Adenosine-5′-triphosphate

Determination with Phosphoglycerate Kinase

Hans Adam

Principle

Phosphoglycerate kinase (PGK) catalyses the reaction:

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

The 1,3-diphosphoglycerate formed is reduced by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and reduced diphosphopyridine nucleotide (DPNH). The glyceraldehyde-3-phosphate is trapped as the hydrazone.

Indicator reaction:

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

Trapping reaction:

(3) Glyceraldehyde-3-phosphate + hydrazine ⟶ glyceraldehyde-3-phosphate hydrazone.

Reaction (1) proceeds 8.8 times slower from left to right than from right to left[1]. The equilibrium of reaction (2) lies 65% to the right, so that the 1,3-diphosphoglycerate formed in reaction (1) is further transformed at a sufficiently rapid rate, especially when the phosphoglycerate kinase is saturated with 3-phosphoglycerate.

Reagents*)

1. Triethanolamine, freshly distilled, b. p. 277—279°C/150 mm.
2. Hydrochloric acid, A. R., 2 N
3. Sodium hydroxide, A. R., 2 N
4. Sulphuric acid, A. R., 2 N
5. Magnesium sulphate, $MgSO_4 \cdot 7H_2O$, A. R.
6. Sodium pyrophosphate, $Na_4P_2O_7 \cdot 10H_2O$, A. R.
7. Glutathione, GSH
   commercial preparation, see p. 1018.
8. Perchloric acid, A. R., sp. gr. 1.67, ca. 70% (w/w)
9. Potassium carbonate, K_2CO_3, A. R., 3.75 M
10. Potassium hydroxide, A. R., 2 N
11. Ethylene-diamine-tetra-acetic acid, EDTA
    disodium salt, EDTA-$Na_2H_2 \cdot 2H_2O$, *e.g.* Titriplex III**)
12. Hydrazine sulphate, A. R.
13. D-3-Phosphoglyceric acid, 3-PGA
    crystalline barium salt·$2H_2O$; commercial preparation, see p. 1025.
14. Reduced diphosphopyridine nucleotide, DPNH
    disodium salt, DPNH-Na_2; commercial preparation, see p. 1011.
15. Adenosine-5′-triphosphate, ATP
    crystalline disodium salt, ATP-$Na_2H_2 \cdot 3H_2O$; commercial preparation, see p. 1006.

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*) Complete reagent kits are available commercially, see p. 1035.
**) from E. Merck, Darmstadt (Germany)

1) *Th. Bücher*, Biochim. biophysica Acta *1*, 292 [1947].

16. Glyceraldehyde-3-phosphate dehydrogenase, GAPDH
crystalline, from skeletal muscle, suspension in 2.5 M ammonium sulphate solution (pH 7.5); 10 mg. protein/ml. Commercial preparation, see p. 979.
17. Phosphoglycerate kinase, PGK
crystalline, from yeast, suspension in 2.4 M ammonium sulphate solution (pH 7); 10 mg. protein/ml. Commercial preparation see p. 994.

Purity of the enzyme preparations

The specific activity of GAPDH should be at least 3300 units*)/mg. and PGK at least 6000 units*)/mg. Both preparations must be free of myokinase (*i.e.* $<0.01\,\%$ myokinase relative to the activities of GAPDH and PGK). The ATPase and lactic dehydrogenase content of the preparations must be less than $0.01\,\%$.

Preparation of Solutions

I. Triethanolamine buffer (5×10^{-2} M; pH 7.55):
Dissolve 7.46 g. triethanolamine in *ca.* 700 ml. doubly distilled water, adjust to pH 7.55 with *ca.* 15 ml. 2 N HCl (glass electrode), dilute with double distilled water to 1000 ml.

II. Magnesium sulphate (0.5 M):
Dissolve 12.3 g. $MgSO_4\cdot7H_2O$ in doubly distilled water and make up to 100 ml.

III. Ethylene-diamine-tetra-acetate (100 mg./ml.):
Dissolve 10 g. EDTA-$Na_2H_2\cdot2H_2O$ in doubly distilled water, neutralize with 2 N NaOH and dilute to 100 ml. with doubly distilled water.

IV. Hydrazine (0.1 M):
Dissolve 1.30 g. hydrazine sulphate in doubly distilled water, neutralize with 2 N NaOH, dilute to 100 ml. with doubly distilled water. Prepare freshly each day!

V. D-3-Phosphoglyceric acid (*ca.* 5×10^{-2} M):
Dissolve 200 mg. barium-3-phosphoglycerate$\cdot2H_2O$ in *ca.* 2 ml. 2 N HCl, add 2 ml. 2 N H_2SO_4 to remove Ba^{2+}, mix well, centrifuge for 10 min. at *ca.* 3000 r.p.m., wash $BaSO_4$ precipitate with 1 ml. doubly distilled water, adjust pH of combined supernatants to 6.5 with *ca.* 4 ml. 2 N NaOH and dilute to 10 ml. with doubly distilled water. Determine the 3-PGA content of the solution enzymatically (p. 224).

VI. Reduced diphosphopyridine nucleotide (*ca.* 10^{-2} M β-DPNH):
Dissolve 20 mg. DPNH-Na_2 in 2 ml. doubly distilled water. Determine the DPNH concentration of the solution enzymatically (p. 531).

VII. Adenosine triphosphate (*ca.* 10^{-2} M ATP):
Dissolve 10 mg. ATP-$Na_2H_2\cdot3H_2O$ in 2 ml. doubly distilled water.

VIII. Glyceraldehyde-3-phosphate dehydrogenase**), GAPDH (10 mg. protein/ml.):
Centrifuge 0.1 ml. crystalline suspension (10 mg. protein/ml.), remove the supernatant

*) According to *Bücher* et al.[2] 1 unit is the amount of enzyme dissolved in 1 ml. which decreases the optical density of DPNH by 0.100 in 100 sec. at 366 mμ and 25°C with a 1 cm. light path.

**) To avoid inhibition of reaction (1) by ammonium sulphate[2], GAPDH and PGK are used as solutions relatively low in ammonium sulphate. The magnesium concentration of the test mixture gives optimal PGK activity; the inhibition of GAPDH by higher magnesium concentrations has not been observed in this range[3].

[2] *G. Beisenherz, H. J. Boltze, Th. Bücher, R. Czok, K. H. Garbade, E. Meyer-Arendt* and *G. Pfleiderer*, Z. Naturforsch. *8b*, 555 [1953].

[3] *H. Adam*, unpublished.

with a capillary pipette and discard. Dissolve sediment in 0.1 ml. of ice-cold buffer (pH 7.5) having the following composition: 2.23 g. $Na_4P_2O_7 \cdot 10H_2O$, 1.655 ml. 2 N HCl in 100 ml. doubly distilled water; prepare freshly each day 1 ml. of buffer + 3.07 mg. glutathione.

IX. Phosphoglycerate kinase, PGK (2 mg. protein/ml.):
Centrifuge 0.1 ml. crystalline suspension (10 mg. protein/ml.), remove the supernatant with a capillary pipette and discard. Dissolve the sediment in 0.5 ml. ice-cold doubly distilled water.

X. Perchloric acid
a) 0.9 N: dilute 7.7 ml. perchloric acid to 100 ml. with doubly distilled water.
b) 0.2 N: dilute 1.7 ml. perchloric acid to 100 ml. with doubly distilled water.

Stability of the solutions

The PGK solution (IX) is stable only for a few hours without a large loss of activity. Very dilute aqueous solutions containing *ca.* 10 μg. enzyme/ml. lose about 25% of their activity after 3 hours. In the test mixture PGK is virtually saturated with its substrate, 3-PGA, and is therefore stabilized. The GAPDH solution (VIII) should be prepared freshly each day. Frozen aqueous solutions of DPNH are stable for *ca.* 14 days, and after thawing can be used for one day. All other solutions are stable for several months when stored in a refrigerator.

