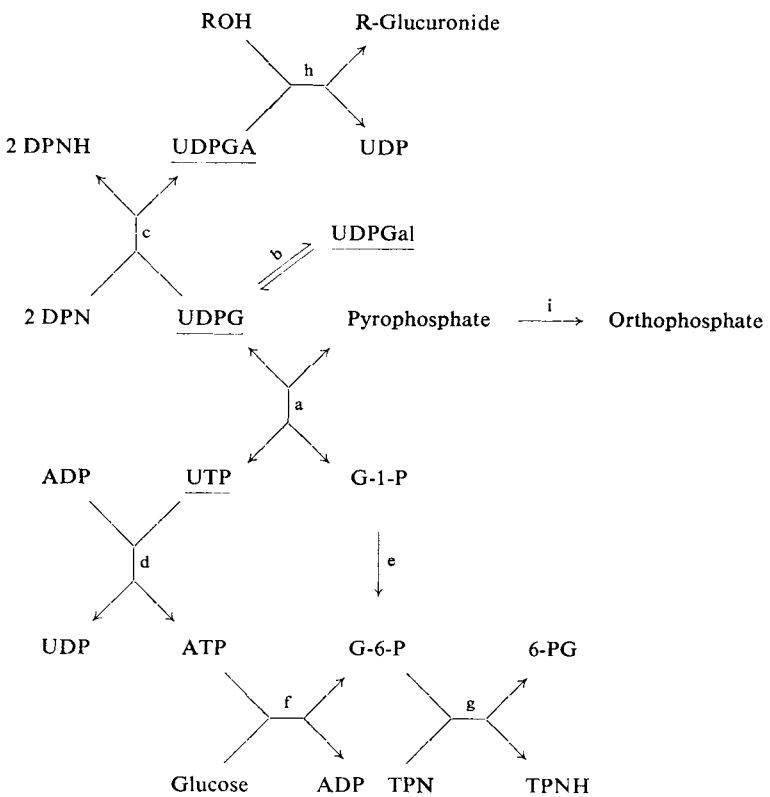
Uridine Diphosphoglucose, Uridine Diphosphogalactose, Uridine Triphosphate and Uridine Diphosphoglucuronic Acid

George T. Mills and Evelyn E. B. Smith

The reactions used for the enzymatic determination of the four compounds mentioned in the title are summarized in Scheme 1. With the exception of the determination of UDPGA*), all of the methods described here are based on the spectrophotometric measurement of the reduction of DPN or TPN.



Scheme 1. Reactions employed in the determination of uridine diphosphoglucose (UDPG), uridine diphosphogalactose (UDPGal), uridine triphosphate (UTP) and uridine diphosphoglucuronic acid (UDPGA).

Enzymes involved:

a) Uridyl transferase
b) UDPGal-4-epimerase
c) UDPG dehydrogenase
d) Nucleoside diphosphokinase
e) Phosphoglucomutase
f) Hexokinase
g) Glucose-6-phosphate dehydrogenase
h) Glucuronosyl transferase
i) Inorganic pyrophosphatase

*) For meaning of the abbreviations, see footnote on p. 582.

Uridine Diphosphoglucose

A) Determination with Uridyl Transferase, Phosphoglucomutase and Glucose-6-phosphate Dehydrogenase

Principle

Pyrophosphorolytic cleavage of UDPG*) yields UTP and G-1-P (equation 1). The G-1-P is converted to G-6-P (equation 2) and the latter is oxidized to 6-PG by TPN (equation 3).

(1) $$\text{UDPG} + \text{pyrophosphate} \rightleftharpoons \text{UTP} + \text{G-1-P}$$

(2) $$\text{G-1-P} \rightleftharpoons \text{G-6-P}$$

(3) $$\text{G-6-P} + \text{TPN}^+ \rightleftharpoons \text{6-PG} + \text{TPNH} + \text{H}^+$$

$$\text{UDPG} + \text{pyrophosphate} + \text{TPN}^+ \longrightarrow \text{UTP} + \text{6-PG} + \text{TPNH}^+ + \text{H}^+$$

Reaction (1) is catalysed by uridyl transferase, (2) by phosphoglucomutase and (3) by glucose-6-phosphate dehydrogenase. In the presence of excess pyrophosphate the UDPG reacts quantitatively. The increase of optical density at 340 mμ due to the formation of TPNH is a measure of the over-all reaction. The method was first described by *Munch-Petersen et al.*[1] and later elaborated[2].

