D-3-Phosphoglycerate, D-2-Phosphoglycerate, Phosphoenolpyruvate

Rudolf Czok and Lieselotte Eckert

D-3-Phosphoglycerate (3-PG) can be detected by paper chromatography[1]. A polarimetric[2] and two enzymatic, spectrophotometric methods[3,4] are available for the quantitative determination of 3-PG. The last two are described here.

The principle of the spectrophotometric methods consists of the enzymatic conversion of 3-PG either to

1. D-Glyceraldehyde-3-phosphate (GAP)

or to

2. L-Lactate

and the quantitative determination of the reduced diphosphopyridine nucleotide (DPNH) consumed in the process. Which of the two assay methods to choose depends on the type of sample and on the concentration of the substrates in the sample, which are to be determined together with 3-PG. With method 2. (p. 229) pyruvate, phosphoenolpyruvate (PEP), 2-phosphoglycerate (2-PG) and 3-PG can be measured in the same assay, while with method 1. (below) only 2-PG and 3-PG can be measured. Phosphate inhibits the conversion of 3-PG to 2-PG and therefore the determination by method 2. takes longer.

D-2-Phosphoglycerate can also be detected by paper chromatography[1]. The assay for 2-PG described here depends on its enzymatic conversion to phosphoenolpyruvate (PEP) by enolase. This method was first described by *Rodwell et al.*[3].

The chemical methods for the determination of phosphoenolpyruvate are based on the lability of the phosphate bond[5,6]. The phosphate is split off in alkaline iodine solution by mercury salts or by heating at 100° C in 1 N HCl, and determined as inorganic phosphate. PEP absorbs in ultraviolet light[7] and has a molar extinction coefficient at 240 mμ and pH 7 of 1.44×10^6 (cm.^{2}/mole). Analysis by spectrophotometric measurements at 240 mμ is only suitable for pure solutions, because the extinction coefficient varies with the pH and magnesium ion concentration[8,9]. The method described here for the determination of PEP depends on its enzymatic conversion to lactate with pyruvic kinase and lactic dehydrogenase.

D-3-Phosphoglycerate and D-2-Phosphoglycerate

Determination with phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate mutase.

Principle

Phosphoglycerate kinase (PGK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) catalyse the following reactions:

---

1) *E. Gerlach, A. Fleckenstein* and *K. J. Freundt*, Pflügers Arch. ges. Physiol. Menschen Tiere *263*, 682 [1956/57]; *E. Gerlach, A. Fleckenstein* and *E. Gross*, ibid. *266*, 528 [1957/58].
2) *O. Meyerhof*, Biochem. Z. *297*, 60 [1938].
3) *V. W. Rodwell, J. C. Towne* and *S. Grisolia*, J. biol. Chemistry *228*, 876 [1957].
4) *H. J. Hohorst, F. Kreutz* and *Th. Bücher*, Biochem. Z. *332*, 18 [1959].
5) *K. Lohmann* and *O. Meyerhof*, Biochem. Z. *273*, 60 [1934].
6) *G. Schmidt* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1957, Vol. III, p. 223.
7) *O. Warburg* and *W. Christian*, Biochem. Z. *310*, 384 [1941].
8) *F. Wold* and *C. E. Ballou*, J. biol. Chemistry *227*, 301 [1957].
9) *J. F. Bealing, R. Czok, L. Eckert* and *I. Jäger*, unpublished.

(1) D-3-Phosphoglycerate + ATP $\underset{Mg^{2+}}{\overset{PGK}{\rightleftharpoons}}$ 1,3-diphosphoglycerate + ADP

(2) 1,3-Diphosphoglycerate + DPNH + H^+ $\overset{GAPDH}{\rightleftharpoons}$ D-glyceraldehyde-3-phosphate + phosphate + DPN^+

A stoichiometric conversion is obtained by trapping the glyceraldehyde phosphate with hydrazine[4]. By combining reactions (1) and (2) with the reaction catalysed by phosphoglycerate mutase (PGM):

(3) D-2-Phosphoglycerate $\overset{PGM}{\rightleftharpoons}$ D-3-phosphoglycerate

D-2-phosphoglycerate can be determined in the same assay mixture.