Procedure

Deproteinization

Preliminary remarks: The enzymatic activity of the biological material to be examined should be stopped as soon as possible. Changes in the ATP content, occurring within a fraction of a second, are to be expected on alteration of the physiological state. Drop whole blood directly from the cannula into stirred acid; freeze organs *in situ* with metal blocks, which have been cooled to a low temperature, and are of a corresponding form and size to the organ (refer to p. 47). Free from slowly frozen edges and store below −30°C until extraction.

The disintegration, extraction and deproteinization of the frozen tissue should be carried out as quickly as possible to minimize enzymic reactions. Separate cell suspensions rapidly in the cold. To homogenize frozen tissue with acid, either first grind tissue in a deep frozen mortar and/or slowly add it to acid already being stirred in an open homogenizer, so that the tissue will be rapidly disintegrated and then inactivated by the cold acid.

In studies on adenine nucleotides in tissues which contain myosin or other highly active ATP-ases, the following method used for *Physarum polycephalum* has proved of value[4]:

Method: Stop the metabolic activity of the organism with deep frozen metal blocks. Pulverize the frozen material in a well cooled mortar. In a second mortar, deep freeze more than twice the tissue weight of 0.9 N perchloric acid (solution Xa) and grind up. While still in the frozen state weigh tissue powder (1 pt. by wt.) and acid (2 pts. by wt.), mix and grind up in a deep frozen mortar. Allow the powder to thaw by warming from −18 to 0°C over a period of two hours in an ice-acetone bath *).

*) This largely prevents warming of the tissue particles before diffusion of the acid has destroyed enzymatic activity in all parts of the tissue. The heat is transferred by the acid, which because of its higher osmotic activity thaws more quickly than the tissue particles. Thawing of the tissue and protein precipitation therefore occur simultaneously.

4) *H. Adam*, Biochem. Z. *335*, 25 [1961].

Then homogenize for at +2°C and centrifuge for 10 min. at 3000 g. Decant the supernatant. Extract the sediment with 0.2 N perchloric acid (solution Xb) (use a third of the volume used for the first extraction). While stirring vigorously, carefully neutralize the combined supernatants to pH 6.0–6.5 with 2 N KOH or 3.75 M K_2CO_3 (preferred for viscous extracts). The solution must not be over-neutralized, even for a short period. Allow to stand for 1 hour in an ice bath and remove precipitated $KClO_4$ by centrifuging.

Spectrophotometric measurements

Wavelength: 340 or 366 mμ; light path: 1 cm.; final volume: 2.00 ml.; room temperature.

Prepare the following reaction mixture immediately before use:

0.012 ml. magnesium sulphate solution (II)
0.040 ml. EDTA solution (III)
0.024 ml. hydrazine solution (IV)
0.040 ml. 3-PGA solution (V)
0.020 ml. DPNH solution (VI)

or a multiple of the individual volumes.

Pipette successively into the cuvette:

deproteinized sample (extract)
and buffer (I) to 2.00 ml.,
0.136 ml. reaction mixture.

Mix in

0.012 ml. GAPDH solution (VIII)

using a small glass or plastic rod flattened and bent at one end. Read optical density E_1.

Mix in

0.020 ml. PGK solution (IX),

measure the decrease in optical density until the reaction stops (E_2), usually after 5–8 min. To check that the system is functioning correctly, mix in

0.020 ml. ATP solution (VII).

A renewed reaction should occur immediately.

$E_1 - E_2 = \Delta E$ is used for the calculation.

Calculations

Extinction coefficients for DPNH (25°C)

$$\varepsilon_{340} = 6.29 \text{ [cm.}^2/\mu\text{mole]}$$
$$\varepsilon_{366} = 3.30 \text{ [cm.}^2/\mu\text{mole].}$$

$$\frac{\Delta E \times V_A \times V_E}{\varepsilon \times d \times V_P} = \mu\text{moles ATP in total extract.}$$

where

$\Delta E = E_1 - E_2$
V_A = Volume of the test mixture in the cuvette (2.0 ml.)
V_E = Total volume of extract [ml.]
V_P = Volume of extract added to cuvette [ml.]
ε = Extinction coefficient [cm.^{2}/μmole]
d = Light path [1 cm.]

for measurements at 366 mμ

$$\frac{\Delta E \times 2 \times V_E}{3.30 \times 1 \times V_P} = \Delta E \times 0.606 \frac{V_E}{V_P} = \mu\text{moles ATP in total extract}$$

If this value is divided by the fresh weight of tissue taken, then the μmoles ATP/g. tissue is obtained. The results are reproducible to ±1.5% and agree with UV absorption, phosphate and ribose determinations.

As little as 10^{-8} moles ATP can be determined with this accuracy. Microcuvettes allow the determination of 10^{-9} moles ATP.

Specificity

ITP, GTP and UTP react quantitatively in the same system. CTP gives no measurable reaction. There is little difference in the reaction rates with ITP, GTP, UTP and ATP. The time course of the reaction does not differentiate individual nucleotides in samples containing more than one nucleotide. The final change is equivalent to the sum of the nucleotides added[3].

Determination with Hexokinase and Glucose-6-phosphate Dehydrogenase

Walther Lamprecht and Ivar Trautschold

Because of its simplicity compared with paper or column chromatographic methods, especially over a series of analyses, the enzymatic determination of adenosine triphosphate (ATP) by the spectrophotometric method involving pyridine nucleotides has established itself. The question of the specificity of the enzymes used must be examined for work on special problems. In recent years, in addition to the method of *Bücher* et al. (see p. 539), the use of hexokinase and glucose-6-phosphate dehydrogenase (discovered by *O. Warburg* et al.[1-3]) has proved of value in the enzymatic determination of ATP[4].

Principle

Hexokinase phosphorylates glucose with ATP in the presence of Mg^{2+} to give glucose-6-phosphate (G-6-P)[5-7], equation (1). Glucose-6-phosphate dehydrogenase (G6P-DH, zwischenferment) catalyses the oxidation of G-6-P with triphosphopyridine nucleotide (TPN), equation (2)*).

(1) Glucose** + ATP ⟶ glucose-6-phosphate** + ADP

(2) Glucose-6-phosphate + TPN^+ ⇌ 6-phosphoglucono-δ-lactone + TPNH + H^+

Each mole of ATP forms 1 mole of TPNH.

*) The lactone is hydrolysed to the free carboxylic acid, if the hexokinase or glucose-6-phosphate dehydrogenase contains glucono-δ-lactonase[8,9].

**) Pyranose form = α-D-(+)-glucopyranose or α-D-(+)-glycopyranose-6-phosphate ("Robison-ester") (cf. *C. S. Hudson*, Advances in Carbohydrate Chemistry *3*, 1 [1948]).

1) *O. Warburg* and *W. Christian*, Biochem. Z. *242*, 206 [1931]; *287*, 440 [1936]; *287*, 291 [1936].
2) *O. Warburg, W. Christian* and *A. Giese*, Biochem. Z. *282*, 157 [1935].
3) *A. Kornberg*, J. biol. Chemistry *182*, 805 [1950].
4) *W. Lamprecht* and *I. Trautschold*, Hoppe-Seylers Z. physiol. Chem. *311*, 245 [1958]; *I. Trautschold*, Diplom-Arbeit, Techn. Hochschule Munich [1956]; *W. Lamprecht*, Habilitationsschrift, Techn. Hochschule Munich [1957]; *I. Trautschold*, Ph. D.-Thesis, Techn. Hochschule Munich [1958]; *W. Lamprecht* and *Th. Hockerts*, Die Medizinische *8*, 289 [1957]; *W. Lamprecht* and *Th. Hockerts:* Struktur u. Stoffwechsel des Herzmuskels. G. Thieme Verlag, Stuttgart 1959.
5) *O. Meyerhof*, Biochem. Z. *183*, 176 [1927].
6) *O. Meyerhof* and *H. Green*, J. biol. Chemistry *178*, 655 [1949].
7) *R. Robison:* The Significance of Phosphoric Esters in Metabolism. University Press, New York 1932.
8) *C. F. Cori* and *F. Lipmann*, J. biol. Chemistry *194*, 417 [1952]; *A. F. Brodie* and *F. Lipmann*, ibid. *212*, 677 [1955].
9) *F. Eisenberg* and *J. B. Field*, J. biol. Chemistry *222*, 293 [1956].