Reagents

1. Tris-hydroxymethyl-aminomethane, tris
2. Hydrochloric acid, A. R., 1 N
3. Magnesium chloride, $MgCl_2 \cdot 6\ H_2O$
4. Cysteine hydrochloride
5. Potassium hydroxide, A. R., 1 N
6. Potassium pyrophosphate, $K_4P_2O_7 \cdot 3\ H_2O$
7. Triphosphopyridine nucleotide, TPN
   sodium salt, TPN-NaH_2; commercial preparation, see p. 1029.
8. Uridyl transferase
   prepared according to[2] from dried yeast (see p. 594). The ammonium sulphate precipitate is stable as a paste at −10 to −15°C for 2 months.
9. Phosphoglucomutase, PGluM
   prepared according to[3] from rabbit skeletal muscle. Commercial preparation, see p. 992.

*) Abbreviations:

UTP	= uridine-5′-triphosphate	TPN	= triphosphopyridine nucleotide
UDP	= uridine-5′-diphosphate	TPNH	= reduced triphosphopyridine nucleotide
UDPG	= uridine diphosphoglucose		
UDPGal	= uridine diphosphogalactose	ATP	= adenosine-5′-triphosphate
UDPGA	= uridine diphosphoglucuronic acid	ADP	= adenosine-5′-diphosphate
		G-1-P	= α-glucose-1-phosphate
DPN	= diphosphopyridine nucleotide	6-PG	= 6-phosphogluconic acid
DPNH	= reduced diphosphopyridine nucleotide	G-6-P	= glucose-6-phosphate

1) *A. Munch-Petersen, H. M. Kalckar, E. Cutolo* and *E. E. B. Smith*, Nature [London] *172*, 1036 [1953].
2) *A. Munch-Petersen*, Acta chem. scand. *9*, 1523 [1955].
3) *V. A. Najjar*, J. biol. Chemistry *175*, 281 [1948].

10. Glucose-6-phosphate dehydrogenase, G6P-DH
prepared according to[4] from yeast. The lyophilized powder is stable at −10 to −15°C for 6 months. Commercial preparation, see p. 975.

Purity of the enzyme preparations

The uridyl transferase prepared according to[2] satisfies the requirements. The purification of the phosphoglucomutase according to[3] should be carried through the 2nd heat treatment and the filtrate used. The glucose-6-phosphate dehydrogenase preparation should not contain any 6-phosphogluconic dehydrogenase (6-PG-DH). To test for this, add a known amount of G-6-P to the assay system described below, but omit the uridyl transferase and phosphoglucomutase. The ΔE value obtained at 340 mμ should be 6.22 per μmole G-6-P (1 cm. light path) if the glucose-6-phosphate dehydrogenase is free from 6-PG-DH.

Preparation of Solutions

I. Tris buffer (0.1 M; pH 7.8):
Dissolve 6.06 g. tris-hydroxymethyl-aminomethane in 200 ml. distilled water, add 35 ml. 1 N HCl and make up to 500 ml. with distilled water.

II. Magnesium chloride (*ca.* 0.5 M):
Dissolve 20.3 g. $MgCl_2 \cdot 6\ H_2O$ in 200 ml. distilled water.

III. Cysteine (*ca.* 0.25 M):
Dissolve 40 mg. cysteine hydrochloride in 1 ml. distilled water and adjust to pH 7 with 1 N KOH (indicator paper). Prepare the solution freshly each day.