Reagents

1. Triethanolamine hydrochloride, A. R.
2. Magnesium sulphate, $MgSO_4 \cdot 7H_2O$, A. R.
3. Glutathione, GSH
4. Hydrazine sulphate, A. R.
5. Adenosine triphosphate, ATP
   sodium salt, ATP-$Na_2H_2 \cdot 3H_2O$. Commercial preparation, see p. 1006.
6. Reduced diphosphopyridine nucleotide, DPNH
   sodium salt, $DPNH_2$-Na_2. Commercial preparation, see p. 1011.
7. D-Glyceraldehyde-3-phosphate dehydrogenase, GAPDH
   crystalline, from rabbit skeletal muscle[10]; suspension in 2.5 M ammonium sulphate solution. Commercial preparation, see p. 979.
8. Phosphoglycerate kinase, PGK
   crystalline, from rabbit skeletal muscle[11] or yeast[12]; suspension in 2.4 M ammonium sulphate solution containing 0.04 M $Na_4P_2O_7$. Commercial preparation, see p. 994.
9. Perchloric acid, A. R., sp. gr. 1.67; *ca.* 70% (w/w)
10. Potassium hydroxide, A. R.
11. Ethylene-diamine-tetra-acetic acid, EDTA
    disodium salt, EDTA-$Na_2H_2 \cdot 2H_2O$ (Titriplex III, Trilon B, Versene).
12. 2,3-Diphosphoglycerate, 2,3-di-PG
    $(brucine)_5$-salt prepared from pig blood according to[13], from D-3-phosphoglycerate and ATP with an extract of acetone-dried chicken breast muscle according to[14] or as the Ba_5-salt according to[15].
13. Phosphoglycerate mutase, PGM
    crystalline, from rabbit muscle[16, 17]; suspension in 2.4 M ammonium sulphate solution. Commercial preparation, see p. 995.
14. Hydrochloric acid, A. R., 1 N
15. Sodium hydroxide, A. R., 1 N
16. Diethyl ether, A. R.

---

[10] *G. Beisenherz, H. J. Boltze, Th. Bücher, R. Czok, K. H. Garbade, E. Meyer-Arendt* and *G. Pfleiderer*, Z. Naturforsch. *8b*, 555 [1953].
[11] *F. W. Bube, R. Czok* and *I. Jäger*, unpublished.
[12] *Th. Bücher*, Biochim. biophysica Acta *1*, 292 [1947].
[13] *J. Greenwald*, J. biol. Chemistry *63*, 339 [1925].
[14] *S. Grisolia* and *B. K. Joyce*, J. biol. Chemistry *233*, 18 [1958].
[15] *E. Baer*, J. biol. Chemistry *185*, 763 [1950].
[16] *R. Czok, L. Eckert* and *I. Jäger*, unpublished.
[17] *R. Czok* and *Th. Bücher*, Advances Protein Chem. *15*, 315 [1960].

Purity of the enzyme preparations

GAPDH: The preparation should have a specific activity of at least 1400 units*)/mg. protein.

PGK: The preparation should have a specific activity of at least 1.7×10^4 units*)/mg. protein. PGK commercial preparations may be contaminated with sufficient phosphoglycerate mutase to cause interference. The PGM can be removed from the yeast enzyme as follows: centrifuge 5 ml. of a crystalline suspension containing 3.2×10^5 units*) PGK/ml. and 875 units*) PGM/ml., take up the sediment in 10 ml. of a mixture[12] of 6 ml. saturated ammonium sulphate solution (20° C), 2 ml. 0.2 M Na-pyrophosphate solution, 0.17 ml. 2 N NH_4OH and distilled water to 10 ml. With thorough mixing, slowly add 2 ml. distilled water until only a very slight turbidity remains. Centrifuge this off and discard. 30% PGK and 45% PGM is lost. Slowly add solid ammonium sulphate (recrystallized according to[10]) to the clear supernatant until the concentration is 3.0 M and then allow to stand overnight. Centrifuge, the clear supernatant still contains 30% of the PGK, but no PGM. The protein concentration is 3.2 mg./ml. Add solid ammonium sulphate to the supernatant to bring the concentration up to 3.25 M (until turbid and crystallization starts). The activity of the PGK obtained in this way is 1.7×10^4 units/mg. protein. The preparation is free from PGM.