With equivalent glucose and Mg^{2+} concentrations ATP is virtually quantitatively converted to ADP by hexokinase[10]. When the enzyme is saturated with substrate 13000 moles glucose/10^5 g. enzyme are esterified per minute (30°C; pH 7.5)[11]. The values given for the Michaelis constant vary: for glucose values of 1.5×10^{-4} M[12], 1×10^{-3} M[13] and 5×10^{-4} M[14,15] have been found, for ATP (glucose) 9.5×10^{-5} M[12] and 1.2×10^{-3} M[16], for Mg^{2+} 2.6×10^{-3} M[16,*].

More recent measurements give the K_M for glucose as 0.31×10^{-7} M, for ATP 0.33×10^{-6} M[17]. The pH optimum of hexokinase (yeast) is 8–9[15,16]; for dilute enzyme solutions in tris-hydroxymethyl-aminomethane buffer (0.05 M) the optimum has been found to be pH 8.4[17].

The equilibrium of the glucose-6-phosphate dehydrogenase reaction lies in favour of the gluconolactone. With sufficient substrate, 1 mole of enzyme converts 12000 moles substrate/min. at 25°C and pH 8**). The values for the Michaelis constants*) for G-6-P are 1.76×10^{-4} M[20] or 0.69×10^{-4} M[21], for TPN^+ 2.6×10^{-5} M[20] or 3.3×10^{-5} M[21]; the pH optimum is 8.5[19,20].

Reagents

1. Triethanolamine
   doubly distilled or as the hydrochloride, A. R.
2. Triphosphopyridine nucleotide, TPN
   sodium salt, $TPN\text{-}NaH_2$; commercial preparation, see p. 1029.
3. Magnesium chloride, $MgCl_2\cdot6H_2O$, A. R.
4. Glucose, A. R.
5. Glucose-6-phosphate dehydrogenase (G6P-DH, zwischenferment)
   from yeast, suspension in 3.3 M ammonium sulphate solution, pH *ca.* 6, or lyophilized preparation. Commercial preparation, see p. 975.
6. Hexokinase, HK
   from yeast, lyophilized preparation. Commercial preparation, see p. 983.
7. Perchloric acid, A. R.
   sp. gr. 1.67; *ca.* 70% w/w or sp. gr. 1.54; *ca.* 60% w/w.
8. Potassium carbonate, K_2CO_3, A. R.
9. Methyl orange indicator

Purity of the enzyme preparations

Hexokinase is commercially available in several grades of purity, but can be prepared relatively easily from baker's yeast by well tried methods[11,15,22,23]. However, on crystallization the yields are low.

*) In the hexokinase reaction (two substrate reaction) the values for K_M depend on the concentration of both substrates[20], therefore the different measurements cannot be compared directly.

**) The exergonic hydrolysis of the lactone gives virtually a quantitative yield of gluconate[18].

10) *J. L. Gamble* jr. and *V. A. Najjar*, Science [Washington] *120*, 1023 [1954]; J. biol. Chemistry *217*, 595 [1955].
11) *L. Berger, M. W. Slein, S. P. Colowick* and *C. F. Cori*, J. gen. Physiol. *29*, 379 [1946].
12) *M. W. Slein, G. T. Cori* and *C. F. Cori*, J. biol. Chemistry *186*, 763 [1950].
13) *M. Dixon* and *D. M. Needham*, Nature [London] *158*, 432 [1946].
14) *J. Wajzer*, C. R. hebd. Séances Acad. Sci. *236*, 2116 [1953].
15) *M. Kunitz* and *M. R. McDonald*, J. gen. Physiol. *29*, 393 [1946].
16) *D. M. Greenberg:* Chemical Pathways of Metabolism. Academic Press, New York 1954, p. 74.
17) *W. Lamprecht* and *J. Sellmair*, unpublished results; *J. Sellmair*, Zulassungsarbeit z. wissenschaftl. Prüfung f. d. Lehramt, Universität Munich 1957.
18) *B. L. Horecker* and *P. Z. Smyrniotis*, Biochim. biophysica Acta *12*, 98 [1953].
19) *E. Negelein* and *W. Gerischer*, Biochem. Z. *284*, 289 [1936].
20) *W. Lamprecht* and *G. Michal*, unpublished results; *G. Michal*, Diplom-Arbeit, Techn. Hochschule Munich 1957.
21) *L. Glaser* and *D. Brown*, J. biol. Chemistry *216*, 67 [1955].
22) *K. Bailey* and *E. C. Webb*, Biochem. J. *42*, 60 [1948].
23) *M. R. McDonald:* Methods in Enzymology. Academic Press, New York 1955, Vol. I, p. 269.

Racker mentions a better preparation (still unpublished, see *Colowick* et al. [24]). The enzyme preparation should have a specific activity of at least 400 units/mg. according to *Bücher* or *ca.* 33 units/mg. according to *Bailey* et al. [22, *]. It must contain less than 0.01% of myokinase, ATP-ase, glucose-6-phosphatase, 6-phosphogluconic dehydrogenase (also refer to p. 117), TPNH oxidase, phosphohexoisomerase, creatine kinase and glutathione reductase.

Glucose-6-phosphate dehydrogenase: Highly purified enzyme suspensions are commercially available. For descriptions of the purification from yeast, see [3, 21, 24, 26]. The yields of the purified enzyme vary and are relatively low. Preparations obtained according to the methods of *Kornberg* [3] and *Horecker* [26] contain *ca.* 10% TPNH oxidase and glutathione reductase. *Frunder* et al. [27] have described a method for the removal of glutathione reductase.
The glucose-6-phosphate dehydrogenase preparation should have a specific activity of at least 4000 units/mg. according to *Bücher* [25], corresponding to *ca.* 70 units/mg. according to *Racker* [24]. The limits of the contaminating activities given for hexokinase should not be exceeded and the amount of hexokinase in the glucose-6-phosphate dehydrogenase must not be more than 0.2%.

Preparation of Solutions

Prepare all solutions with fresh, doubly distilled water.

I. Triethanolamine buffer (0.05 M; pH 7.5–7.6):
Dissolve 4.65 g. triethanolamine hydrochloride in *ca.* 200 ml. distilled water, add 11 ml. 1 N NaOH and after cooling dilute to 500 ml. with distilled water.

II. Triphosphopyridine nucleotide (*ca.* 7×10^{-3} M β-TPN):
Dissolve 7.5 mg. TPN-NaH_2 in distilled water and make up to 1.5 ml.

III. Magnesium chloride (0.1 M):
Dissolve 2.03 g. $MgCl_2 \cdot 6H_2O$ in distilled water and make up to 100 ml.

IV. Glucose (0.5 M):
Dissolve 9.91 g. glucose ($C_6H_{12}O_6 \cdot H_2O$) in distilled water and make up to 100 ml.

V. Glucose-6-phosphate dehydrogenase, G6P-DH
a) According to the specific activity, dissolve 10–15 mg. lyophilized enzyme in 1.0 ml. distilled water; or
b) (200 μg. protein/ml.): dilute 0.3 ml. enzyme suspension (1 mg./protein/ml. in 3.3 M ammonium sulphate solution) to 1.5 ml. with distilled water.

VI. Hexokinase, HK (*ca.* 10–15 mg. protein/ml.):
Depending on the specific activity, dissolve 20–30 mg. lyophilized enzyme in 2.0 ml. distilled water.

VII. Perchloric acid (6% w/v):
dilute 5.2 ml. $HClO_4$, sp. gr. 1.67, to 150 ml. with distilled water; or
dilute 6.6 ml. $HClO_4$, sp. gr. 1.54, to 150 ml. with distilled water.