IV. Potassium pyrophosphate (*ca.* 0.1 M):
Dissolve 3.84 g. $K_4P_2O_7 \cdot 3\ H_2O$ in 100 ml. distilled water.

V. Triphosphopyridine nucleotide (*ca.* 10^{-2} M β-TPN):
Dissolve 8.7 mg. TPN-NaH_2 in 1 ml. distilled water.

VI. Uridyl transferase:
Dissolve the ammonium sulphate paste prepared according to[2] in sufficient tris buffer (solution I), so that 10 μl. of the enzyme solution gives an initial ΔE/min. of 0.100 with 0.1 μmole of pure UDPG in the assay system described below.

VII. Phosphoglucomutase, PGluM (3 mg. protein/ml.):
Dilute the filtrate from the 2nd heat treatment of the method according to[3] with 1.34 M ammonium sulphate or the commercial preparation (see p. 992) with 2.5 M ammonium sulphate.

VIII. Glucose-6-phosphate dehydrogenase, G6P-DH (10 mg. protein/ml.):
Dissolve the dry powder prepared according to[4] in tris buffer (solution I) or dilute the commercial preparation with 3.3 M ammonium sulphate solution.

Stability of the solutions

Prepare the cysteine and uridyl transferase solution freshly each day. The pyrophosphate solution keeps for 1 month at 4°C, and the TPN solution 2 months at −10 to −15°C. The phosphoglucomutase solution keeps for 1 month at −10 to −15°C, while the crystalline suspension in ammonium sulphate solution keeps for longer than 1 year. The glucose-6-phosphate dehydrogenase solution keeps for 1 week at −15°C and the crystalline suspension in ammonium sulphate solution is stable for more than 1 year.

[4] *A. Kornberg*, J. biol. Chemistry *182*, 805 [1950].

Procedure

Experimental material

The samples for analysis should be in aqueous solution (pH 7—8). Homogenize tissue in ice-cold perchloric acid (final concentration 2% w/v), centrifuge and adjust the supernatant to pH 7 with 3 N KOH. Allow to stand for 1 hour at 0°C, centrifuge off the precipitated $KClO_4$ and use the supernatant for the analysis.

Spectrophotometric measurements

Wavelength: 340 mμ; silica cuvettes, light path: 1 cm.; final volume 1 ml. Measure against the control cuvette.

Pipette into the experimental and control cuvette:

0.1 ml. sample
0.80 ml. tris buffer (solution I)
0.01 ml. $MgCl_2$ solution (II)
0.01 ml. cysteine solution (III)
0.025 ml. TPN solution (V)
0.025 ml. PGluM solution (VII)
0.01 ml. G6P-DH solution (VIII)
0.01 ml. uridyl transferase solution (VI).

Read the optical density (should be constant: E_1). Mix into the experimental cuvette

0.01 ml. $K_4P_2O_7$ solution (IV)

and read the optical density every minute until constant: E_2. The optical density difference $\Delta E = E_2 - E_1$ is used for the calculations.

Calculations

$$\frac{\Delta E}{6.22} = \mu\text{moles UDPG/reaction mixture}$$

where

$\Delta E = E_2 - E_1$

6.22 = extinction coefficient of TPNH at 340 mμ [cm.^{2}/μmole]

Specificity and Sources of Error

The method is specific for UDPG. The presence of G-1-P and G-6-P in the sample leads to increased formation of TPNH, but this does not interfere because the same increase in optical density occurs in the control cuvette. The presence of inorganic pyrophosphate in the sample interferes.

B) Determination with UDPG Dehydrogenase

Principle

UDPG dehydrogenase catalyses the reaction[5]:

(4) $$\text{UDPG} + 2\,\text{DPN}^+ \longrightarrow \text{UDPGA} + 2\,\text{DPNH} + 2\,\text{H}^+$$

The reaction is irreversible[6]. The pH optimum is 8.7. The increase of optical density at 340 mμ due to the formation of DPNH is a measure of the reaction.

[5] *J. L. Strominger, H. M. Kalckar, J. Axelrod* and *E. S. Maxwell*, J. Amer. chem. Soc. *76*, 6411 [1954].
[6] *J. L. Strominger, E. S. Maxwell, J. Axelrod* and *H. M. Kalckar*, J. biol. Chemistry *224*, 79 [1957].