PGM: The specific activity should be at least 30000 units*)/mg.

PGK and PGM should be free from enolase, pyruvic kinase and lactic dehydrogenase. GAPDH and PGK should be free from PGM.

Preparation of Solutions

I. Triethanolamine buffer (0.2 M; pH 7.6):
Dissolve 9.3 g. triethanolamine hydrochloride in *ca.* 200 ml. doubly distilled water, add 3.7 g. EDTA-$Na_2H_2 \cdot 2H_2O$, adjust to pH 7.6 with *ca.* 9 ml. 2 N NaOH and dilute with doubly distilled water to 250 ml.

II. Magnesium sulphate (0.5 M):
Dissolve 12.3 g. $MgSO_4 \cdot 7H_2O$ in doubly distilled water and make up to 100 ml.

III. Glutathione (0.05 M):
Dissolve 15.4 mg. GSH in 1 ml. buffer (solution I).

IV. Hydrazine (0.2 M):
Dissolve 3.12 g. hydrazine sulphate in doubly distilled water and make up to 100 ml.; just before use neutralize 1 ml. of this solution with 0.2 ml. 1 N NaOH.

V. Adenosine triphosphate (*ca.* 0.15 M ATP):
Dissolve 100 mg. ATP-$Na_2H_2 \cdot 3H_2O$ in 1 ml. doubly distilled water, neutralize to between pH 7.0 and 7.4 with about 0.03 ml. 10 N KOH (indicator paper).

VI. Reduced diphosphopyridine nucleotide (*ca.* 0.01 M β-DPNH):
Dissolve 7 mg. DPNH-Na_2 in 1 ml. doubly distilled water or buffer (solution I).

VII. 2,3-Diphosphoglycerate (*ca.* 0.01 M 2,3-di-PG):
Suspend 30 mg. $(brucine)_5$-salt (molecular weight 2260) in 1.5 ml. doubly distilled water. Precipitate the brucine with 0.06 ml. 1 N NaOH (curd-like precipitate) and centrifuge for 5 min. (3000 g). Wash the precipitate with 0.5 ml. doubly distilled water, centrifuge, extract the combined supernatants twice with 10 ml. portions of diethyl ether (shake for 5 min.). Separate off the aqueous phase and free from residual ether by evacuating (water pump). Adjust to pH 6–7 with about 0.01 ml. 1 N HCl (universal indicator paper, Merck).

*) Definition of the units, see p. 33.

VIII. Glyceraldehyde-3-phosphate dehydrogenase, GAPDH (*ca.* 10 mg. protein/ml.):
Use as a suspension or centrifuge and dissolve the sediment in the original volume of doubly distilled water.

IX. Phosphoglycerate kinase, PGK (*ca.* 10 mg. protein/ml.):
Use as a suspension or centrifuge and dissolve the sediment in the original volume of doubly distilled water.

X. Phosphoglycerate mutase, PGM (*ca.* 10 mg. protein/ml.):
Use as a suspension or centrifuge and dissolve the sediment in the original volume of doubly distilled water.

XI. Perchloric acid (*ca.* 6% w/v):
Dilute 52 ml. 70% (w/w) $HClO_4$ to 1000 ml. with doubly distilled water.

XII. Potassium hydroxide (*ca.* 10 N):
Dissolve 56 g. KOH in doubly distilled water and make up to 100 ml.

Stability of the solutions

The ATP solution is stable for several weeks at pH 7. Prepare the GSH and hydrazine solutions freshly for each series of measurements. The acid hydrazine solution is stable indefinitely. Prepare the DPNH solution freshly each week. All the other solutions are stable practically indefinitely at between 0 and 5° C.

Procedure

Experimental material and deproteinization

Deproteinize samples with perchloric acid solution (XI) and then adjust to pH 3—4 with KOH (solution XII) (cool in ice). For a complete description, see p. 254. For correction of the analytical results for the blood content of the tissue, see p. 549.