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*) Conversion factors [4]: 1 International Unit corresponds to 55.5 units acc. to Bücher; approx. 22.4 units acc. to Berger et al. and Kunitz-McDonald; 4.6 units acc. to Bailey-Webb.

[24] *P. Srere, J. R. Cooper, M. Tabachnik* and *E. Racker*, Arch. Biochem. Biophysics *74*, 295 [1959]; *R. A. Darrow* and *S. P. Colowick*, unpublished results.

[25] *Th. Bücher, G. Beisenherz, H. J. Boltze, R. Czok, K. H. Garbade, E. Meyer-Arendt* and *G. Pfleiderer*, Z. Naturforsch. *8b*, 555 [1953].

[26] *B. L. Horecker* and *P. Z. Smyrniotis:* Methods in Enzymology. Academic Press, New York 1955, Vol. I, p. 323.

[27] *N. Sönnichsen, H. Frunder, H. Börnig* and *G. Richter*, Hoppe-Seylers Z. physiol. Chem. *316*, 209 [1959].

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

IX. Methyl orange indicator :
Dissolve *ca.* 50 mg. in 100 ml. distilled water.

Stability of the solutions

Store all solutions, stoppered, in a refrigerator at 1—4°C. Prepare TPN and glucose solutions freshly each week, enzyme solutions from dry powder daily, and enzyme suspensions diluted with water every 2—3 days.

Procedure

Experimental material

Collection of blood and separation of the components are described in the chapter "Determination of Pyruvate", p. 253. Plasma normally contains no ATP and the ATP of blood is located in the erythrocytes[4,29]. Hexose monophosphate rarely occurs in whole blood (rat)[4]. For the determination of ATP in tissues it is essential that the samples are frozen within a fraction of a second. *Wollenberger*[28] and *Bücher*[29] have successfully used "jaws" made of aluminium or light metal blocks, which were cooled with liquid air. It is well known that the metabolite concentration in a tissue depends on the speed with which the organ is removed and on the manipulations during the killing of the animal (ether narcosis, decapitation, "quick-freezing", *etc.*).

Deproteinization

The sample is deproteinized with perchloric acid solution. The ratio of total fluid volume to original weight of organ should be 4 : 1. The amount of perchloric acid is calculated according to the water content of the sample (see p. 254).

Method: Whole blood is deproteinized according to the method of *Bücher* (see p. 255). Variations in the ratio of perchloric acid to blood from 4 : 1 results in incorrect values for the ATP content. For example: Whole blood was deproteinized with perchloric acid in the ratio 1 : 1 (v/v) and centrifuged for 10 min. at 3000 r.p.m. at 2°C yielding supernatant I. The large, gelatinous, residual precipitate retained varying amounts of ATP; washing this residue three times with 4 ml. portions 6% perchloric acid (by homogenization and centrifugation) yielded supernatants II—IV:

Amount of ATP in supernatant I	8.35 mg.%
supernatant II	6.75 mg.%
supernatant III	2.50 mg.%
supernatant IV	0.79 mg.%
Total	18.39 mg.%

In comparison the ATP concentration in supernatant I, calculated for whole blood, would be 16.7 mg.%

yeast cells: These are only partially lysed on deproteinization with perchloric acid or trichloroacetic acid[30]. The following deproteinization and disintegration method has proved

[28] *A. Wollenberger*, Naturwissenschaften *45*, 294 [1958].
[29] *H. J. Hohorst*, *F. H. Kreutz* and *Th. Bücher*, Biochem. Z. *332*, 18 [1959].
[30] *W. Lamprecht* and *D. Lommer*, unpublished results; *D. Lommer*, Diplom-Arbeit, Techn. Hochschule Munich 1960.

successful[30]: Disintegrate cells according to *Merkenschlager* et al.[31, *)] with a "mechanical cell homogenizer" in 30 ml. Duran tubes*) with glass beads (size 31/8)*).

Pipette 20 ml. of a 5.4% yeast suspension (pressed, fresh yeast) into a mixture of 2.0 ml. 60% perchloric acid and 20 ml. glass beads, which has been previously cooled in the homogenizer tubes with ice water. Immediately shake vigorously and homogenize for 30 seconds at a frequency of 4000/min. without further cooling (temperature of the homogenate after 30 sec. is *ca.* 12°C) and then centrifuge in the cold at *ca.* 4000 g.

Organs or tissue samples are quickly pressed between the "jaws" of the "quick-freeze" tongs, which have been cooled with liquid air. Cut or break off any portions of the sample projecting over the edges of the blocks and once again immerse in liquid air. Quickly weigh the sample**) (g. tissue = V_1) and powder in a porcelain mortar with repeated additions of liquid air (*ca.* 10 ml. portions). Take care that the piece of tissue does not thaw at any time during the manipulations. Slowly add the calculated amount of perchloric acid (6.5 ml. $HClO_4$ to 2 g. tissue; V_1 + g. $HClO_4$ = V_2) and grind with the tissue to form a powder.

Homogenization: either a) allow tissue powder in mortar to warm up until temperature reaches *ca.* 2–4°C, and then homogenize 30 seconds***), or b) after evaporation of the liquid air quickly transfer the still dry powder into a glass homogenizer, removing the last traces with a small rubber policeman or plastic spatula then when the mixture just becomes fluid, homogenize for 30 seconds†), while cooling in ice.

Take portions (2 to 4 ml.) (= V_3) of the centrifuged perchloric acid extract for duplicate determinations. Either titrate with 5 M K_2CO_3 solution (VIII) using a 0.2 ml. capillary pipette[29] and methyl orange as indicator, while stirring magnetically or bubbling nitrogen through, or adjust to pH 7.4 by means of the more sensitive end-point titration using a "automatic Titrigraph"+) (V_3 + ml. K_2CO_3 required = V_4). A total of about 0.12–0.15 ml. carbonate solution is required. Allow the neutral solution to stand for 10 min. in ice water, decant from the sediment of $KClO_4$ and immediately analyse 0.1 ml. or 0.2 ml. (= V_5)++); the ATP is hydrolysed on standing.

Spectrophotometric measurements

Preliminary remarks: The volume of sample (V_5) is so arranged that the maximum optical density change is 0.150 and the assay is complete in 15 min. Read against a control cuvette containing 4.0 ml. buffer (solution I).

Method:

Wavelength: 366 mμ; light path: 2 cm.; final volume for determination of ATP: 5.00 ml., for hexose monophosphate or glucose-6-phosphate: 4.53 ml.

Pipette the solutions into the cuvettes in the following order.

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*) B. Braun, Melsungen (Germany).

**) Do not place the tissue sample on metal weighing pans; the use of small plastic dishes, glazed paper or strips of film as supports avoids the sample freezing to the pan. Weighing in a cold room is recommended.

***) Ultra-Turrax, Type 18/2, Janke & Kunkel & Co., Stauffen i. B. (Germany).

†) Homogenizer for scientific purposes from Bühler & Co., Tübingen (Germany).

+) Radiometer & Co., Copenhagen (Denmark).

++) Store at 0°C; freezing or lyophilization usually leads to a lowering of the ATP values.

31) *M. Merkenschlager, K. Schlossmann* and *W. Kurz*, Biochem. Z. *329*, 332 [1957].

Take readings of the initial optical density E_1 for 1—3 min. until constant. Stir into the experimental cuvettes the stated amounts of the enzymes with a small plastic spoon. After the first enzyme addition (0.02 ml. glucose-6-phosphate dehydrogenase soln.) follow the optical density for 5 min. (final value = E_2) and then add a further 0.02 ml. G6P-DH solution to the cuvettes. After 1 min. read E_3.

