 min. intervals. The changes in the optical densities E_1, E_2 and E_3 during 3 min. are usually small in comparison to the optical density differences $\Delta E_{DAP} = E_1 - E_2$ or $\Delta E_{FDP} = E_2 - E_3$ and can be neglected. If necessary, apply a correction (see p. 256).

To check that the assay is functioning correctly, on completion of the reaction, mix

0.01 ml. 10^{-3} M FDP solution

into the experimental cuvette. The decrease in optical density should be complete in 6 min. Read the optical density E_4 two or more times at 3 min. intervals and calculate the difference $\Delta E' = E_3 - E_4$. $\Delta E'$ should be 0.138 at 340 mμ and 0.134 at 334 mμ.

Calculations

Dihydroxyacetone phosphate and fructose-1,6-diphosphate react quantitatively and therefore the DAP and FDP content of the sample can be calculated from the optical density differences ΔE. However, it should be noted that for each mole of FDP 2 moles of DPNH are oxidized. If constant dilution ratios are used then the usual formula:

$$\frac{E \times \text{dil.}}{\varepsilon \times d} = \mu\text{moles/g. tissue}$$

where

ΔE = optical density difference ($E_1 - E_2$ or $E_2 - E_3$)
dil. = total dilution of the sample in the assay
ε = extinction coefficient [cm.^{2}/μmole]
d = light path [cm.]

simplifies to $\Delta E \times F$ = μmoles DAP or FDP/g. tissue, where $F = \frac{\text{dil.}}{\varepsilon \times d}$.

Under the conditions described above the total dilution of the sample in the assay mixture is 15 : 1. Therefore the following values for F are obtained:

Dihydroxyacetone phosphate: at 334 mμ F = 0.494, 340 mμ F = 0.478
Fructose-1,6-diphosphate*): at 334 mμ F = 0.297, 340 mμ F = 0.239

*) In the calculation of the F values the relationship 2 moles DPNH/1 mole FDP was taken into account. Therefore the product of $\Delta E \times F$ ($\Delta E = E_2 - E_3$) gives the FDP content of the tissue directly.

Spectrophotometric measurements of GAP

Preliminary remarks: All tissues so far examined contain extremely little glyceraldehyde-3-phosphate. The determination of GAP in tissue extracts is therefore only possible with highly sensitive, recording instruments. It can be assumed that, because of the high triosephosphate isomerase activity in tissues, GAP and DAP will be present in a ratio corresponding to the equilibrium of this reaction, *i. e.* the GAP content will be about 1/20 of the DAP content. Determinations on rat abdominal muscle gave values[9] of 2×10^{-9} moles GAP/g. and 25×10^{-9} moles DAP/g. fresh weight.

Method: The description is for the Beckmann DK spectrophotometer. First determine DAP according to the method given above. On completion of the reaction caused by the addition of GDH the light transmission (I/I_0) should be 90–95%.

If necessary, mix a little potassium dichromate solution (IX) or water into the control cuvette. After switching the scale to 90–110% transmission record the initial value (T_1) for 3–4 min. at the slowest chart speed. Stir the cuvette contents frequently until either the transmission or the rate of change in transmission remains constant. Start the reaction by mixing

0.005 ml. TIM solution (VII)

into the experimental cuvette and record the increase in transmission. On completion of the reaction (3–5 min.) record the final value T_2 for 3–4 min. By stirring in a further

0.005 ml. TIM solution (VII)

obtain the correction for the absorption change on addition of the enzyme (T_3).

Calculations

The GAP content of the tissue is calculated according to the formula:

$$\frac{(\log T_2 - \log T_1)}{\varepsilon \times d} \times \text{dil.} = (\log T_2 - \log T_1) \times F = \mu\text{moles GAP/g. tissue}$$

where

dil. = total dilution of the sample in the assay (here 15 : 1)

ε = extinction coefficient [cm.²/μmole]

d = light path [cm.]

Under the conditions described here the same values for F apply as those stated above (see p. 250) for the calculation of the DAP content.

Example

The volume of a neutralized extract from 0.923 g. rat liver was 7.65 ml. The following values were measured spectrophotometrically (wavelength: 340 mμ; light path: 5 cm.):

before addition of GDH	0 min. E_1	= 0.875
	3 min. E'_1	= 0.872
	6 min. E''_1	= 0.870
after addition of GDH	12 min. E_2	= 0.784
	15 min. E'_2	= 0.781

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[9] *H. J. Hohorst* and *M. Reim*, unpublished.

$\Delta E_{DAP} = E''_1 - E_2 = 0.086$. The decrease in optical density before the start of the reaction was 0.005 ($E_1 - E''_1$) for a period of 6 min. Therefore the corrected optical density difference $\Delta E_{corr.} = 0.086 - 0.005 = 0.081$. From this it follows that the dihydroxyacetone phosphate content of the tissue is

$$0.081 \times 0.478 = 0.039 \ \mu\text{moles DAP/g. tissue.}$$

Other Determinations

Other metabolites can be determined in the same assay mixture by addition of specific enzymes, *e.g.* pyruvate with lactic dehydrogenase[1,4,8] (refer to p. 253) and oxaloacetate with malic dehydrogenase (refer to p. 335). Oxaloacetate must be determined before DAP and FDP, while it is better to determine pyruvate last.

Sources of Error

1. If the final value is not reached within 4 min. the activity of the enzymes is too low. Check the activity of the enzymes and, if necessary, use larger amounts or a new enzyme preparation.

2. The initial and final optical densities show large changes (especially when carrying out measurements with glycogen-rich liver extracts). The remedy is to first centrifuge the extracts at high speed or to obtain the difference between the initial and final optical densities by extrapolation (see "Example" and p. 39).

Specificity

The assay is specific for dihydroxyacetone phosphate or fructose-1,6-diphosphate; fructose-6-phosphate and fructose-1-phosphate do not interfere with the determination of FDP.