Spectrophotometric measurements

Wavelength: 366 mμ; light path: 1 cm.; final volume: 2 ml. Measure against air.
Pipette successively into the cuvette:

0.500 ml. buffer (solution I)
0.032 ml. $MgSO_4$ solution (II)
0.100 ml. GSH solution (III)
0.030 ml. neutralized hydrazine solution (IV)
0.100 ml. ATP solution (V)
0.040 ml. DPNH solution (VI)
up to 1.200 ml. deproteinized sample.

If 2-phosphoglycerate is to be determined in the same assay add

0.025 ml. 2,3-di-PG solution (VII)

and take correspondingly less sample.

Equilibrate the mixture at 25° C. Mix in

0.040 ml. GAPDH suspension or solution (VIII) (30 units)

and read the initial optical density E_1. There should be no significant change within 3—5 min. If a constant "drift" occurs, which is repeated after the complete reaction of the 3-PG and 2-PG, extrapolate to zero time (refer to p. 39). Start the reaction by mixing in

0.002 ml. PGK suspension or solution (IX) (50 units)

and after 4—5 min. read the optical density E_2. To determine 2-phosphoglycerate mix in

0.002 ml. PGM suspension or solution (X) (*ca.* 100 units)

and after 5—10 min. read the optical density E_3.

$E_1 - E_2 = \Delta E_{3\text{-PG}}$ and $E_2 - E_3 = \Delta E_{2\text{-PG}}$ are used for the calculations.

The optimum concentration of both substrates is 0.06 to 0.4 μmoles/assay mixture. For measurements at 340 mμ or 334 mμ take only 0.020 ml. DPNH solution (optimum concentration range: 0.03 to 0.2 μmoles 3-PG and 2-PG/assay mixture).

An illustration of the course of the reaction is given in Fig. 1.

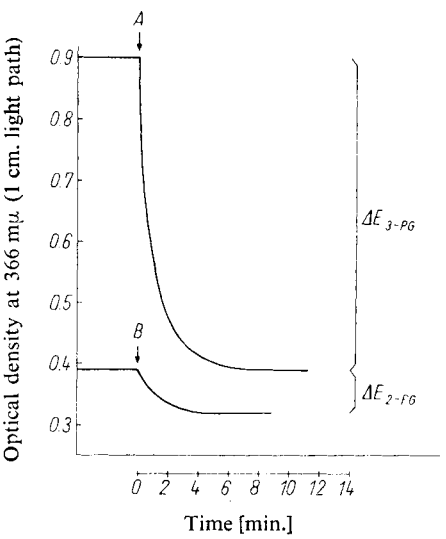

Fig. 1. Determination of 3-phosphoglycerate (3-PG) and 2-phosphoglycerate (2-PG) in a neutralized extract of rat liver; 1 g. liver (wet weight) in 5.2 ml. extract; 1.5 ml. extract/2 ml. assay mixture.
A: Addition of phosphoglycerate kinase
B: Addition of phosphoglycerate mutase

Calculations

$$\frac{\Delta E \times V}{\varepsilon \times V_D \times d} = \text{μmoles 3-PG or 2-PG/ml. deproteinized sample}$$

where

V = assay volume

V_D = deproteinized sample taken for assay [ml.]

d = light path of the cuvette [cm.]

ε = extinction coefficient of DPNH [cm.²/μmole]

$\varepsilon_{366} = 3.30$ [18]

$\varepsilon_{340} = 6.22$ [19]

$\varepsilon_{334} = 6.09$ [20]

Specificity

No work has been done on the specificity of the determination.

---

18) *H. J. Hohorst*, Biochem. Z. *328*, 509 [1957].

19) *A. Kornberg* and *W. E. Pricer* in *E. E. Snell:* Biochemical Preparations. Wiley, New York 1953, Vol. III, p. 20.

20) *G. Beisenherz, Th. Bücher* and *K. H. Garbade* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1955, Vol. I, p. 391.