Solutions *)	Experimental cuvettes (I)	(II)	Optical density (E) E after	change *)	ΔE
buffer (soln. I)	4.00 ml.	3.90 ml.			
TPN soln. (II)	0.06 ml.	0.06 ml.			
$MgCl_2$ soln. (III)	0.35 ml.	0.35 ml.			
deproteinized soln.	0.10 ml.	0.20 ml.	3 min; E_1		
G6P-DH soln. (V)	0.02 ml.	0.02 ml.	5 min; E_2	E_{HMP}	ΔE_{HMP}
G6P-DH soln. (V)	0.02 ml.	0.02 ml.	1 min; E_3	$E_{G6P\text{-}DH}$	
glucose soln. (IV)	0.40 ml.	0.40 ml.	0.5 min; E_4	$E_{gluc.}$	
HK soln. (VI)	0.05 ml.	0.05 ml.	15 min; E_5	E_{ATP}	ΔE_{ATP}
HK soln. (VI)	0.05 ml.	0.05 ml.	1 min; E_6	E_{HK}	

Subtract the optical density change E_3-E_2 (*i. e.* $E_{G6P\text{-}DH}$ caused by the addition of G6P-DH) from the difference $E_2-E_1 = E_{HMP}$; $(E_2-E_1)-(E_3-E_2) = E_{HMP}-E_{G6P\text{-}DH} = \Delta E_{HMP}$ is the optical density change corresponding to the hexose monophosphate or glucose-6-phosphate content.

On addition of the glucose solution read the small optical density decrease due to dilution of experimental cuvette content after only 30 seconds: E_4 **). Start the ATP reaction by addition of 0.05 ml. hexokinase solution. It is usually complete in *ca.* 12 min. Follow the optical density change for 15 min. (final value = E_5). A further 0.05 ml. hexokinase solution gives the absorption due to this enzyme; read 1 min. after addition: E_6 †).

Subtract $E_6-E_5 = E_{HK}$ from the difference $E_5-E_4 = E_{ATP}$: $(E_5-E_4)-(E_6-E_5) = E_{ATP}-E_{HK} = \Delta E_{ATP}$.

Δ_{HMP} and ΔE_{ATP} are used for the calculations.

Calculations

Under the stated conditions the reactions are stoichiometric, and within experimental error, double the amount of deproteinized sample gives double the value for ΔE_{HMP} and ΔE_{ATP}. The amount of HMP and ATP is calculated as follows:

$$(3) \qquad \frac{\Delta E_{ATP} \times V_K \times V_2 \times V_4}{\varepsilon \times d \times V_1 \times V_3 \times V_5} = \mu\text{moles ATP/g. or ml. tissue}$$

*) Abbreviations: HMP = hexose monophosphate, G6P-DH = glucose-6-phosphate dehydrogenase, HK = hexokinase.

**) In spite of a hexokinase contamination in the glucose-6-phosphate dehydrogenase preparation of only $< 0.2\%$, the addition of glucose causes a small but significant ATP reaction (ΔE *ca.* 0.003 per min.). The optical density change is to be observed after 3—5 min.; therefore the hexokinase should be added soon after the glucose, certainly not longer than 1 min. This is also the reason why glucose is only added after the glucose-6-phosphate dehydrogenase.

†) A decrease in optical density can often be observed after 20—25 min. (TPNH oxidase) but does not interfere with the determination of ΔE_{ATP}.

$$(4) \qquad \frac{\Delta E_{HMP} \times V_K \times V_2 \times V_4}{\varepsilon \times d \times V_1 \times V_3 \times V_5} = \mu\text{moles hexose monophosphate/g. or ml. tissue}$$

where V_K = final volume in the cuvette after the last enzyme addition.
V_1 = weight [g.] or volume [ml.] of the tissue.
V_2 = V_1 + g. [ml.] perchloric acid required for deproteinization.
V_3 = volume of the perchloric acid extract before neutralization.
V_4 = V_3 + ml. K_2CO_3 required.
V_5 = volume of deproteinized solution in the cuvette.
ε = extinction coefficient for TPNH; $\varepsilon_{366} = 3.3$ cm.^{2}/μmole.
d = light path in cm.

If the amount of ATP is to be calculated per g. tissue, then both V_1 and V_2 are expressed in g. For 6% perchloric acid: 1 ml. = 1.035 g. Therefore V_2 [g.] = V_1 [g.] + (ml. perchloric acid) × 1.035. If the amount of ATP is to be calculated per ml. tissue, then V_2 and V_1 are expressed in ml. The weight of tissue divided by the density of the tissue is V_1 [ml.]. This value is then used to calculate V_2 [ml.].
If the amount is to be given in μg. instead of μmoles, then the result must be multiplied either by the molecular weight of ATP (507.2) or by the molecular weight of G-6-P (260.2).

Corrections

Corrections for blood: To obtain the ATP content of the cells of a tissue, the amount of ATP present in the occluded blood of the tissue must be subtracted from the total ATP content. For this correction the following equation is valid[29]:

(5) ATP content of the cells = [(total ATP content of the tissue) − (the fraction by weight of blood in the tissue × ATP content of the blood)] : [1 − fraction by weight of blood in the tissue]

This same formula is obviously valid for other metabolites.

The fraction by weight of blood in the tissue is determined according to *Bücher* et al.[29] from optical density measurements at 578, 560 and 540 mμ. Assuming that the proportion of oxyhaemoglobin (HbO_2) in the circulating blood and the tissue is virtually the same, it follows that the fraction of blood x in the tissue is

$$(6) \qquad x = \frac{\Delta E_{HbO_2} \times \text{dilution} \times d_1}{\Delta E'_{HbO_2} \times \text{dilution} \times d_2} \times 100\ [\%\ \text{wt.}]$$

where ΔE_{HbO_2} = optical density difference of the tissue extract.
$\Delta E'_{HbO_2}$ = optical density difference of the blood dilution
d_1 and d_2 = light path of the cuvettes

$\Delta E'_{HbO_2}$ and $\Delta E'_{HbO_2}$ are calculated[29] without the use of graphical methods, from the measurements of optical density at 578, 560 and 540 mμ according to the formula

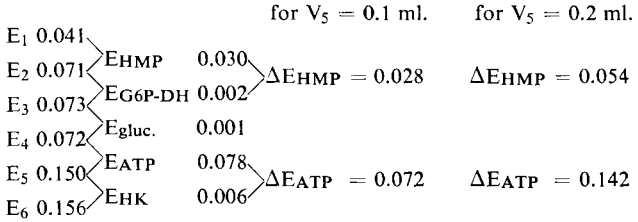
$$(7) \qquad \Delta E_{HbO_2} \text{ or } \Delta E'_{HbO_2} = (E_{578} - E_{560}) + [(E_{540} - E_{578}) \times 0.47]$$

Example

2.8035 g. liver from a normal fed rat were deproteinized with 9.10 ml. perchloric acid solution (VII). Each 4.0 ml. perchloric acid extract required 0.14 ml. K_2CO_3 solution (VIII) for neutralization.

V_1 = 2.8035 g.
V_2 = 2.8035 + (9.10 × 1.035) = 12.222 g.
V_3 = 4.0 ml.
V_4 = 4.14 ml.
V_5 = 0.1 and 0.2 ml.

Optical density values



By substitution in equations (3) and (4) the following values are obtained:

2.458 μmoles ATP/g. liver (V_5 = 0.1 ml.)
2.424 μmoles ATP/g. liver (V_5 = 0.2 ml.)
0.879 μmoles hexose monophosphate/g. liver (V_5 = 0.1 ml.)
0.848 μmoles hexose monophosphate/g. liver (V_5 = 0.2 ml.)

Determination of ATP in the blood of the same rat: Since 6.4 ml. of perchloric acid are required for each 2.0 g. of blood a total of 9.55 ml. was used for the 3.5485 g. of blood obtained. 4.0 ml. of the centrifuged perchloric extract required 0.14 ml. 5 M K_2CO_3-solution for neutralization. V_5 = 0.1 ml. and 0.2 ml.

The optical density differences obtained: for V_5 = 0.1 ml.: $\Delta E_{ATP} = 0.015$; $\Delta E_{HMP} = 0$; for V_5 = 0.2 ml.: $\Delta E_{ATP} = 0.030$; $\Delta E_{HMP} = 0$.

This is equivalent to 0.518 μmoles ATP/g. blood.

Amount of blood in the liver of the same animal:

Dilution of the blood sample 1 :125,

Dilution of the liver sample 1 :12.5.

All measurements in cuvettes with 1 cm. light path.

The optical densities obtained by measurements at 578, 560, and 540 mμ give according to equation (7)

for blood $\Delta E'_{HbO_2} = 0.237$
for liver $\Delta E'_{HbO_2} = 0.168$

The amount of blood in the liver according to equation (6) is

$$x = \frac{0.168 \times 12.5 \times 1}{0.237 \times 125 \times 1} \times 100 = 7.1\,\%$$

Corrections for blood: Substitution in equation (5) gives the following values:

1. ATP $\quad \dfrac{2.458 - (0.071 \times 0.518)}{1 - 0.071} = 2.60$ μmoles ATP/g. liver

2. ATP $\quad \dfrac{2.424 - (0.071 \times 0.518)}{1 - 0.071} = 2.58$ μmoles ATP/g. liver

1. Hexose monophosphate $\quad \dfrac{0.879}{1 - 0.071} = 0.93$ μmoles hexose monophosphate/g. liver

2. Hexose monophosphate $\quad \dfrac{0.848}{1 - 0.071} = 0.91$ μmoles hexose monophosphate/g. liver

Further Determinations

The optical density difference ΔE_{HMP} corresponds to the amount of hexose monophosphate in the sample. Only with highly purified glucose-6-phosphate dehydrogenase can a specific reaction with glucose-6-phosphate be expected *). With most of the commercial preparations the sum of the hexose monophosphates is obtained.

*) In this case, the fructose-6-phosphate can be estimated as well, by addition of 0.01 ml. phosphohexoisomerase solution (25–50 μg. enzyme), without interfering with the ATP determination.

On completion of the ATP reaction, phosphocreatine can be determined specifically by the addition of creatine kinase and ADP[32], refer to p. 610.

Sources of Error

1. Almost without exception, interference can be traced to contamination of the hexokinase or glucose-6-phosphate dehydrogenase with other enzymes (especially with TPNH oxidase, glutathione reductase or too large an amount of hexokinase in the glucose-6-phosphate dehydrogenase).

2. In the presence of large amounts of PO_4^{3-} (*e.g.* deproteinized solutions from incubations carried out in Krebs-Ringer phosphate saline) a fine crystalline precipitate of magnesium ammonium phosphate often appears in the cuvette during the measurements resultings in an apparent increase in optical density.

3. Enzyme solutions diluted with water, if several days old, give incorrect results.

Specificity

Systematic studies of the specificity of hexokinase (yeast) towards nucleotide triphosphates are lacking Inosine triphosphate (ITP) reacts with yeast hexokinase, but at a slower rate than ATP[33]. Interference by ITP must be considered when using glucose-6-phosphate dehydrogenase/hexokinase for the enzymatic determination of ATP. *Bücker* and co-workers[29] have shown with chromatographic methods that the maximum error to be expected in the determination of ATP in liver extracts is about 20%. Comparison of ATP determinations by the phosphoglycerate kinase method (see p. 539) and the glucose-6-phosphate dehydrogenase/hexokinase method showed that the values were on average 10–15% higher with the latter method. The difference between the results obtained with the two enzymatic methods approaches the error expected from the studies of *Bücher*. Glucose-6-phosphate dehydrogenase is completely specific for glucose-6-phosphate, and TPN cannot be replaced by diphosphopyridine nucleotide (DPN).

Other Methods for the Determination of ATP

1. The enzymatic method of *Bücher* et al.[29] with phosphoglycerate kinase is described on p. 539.
2. The fluorimetric, enzymatic analysis with luciferase (*McElroy* et al.[34]) detects less than 1 μg. ATP/ml. sample and is extremely specific. It is described on p. 559.
3. Using potato apyrase, adenylic kinase and 5'-AMP deaminase, ATP, ADP and AMP can be determined in one operation according to the method of *Kalckar* et al.[35] by the spectrophotometric measurement of the optical density at 265 mμ.

Determination by Fluorimetry

Paul Greengard

Principle

Reduced diphosphopyridine nucleotide (DPNH) and reduced triphosphopyridine nucleotide (TPNH) fluoresce, while DPN and TPN do not. The fluorescence can be activated by the 365 mμ mercury line and has its maximum about 460 mμ. The fluorimetric determination of both coenzymes is about a 100 times more sensitive than the spectrophotometric. The two methods described here for the fluorimetric determination of adenosine triphosphate (ATP) are based on the following reactions:

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[32] *W. Lamprecht* and *P. Stein*, unpublished results.
[33] *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1957, Vol. III, p. 874.
[34] *B. L. Strehler* and *W. D. McElroy:* Methods in Enzymology. Academic Press, New York 1957, Vol. III, p. 871.
[35] *A. Munch-Petersen* and *H. M. Kalckar:* Methods in Enzymology. Academic Press, New York 1957, Vol. III, p. 869.

Method A:

(1) $$\text{ATP} + \text{glucose} \xrightarrow{\text{hexokinase}} \text{ADP} + \text{glucose-6-phosphate}$$

(2) $$\text{Glucose-6-phosphate} + \text{TPN}^+ \xrightarrow[\text{dehydrogenase}]{\text{glucose-6-phosphate}} \text{6-phosphogluconic acid} + \text{TPNH} + \text{H}^+$$

Method B:

(3) $$\text{ATP} + \text{3-phosphoglyceric acid} \xrightleftharpoons{\text{phosphoglycerate kinase}} \text{ADP} + \text{1,3-diphosphoglyceric acid}$$

(4) $$\text{1,3-Diphosphoglyceric acid} + \text{DPNH} + \text{H}^+ \xrightleftharpoons[\text{dehydrogenase}]{\text{glyceraldehyde-3-phosphate}} \text{glyceraldehyde-3-phosphate} + \text{DPN}^+ + \text{phosphate}$$

Glyceraldehyde-3-phosphate is "trapped" with cysteine.

These enzyme systems correspond to those used by *Kornberg*[1] and *Thorn* et al.[2] for spectrophotometric determination of ATP. As little as 2×10^{-4} μmoles ATP/ml. can be determined with the aid of the fluorimetric methods described here.

Fluorimeter

For most of our measurements we have used a fluorimeter built by ourselves[3]. The filter fluorimeter commercially available from the Farrand Optical Co., New York, has also been used and found satisfactory. Primary glass filter: Corning 7-37 (365 mμ); secondary glass filter: Corning 4-70 and 3-73 (460 mμ).

Experimental Material

The method was developed for the determination of ATP in peripheral nerve fibres[4], and it has not been tried on other tissues.

Plunge the nerve bundle into a centrifuge tube containing 1.5 ml. 0.1 M triethanolamine buffer (pH 8.0) which has been heated to 100° C in a boiling water bath. After 40 seconds, cool the tube rapidly to 0° C and homogenize the contents. Add 1.5 ml. alcohol-free chloroform to the homogenate, stopper the tube with a ground-glass stopper, shake vigorously for 3 minutes and centrifuge for 10 minutes at 3000 g. Use the clear supernatant for the analysis.

Method A

Reagents

1. Glucose
2. Magnesium chloride, $MgCl_2 \cdot 6H_2O$
3. Ethylene-diamine-tetra-acetic acid, EDTA disodium salt, EDTA-$Na_2H_2 \cdot 2H_2O$
4. Triethanolamine hydrochloride,
5. Sodium hydroxide, 0.1 N, A. R.
6. Sulphuric acid, 0.1 N, A. R.