D-3-Phosphoglycerate, D-2-Phosphoglycerate, Phosphoenolpyruvate

Determination with phosphoglycerate mutase, enolase, pyruvic kinase and lactic dehydrogenase

Principle

3-Phosphoglycerate (3-PG), 2-phosphoglycerate (2-PG) and phosphoenolpyruvate (PEP) can be determined by means of the following reactions:

(1) $$\text{3-PG} \xrightleftharpoons{\text{PGM}} \text{2-PG}$$

(2) $$\text{2-PG} \xrightleftharpoons[Mg^{2+}]{\text{enolase}} \text{PEP}$$

(3) $$\text{PEP} + \text{ADP} \xrightleftharpoons[Mg^{2+},\ K^{+}]{\text{PK}} \text{pyruvate} + \text{ATP}$$

(4) $$\text{Pyruvate} + \text{DPNH} + H^{+} \xrightleftharpoons{\text{LDH}} \text{lactate} + DPN^{+}$$

The measure of the over-all reaction is the decrease in optical density at 366 mμ due to the oxidation of DPNH (last step in the series of reactions). Quantitative conversion is assured because of the positions of the equilibria of the reactions catalysed by pyruvic kinase (PK) and lactic dehydrogenase (LDH) [4, 21].

Biological material may contain 10 times more 3-PG than 2-PG, PEP and pyruvate. In such cases, the accuracy of the assay of all four compounds is not very high. The accuracy can be increased by determining the 2-PG and PEP separately in larger samples or by carrying out the combined assay at 340 mμ, thus increasing the sensitivity of the measurements of optical density.

3-PG can only be determined under the conditions described here if the assay mixture contains less than 10^{-3} M inorganic phosphate [16]. However, the inhibitory effect of phosphate can be considerably reduced if the same volume of $MnSO_4$ solution (IV) is added to the assay mixture instead of the $MgSO_4$ solution. The determination of 2-PG, PEP and pyruvate is not affected by phosphate.

Reagents

1. Triethanolamine hydrochloride, A. R.
2. Potassium chloride, A. R.
3. Magnesium sulphate, $MgSO_4 \cdot 7H_2O$, A. R.
4. Manganous sulphate, $MnSO_4 \cdot 4H_2O$, A. R.
5. Adenosine diphosphate, ADP
   sodium salt, ADP-Na_3; commercial preparation, see p. 1004.
6. 2,3-Diphosphoglycerate, 2,3-di-PG
   (brucine)$_5$-salt prepared from pig blood according to [13], from D-3-phosphoglycerate and ATP with an extract of acetone-dried chicken breast muscle according to [14] or as the Ba_5-salt according to [15].
7. Reduced diphosphopyridine nucleotide, DPNH
   sodium salt, DPNH-Na_2. Commercial preparation, see p. 1011.
8. Ethylene-diamine-tetra-acetic acid, EDTA
   disodium salt, EDTA-$Na_2H_2 \cdot 2H_2O$ (Titriplex III, Trilon B, Versene).

---

21) *J. T. McQuate* and *M. F. Utter*, J. biol. Chemistry *234*, 2151 [1959].

9. Lactic dehydrogenase, LDH
   crystalline, from rabbit skeletal muscle[10], suspension in 2.2 M ammonium sulphate solution. Commercial preparation, see p. 986.
10. Pyruvic kinase, PK
    crystalline, from rabbit skeletal muscle[10], suspension in 2.1 M ammonium sulphate solution. Commercial preparation, see p. 997.
11. Enolase
    crystalline, from rabbit skeletal muscle[9, 17], suspension in 2.6 M ammonium sulphate solution. Commercial preparation, see p. 973.
12. Phosphoglycerate mutase, PGM
    crystalline, from rabbit skeletal muscle[16, 17], suspension in 2.4 M ammonium sulphate solution. Commercial preparation, see p. 995.
13. Perchloric acid, A. R., sp. gr. 1.67; *ca.* 70% (w/w)
14. Potassium hydroxide, A. R.
15. Hydrochloric acid, A. R., 1 N
16. Sodium hydroxide, A. R., 1 N
17. Diethyl ether, A. R.

Purity of the enzyme preparations

The preparations should have the specific activities stated in the following Table and the amounts of the contaminants should not be greater than those laid down in columns 2 to 5.