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[1] *A. Kornberg*, J. biol. Chemistry *182*, 779 [1950].
[2] *W. Thorn, G. Pfleiderer, R. A. Frowein* and *I. Ross*, Pflügers Arch. ges. Physiol. Menschen Tiere *261*, 334 [1955].
[3] *P. Greengard*, Bulletin of the Photoelectric Spectrometry Group No. *11*, 292 [1958].
[4] *P. Greengard* and *R. W. Straub*, J. Physiology *148*, 353 [1959].

7. Quinine sulphate
8. Triphosphopyridine nucleotide, TPN
   sodium salt, TPN-NaH_2; commercial preparation, see p. 1029.
9. Adenosine triphosphate, ATP.
   crystalline sodium salt, ATP-$Na_2H_2 \cdot 3H_2O$; commercial preparation, see p. 1006.
10. Glucose-6-phosphate dehydrogenase/hexokinase, G6P-DH/HK
    from brewer's yeast[5,*]; commercial preparation, see p. 975 and 983.

Purity of the enzyme preparations

The G6P-DH preparation obtained from brewer's yeast had a specific activity of 2.1 units**/mg. and contained sufficient hexokinase to allow completion of the coupled test reactions in 20—30 minutes. Crystalline hexokinase (commercial preparation, see p. 983) must be added if the hexokinase content of the preparation is insufficient.

Preparation of Solutions

I. Glucose (1.0 M):
Dissolve 1.8 g. of anhydrous glucose in distilled water and make up to 10 ml.

II. Magnesium chloride (0.15 M):
Dissolve 305 mg. $MgCl_2 \cdot 6H_2O$ in distilled water and make up to 10 ml.

III. Ethylene-diamine-tetra-acetate (0.02 M; pH 7.4):
Dissolve 774 mg. EDTA-$Na_2H_2 \cdot 2H_2O$ in *ca.* 75 ml. distilled water, adjust pH to 7.4 with 0.1 N NaOH, and dilute with distilled water to 100 ml.

IV. Triethanolamine buffer (0.1 M; pH 8.0):
Dissolve 1.857 g. triethanolamine hydrochloride in a little distilled water, adjust pH to 8.0 with 63 ml. 0.1 N NaOH, and dilute with distilled water to 100 ml. Check pH with a glass electrode.

V. Triphosphopyridine nucleotide (*ca.* 5×10^{-5} M β-TPN):
Stock solution (*ca.* 2.5×10^{-3} M): Dissolve 20 mg. TPN-NaH_2 in distilled water and make up to 10 ml. Just before use dilute 1 : 50 with distilled water.

VI. Adenosine triphosphate (10^{-5} M ATP):
Stock solution (10^{-2} M): Dissolve 12 mg. ATP-$Na_2H_2 \cdot 3H_2O$ in distilled water and make up to 2 ml. Just before use dilute 1 : 1000 with distilled water.

VII. Glucose-6-phosphate dehydrogenase/hexokinase, G6P-DH/HK (0.7 mg. protein/ml.):
Pipette 1 ml. of the enzyme preparation (0.7 mg. protein/ml.) obtained according to[5] into ampoules, freeze, lyophilize, displace air with nitrogen, and seal ampoule. Just before use open an ampoule and dissolve the enzyme in 1 ml. ice-cold distilled water.

VIII. Quinine sulphate (3×10^{-8} M):
Dissolve 13.4 mg. quinine sulphate in 0.1 N H_2SO_4 and make up to 1000 ml. Make a 1 : 500 dilution in 0.1 N H_2SO_4 weekly.

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*) *Racker* et al.[6] have described a preparation of glucose-6-phosphate dehydrogenase which is purer than that of *Kornberg*[5] and is virtually free of TPNH oxidase. See section on "Sources of Error" (p. 554).

**) A unit is defined according to[5]. For conversion to other units, see p. 545.

5) *A. Kornberg*, J. biol. Chemistry *182*, 805 [1950].
6) *P. A. Srere, J. R. Cooper, M. Tabachnick* and *E. Racker*, Arch. Biochem. Biophysics *74*, 295 [1958].

Stability of the solutions

The TPN should be prepared freshly every fortnight, and the dilute solution (1 : 50) freshly every day. The lyophilized enzyme preparation is stable for at least two years at 4° C.

Procedure

Fluorimetric measurements

Wavelength: 460 mμ; excitation wavelength: 365 mμ; Pyrex test tubes (1 cm. diameter, 7.5 cm. long); final volume 1 ml.; room temperature. Set fluorimeter with quinine sulphate solution (VIII).

Prepare: experimental solution (contains the sample), standard solutions (contain known amounts of ATP) and reagent blank (ATP-free). Up to 8 experimental solutions can be analysed successively. In order to save time and to increase the accuracy, prepare the following reaction mixture freshly (sufficient for 8 samples, 3 standards and 1 reagent blank):

0.2 ml. glucose solution (I)
2.0 ml. TPN solution (V)
0.2 ml. $MgCl_2$ solution (II)
0.6 ml. EDTA solution (III).

Pipette successively into the test tubes:

0.15 ml. reaction mixture
sample or 0.1 to 0.3 ml. ATP standard solution (VI)
0.120 ml. buffer (solution IV); take less in the experimental tubes according to volume of the sample, since the deproteinization was carried out in this buffer
distilled water to 0.95 ml.

Measure the fluorescence and multiply the reading by the dilution factor 0.95. Mix into all the test tubes

0.05 ml. enzyme solution (VII),

follow the increase of fluorescence and read the maximum value.

Calculations

In contrast to the measurement of light absorption there exists no fluorescence coefficient analogous to the extinction coefficient[3]. Therefore a standard curve must be prepared with solutions containing known amounts of ATP. The reagent blank corrects for fluorescence due to the enzyme and the slight reaction without ATP.

The increase in intensity of fluorescence is proportional to the ATP concentration in the reaction mixture and the concentration is read off from a standard curve.

Sources of Error

Glucose-6-phosphate (G-6-P) in the experimental material is estimated as apparent ATP, resulting in high values. A correction for the amount of G-6-P present is necessary. The G-6-P is determined using the same reaction mixture, but omitting glucose and $MgCl_2$ solution. Under these conditions the G6P-DH activity is virtually unaltered, whereas the hexokinase is inactive, so that no ATP reacts.

Glucose dehydrogenase which reduces TPN about 10 times as rapidly as DPN may be present in G6P-DH preparations. Fortunately, the affinity of this enzyme for glucose is much smaller than the affinity between glucose and hexokinase. This side reaction is avoided by using low concentrations of glucose in the reaction mixture (0.01 M).

Alcohol dehydrogenase was also present in the G6P-DH preparation. This enzyme is activated by Mg^{2+} and catalyses the reduction of TPN by ethanol. The chloroform used in the extraction of experimental material should be washed with water, just before use, to remove alcohol, and so eliminate any interference due to alcohol dehydrogenase.

TPNH oxidase is the most troublesome contaminant of the G6P-DH preparations. It interferes to a greater extent with the fluorimetric determination of ATP than with the spectrophotometric method because of the smaller amount of substrate and the great affinity between enzyme and TPNH. Nevertheless, the quantitative determination of ATP according to the method described here is possible, because the maximum of the intensity of fluorescence is proportional to the ATP concentration. Pre-incubation of the enzyme preparation for one hour at 37° C greatly reduces the TPNH oxidase activity, with only a slight decrease in hexokinase activity. *Racker* et al.[6] have described a G6P-DH preparation which has a specific activity several times greater than the *Kornberg* preparation and is substantially free from TPNH oxidase. The use of this preparation will undoubtedly improve the determination of ATP described here.

Of the nucleotides so far tested only ATP reacts. With UTP, GTP or CTP no reaction occurs.

Further Determinations

Creatine phosphate can be determined by the addition of creatine phosphate-ADP transphosphorylase together with G6P-DH/HK to the usual reaction mixture. The sum of ATP and creatine phosphate and the value for ATP alone are determined in two separate estimations[4].

Method B

Reagents

1. Magnesium sulphate, $MgSO_4 \cdot 7H_2O$
2. Cysteine hydrochloride
3. Triethanolamine hydrochloride
4. Sodium hydroxide, 0.1 N and 1 N, A. R.
5. Ethylene-diamine-tetra-acetic acid, EDTA
   disodium salt, EDTA-$Na_2H_2 \cdot 2H_2O$.
6. D-3-Phosphoglyceric acid, 3-PG
   calcium or crystalline tricyclohexylammonium salt $\cdot 3H_2O$; commercial preparation, see p. 1025.
7. Reduced diphosphopyridine nucleotide, DPNH
   sodium salt, DPNH-Na_2; commercial preparation, see p. 1011.
8. Adenosine triphosphate, ATP
   sodium salt, ATP-$Na_2H_2 \cdot 3H_2O$; commercial preparation, see p. 1006.
9. Glyceraldehyde-3-phosphate dehydrogenase*) GAPDH
   crystalline, from skeletal muscle; commercial preparation**), see p. 979.
10. Phosphoglycerate kinase*), PGK
    crystalline, from yeast; commercial preparation**), see p. 994.

Purity of the enzyme preparations

The purity of the enzymes has not been specially examined. If 3-phosphoglyceric acid is omitted from the reaction mixture no reaction occurs when nerve extracts are used as experimental material. Contamination of the enzyme preparations with myokinase did not interfere with the method described here (see section on "Sources of Error").

*) Both enzymes were obtained through the generosity of Professor *M. Schneider* and Dr. *W. Thorn*.
**) Crystalline glyceraldehyde-3-phosphate dehydrogenase and crystalline phosphoglycerate kinase obtained from C. F. Boehringer & Soehne were used in some experiments and found satisfactory.

Preparation of Solutions

I. Magnesium sulphate (0.015 M):
Dissolve 37 mg. $MgSO_4 \cdot 7H_2O$ in distilled water and make up to 10 ml.

II. Cysteine (0.2 M):
Dissolve 31.5 mg. cysteine hydrochloride in 0.5 ml. distilled water, neutralize with *ca.* 0.2 ml. 1 N NaOH and dilute to 1 ml. with distilled water. Prepare the solution freshly each day.

III. Triethanolamine buffer (0.1 M; pH 7.6):
Dissolve 1.857 g. triethanolamine hydrochloride in a little distilled water, adjust to pH 7.6 with 40.1 ml. 0.1 N NaOH and dilute to 100 ml. with distilled water. Check pH with a glass electrode.

IV. Ethylene-diamine-tetra-acetate (0.004 M; pH 7.6):
Dissolve 149 mg. EDTA-$Na_2H_2 \cdot 2H_2O$ in 75 ml. distilled water, adjust to pH 7.6 with 0.1 N NaOH and dilute to 100 ml. with distilled water.

V. D-3-Phosphoglyceric acid (*ca.* 1.6×10^{-4} M):
Stock solution (*ca.* 8×10^{-3} M): Dissolve 16.7 mg. of the calcium salt or 40 mg. of the tricyclohexylammonium salt in distilled water and make up to 10 ml. Before use dilute the solution 1 : 50.

VI. Reduced diphosphopyridine nucleotide (*ca.* 5×10^{-5} M β-DPNH):
Stock solution (*ca.* 1.5×10^{-3} M): Dissolve 10 mg. DPNH-Na_2 in distilled water and make up to 10 ml. Before use dilute the solution 1 : 30.

VII. Adenosine triphosphate (10^{-5} M ATP):
Stock solution (10^{-2} M): Dissolve 12 mg. ATP-$Na_2H_2 \cdot 3H_2O$ in distilled water and make up to 2 ml. Before use dilute the solution 1 : 1000.

VIII. Glyceraldehyde-3-phosphate dehydrogenase, GAPDH (10 mg. protein/ml.):
If necessary, dilute the enzyme suspension with ice-cold water.

IX. Phosphoglycerate kinase, PGK (10 mg. protein/ml.):
If necessary, dilute the enzyme suspension with ice-cold water.

X. Quinine sulphate (3×10^{-8} M):
see solution VIII on p. 553.

Stability of the solutions

The cysteine solution should be prepared freshly each day, and the DPNH solution each week. Ammonium sulphate suspensions of the enzymes are stable for more than 1 year at 0–4°C. Prepare the dilutions of the enzymes freshly each day and keep cold.

Procedure

Fluorimetric measurements

Wavelength: 460 mμ; excitation wavelength: 365 mμ; Pyrex tubes (1 cm. diameter, 7.5 cm. long); final volume: 1 ml.; room temperature. Set the fluorimeter with quinine sulphate solution (X). Prepare: experimental solution (contains the sample), standard solutions (contain known amounts of ATP) and reagent blank (ATP-free). Up to 8 experimental samples can be analysed successively. In order to save time and to increase the accuracy, prepare

the following reaction mixture freshly (sufficient for 8 samples, 3 standards and 1 reagent blank):

4.0 ml. $MgSO_4$ solution (I)
1.0 ml. DPNH solution (VI)
4.0 ml. phosphoglycerate solution (V)
1.0 ml. EDTA solution (IV)
1.0 ml. cysteine solution (II).

Pipette successively into the test tubes:

0.55 ml. reaction mixture
sample or 0.05 to 0.2 ml. ATP standard solution (VII)
0.09 ml. buffer (solution III); take less in the experimental tubes according to the volume of the sample, since the deproteinization was carried out in this buffer.
distilled water to 0.90 ml.

Measure the fluorescence and multiply the reading by the dilution factor 0.90. Mix into all the test tubes

0.05 ml. GAPDH solution (VIII)
0.05 ml. PGK solution (IX),

follow the decrease in fluorescence and read the minimum value.

Calculations

From the intensity of fluorescence of the standard solutions (minus the fluorescence of the reagent blank) a standard curve is prepared (decrease in fluorescence versus ATP concentration) from which the ATP content of the mixture is obtained. With the experimental material so far examined (nerve fibres from guinea pigs), the ATP values obtained with methods A and B have agreed within a few percent.

Sources of Error

1,3-Diphosphoglyceric acid in the experimental material is estimated as apparent ATP, resulting in high values. It can be determined separately if 3-phosphoglyceric acid, Mg^{2+} and phosphoglycerate kinase are omitted from the test mixture.

If the enzyme preparation contains myokinase, then according to the equation

$$2\ ADP \rightleftharpoons ATP + AMP$$

any ADP, either present in the sample, or formed during the determination (refer to equation (3) see p. 552) may be converted to ATP and therefore interfere in the determination. This is true for the spectrophotometric determination of ATP, but not for the fluorimetric method: with low ADP concentrations the velocity of the myokinase reaction is proportional to the square of the ADP concentration. As the concentration of ATP required in the fluorimetric method is lower by a factor of about 100 compared to that for the spectrophotometric method, the velocity of the myokinase reaction is decreased by about 10000. The interfering myokinase reaction is reduced by dilution.

GTP reacts, whereas UTP and CTP do not interfere.

Other Methods for the Determination of ATP

Micro amounts of ATP can also be determined with the luciferin-luciferase system from fire-flies[7,8] (see p. 559).

A determination of ATP + ADP in amounts as small as 5×10^{-12} moles depends on the catalytic activity of the two adenosine phosphates in the following system:

(1) ATP + creatine ⟶ ADP + creatine phosphate

(2) ADP + phosphoenolpyruvate ⟶ ATP + pyruvate

(3) DPNH + H^+ + pyruvate ⇌ lactate + DPN^+

The rate of the fluorimetrically measured DPNH oxidation is proportional to the ATP + ADP concentration[9].

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7) *W. D. McElroy* and *B. L. Strehler*, Arch. Biochem. Biophysics *22*, 420 [1949].
8) *B. L. Strehler* and *J. R. Totter* in *D. Glick:* Methods of Biochemical Analysis. Interscience Publishers, Inc., New York 1954, Vol. I, p. 341.
9) *P. Greengard*, unpublished.