Enzyme	Specific activity	Contaminants Units as % of the total activity			
		PK	Enolase	PGM	LDH
LDH	17000 units*)/mg. protein	0	0	0	—
PK	6500 units/mg. protein	—	0.001	0	0.04
Enolase	2500 units/mg. protein	0	—	0	0.04
PGM	30000 units/mg. protein	0.2	0	—	0.6

Preparation of Solutions

I. Triethanolamine buffer (0.2 M; pH 7.6):
   Dissolve 9.3 g. triethanolamine hydrochloride in *ca.* 200 ml. doubly distilled water, add 3.7 g. EDTA-Na_2H_2, adjust to pH 7.6 with *ca.* 9 ml. 2 N NaOH and dilute to 250 ml. with doubly distilled water.

II. Potassium chloride (2 M):
   Dissolve 14.9 g. KCl in doubly distilled water and make up to 100 ml.

III. Magnesium sulphate (0.5 M):
   Dissolve 12.3 g. $MgSO_4 \cdot 7H_2O$ in doubly distilled water and make up to 100 ml.

IV. Manganous sulphate (0.005 M):
   Dissolve 1.11 g. $MnSO_4 \cdot 4H_2O$ in doubly distilled water and make up to 1000 ml.

V. Adenosine diphosphate (*ca.* 0.01 M ADP):
   Dissolve 51.1 mg. ADP-Na_3 in doubly distilled water and make up to 10 ml.

*) Definition of the units according to[10], see p. 33.

VI. 2,3-Diphosphoglycerate (*ca.* 0.01 M 2,3-di-PG):
Suspend 30 mg. (brucine)$_5$-salt (molecular weight 2260) in 1.5 ml. doubly distilled water. Precipitate the brucine with 0.06 ml. 1 N NaOH (curd-like precipitate). Centrifuge for 5 min. (3000 g). Wash the precipitate with 0.5 ml. doubly distilled water, centrifuge and extract the combined supernatants twice with 10 ml. portions of diethyl ether (shake for 5 min.). Separate off the aqueous phase and free from residual ether by evacuating (water pump). Adjust to pH 6—7 with about 0.01 ml. 1 N HCl (universal indicator paper, Merck).

VII. Reduced diphosphopyridine nucleotide (*ca.* 0.01 M β-DPNH):
Dissolve 7 mg. DPNH-Na_2 in 1 ml. doubly distilled water or buffer (solution I).

VIII. Lactic dehydrogenase, LDH (*ca.* 15 mg. protein/ml.):
Use as a suspension or centrifuge and dissolve the sediment in the original volume of doubly distilled water.

IX. Pyruvic kinase, PK (*ca.* 10 mg. protein/ml.):
Use as a suspension or centrifuge and dissolve the sediment in the original volume of doubly distilled water.

X. Enolase (*ca.* 5 mg. protein/ml.):
Use as a suspension or centrifuge and dissolve the sediment in the original volume of doubly distilled water.

XI. Phosphoglycerate mutase, PGM (*ca.* 10 mg. protein/ml.):
Use as a suspension or centrifuge and dissolve the sediment in the original volume of doubly distilled water.

XII. Perchloric acid (*ca.* 6% w/v):
Dilute 52 ml. 70% (w/w) $HClO_4$ to 1000 ml. with doubly distilled water.

XIII. Potassium hydroxide (*ca.* 10 N):
Dissolve 40 g. KOH in doubly distilled water and make up to 100 ml.

Stability of the solutions

Prepare the DPNH solution freshly each week. Prepare the $MnSO_4$ solution freshly for each series of determinations. All the other solutions are stable practically indefinitely between 0 and 5°C.

Procedure

Experimental material and deproteinization

Deproteinize samples with perchloric acid (solution XII) and then adjust to pH 3—4 with KOH (solution XIII) (cool in ice). For a complete description, see p. 254. For correction of the analytical results for the blood content of the tissue, see p. 549.

Spectrophotometric measurements

Wavelength: 366 mμ; light path: 1 cm.; final volume: 2.25 ml.*). Measure against air. Pipette successively into the cuvette:

0.500 ml. buffer (solution I)
0.075 ml. KCl solution (II)
0.032 ml. $MgSO_4$ solution (III)
(or $MnSO_4$ solution (IV))

*) The addition of the enzyme solutions or suspensions increases the assay volume by less than 1%.

0.050 ml. ADP solution (V)
0.025 ml. 2,3-di-PG solution (VI)
0.040 ml. DPNH solution (VII)
up to 1.500 ml. deproteinized sample.

Equilibrate the assay mixture at *ca.* 25°C (5 to 10 min. in a constant temperature cuvette holder). Mix in

0.001 ml. LDH suspension or solution (VIII) (130 units),

wait for the end of the reaction and then read the optical density E_1. Mix in

0.002 ml. PK suspension or solution (IX) (about 60 units).

On completion of the reaction (5—10 min.) read the optical density E_2. Mix in

0.02 ml. enolase suspension or solution (X) (40 units).

On completion of the reaction (5 to 10 min.) read the optical density E_3. It should not change significantly within 3—5 min. If a small constant "drift" occurs, which continues after the complete conversion of the 3-PG, extrapolate to zero time (refer to p. 39). Mix in

0.002 ml. PGM suspension or solution (XI) (*ca.* 100 units)

and after 10—15 min. read the optical density E_4.

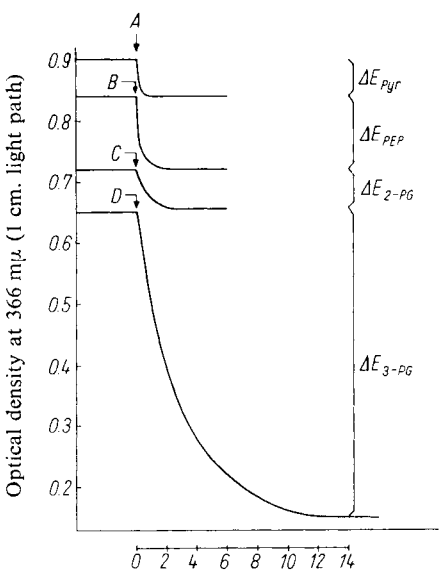

Fig. 2. Determination of 3-phosphoglycerate (3-PG), 2-phosphoglycerate (2-PG), phosphoenolpyruvate (PEP) and pyruvate (Pyr.) in a neutralized extract of rat liver (1 g. liver; wet weight, in 5.2 ml. extract; 1.5 ml. extract/2 ml. assay mixture).
A: Addition of lactic dehydrogenase
B: Addition of pyruvic kinase
C: Addition of enolase
D: Addition of phosphoglycerate mutase

The following are used for the calculations:

$$E_1 - E_2 = \Delta E_{PEP};\ E_2 - E_3 = \Delta E_{2\text{-}PG};\ E_3 - E_4 = \Delta E_{3\text{-}PG}.$$

The optimum concentration of all three metabolites is 0.06 to 0.4 μmoles/assay mixture. The sensitivity of the analysis is doubled if the optical density is read at 334 or 340 mμ (use only 0.020 ml. DPNH solution).

An illustration of the course of the reaction is given in Fig. 2.

Calculations

$$\frac{\Delta E \times V}{\varepsilon \times V_D \times d} = \mu\text{moles 3-PG, 2-PG or PEP/ml. deproteinized sample}$$

where

V = volume of the assay mixture

V_D = volume of the deproteinized sample taken for assay [ml.]

d = light path of the cuvette [cm.]

ε = extinction coefficient of DPNH [cm.^{2}/μmole]

$\varepsilon_{366} = 3.30$ [18]

$\varepsilon_{340} = 6.22$ [19]

$\varepsilon_{334} = 6.09$ [20]

Specificity

The reactions catalysed by enolase and pyruvic kinase guarantee the high specificity of the determination described here[22]. L-2-PG and the homologues of D-2-PG do not react with enolase. Apart from PEP, no substrate of pyruvic kinase is known which can give a reaction product capable of reacting with lactic dehydrogenase.

---

[22] *F. Wold* and *C. E. Ballou*, J. biol. Chemistry *227*, 313 [1957].