Reagents

1. Tris-hydroxymethyl-aminomethane, tris
2. Hydrochloric acid, A. R., 1 N
3. Diphosphopyridine nucleotide, DPN
   free acid; commercial preparation, see p. 1010.
4. UDPG dehydrogenase
   prepared according to[6] from calf liver; commercial preparation, see p. 1001.

Purity of the enzyme preparation

It is sufficient to carry the purification according to[6] as far as step 5. Dissolve the final ammonium sulphate precipitate from this step in 20 ml. distilled water, adjust to pH 5.9 with acetic acid and dialyse against distilled water for 4 hours at 2°C. Use the contents of the dialysis sac.

Preparation of Solutions

I. Tris buffer (0.1 M; pH 8.7):
   Dissolve 6.06 g. tris-hydroxymethyl-aminomethane in 200 ml. distilled water, adjust to pH 8.7 with 10 ml. 1 N HCl and make up to 500 ml. with distilled water.

II. Diphosphopyridine nucleotide (*ca.* 2.5×10^{-2} M β-DPN):
   Dissolve 19.3 mg. DPN in 1 ml. distilled water.

III. UDPG dehydrogenase (25000 units[6]/ml.):
   Use the preparation (see "Purity of the enzyme preparation") obtained according to[6] undiluted. If necessary, dilute the commercial preparation with distilled water.

Stability of the solutions

The enzyme solution prepared according to[6] (see "Purity of the enzyme preparation") is stable for about 6 weeks at −10°C. The DPN solution is stable for 2 months at −10 to −15°C.

Procedure

Experimental material

See p. 584.

Spectrophotometric measurements

Wavelength: 340 mμ; silica cuvettes, light path: 1 cm.; final volume 1.0 ml. Measure against the control cuvette.

Pipette into the experimental and control cuvette:

0.10 ml. sample
0.87 ml. tris buffer (solution I)
0.01 ml. enzyme solution (III).

Read the optical density E_1. Mix into the experimental cuvette

0.02 ml. DPN solution (II)

and read the optical density every minute until constant: E_2. The optical density difference $\Delta E = E_2 - E_1$ is used for the calculations.

Calculations

2 μmoles of DPNH are formed for each μmole of UDPG. Therefore it follows that:

$$\frac{\Delta E}{6.22 \times 2} = \frac{\Delta E}{12.44} = \mu\text{moles UDPG/reaction mixture}$$

where

$\Delta E = E_2 - E_1$

6.22 = extinction coefficient of DPNH at 340 mμ [cm.²/μmole]

Specificity and Sources of Error

The method is specific for UDPG. UDPGal, uridine diphosphoacetylglucosamine, guanosine diphosphomannose, G-1-P and G-6-P do not react. DPN cannot be replaced by TPN. The presence of DPN in the sample interferes, but it can be removed by Dowex 50 (H^+ form)[7].

Uridine Diphosphogalactose

Principle

UDPGal-4-epimerase converts uridine diphosphogalactose (UDPGal) to uridine diphosphoglucose (UDPG)[8–10]. The UDPG is determined by one of the methods described above (p. 582 and 584) (refer to reaction b and c or b and a, e, g in Scheme 1, p. 581). UDPGal-4-epimerase has a wide activity optimum between pH 8.0 and 9.6. Catalytic amounts of DPN (5×10^{-5} M) must be present. If UDPG is estimated according to method A (p. 582), pH 8.0 should be used. With method B (p. 584) the pH is 8.7.

Reagents

See p. 582 or 585. In addition:

For method A:

1. Diphosphopyridine nucleotide, DPN
   free acid; commercial preparation, see p. 1010.

For method A and B:

2. UDPGal-4-epimerase
   prepared according to[10] from calf liver (see p. 595). The lyophilized powder is stable for about 3 months at −15°C.

Preparation of Solutions

See p. 583 or 585. In addition:

For method A:

I. Instead of the tris buffer given on p. 583:
   Tris buffer (0.1 M; pH 8.0):
   Dissolve 6.06 g. tris-hydroxymethyl-aminomethane in 200 ml. distilled water, add 28 ml. 1 N HCl and make up to 500 ml. with distilled water.

II. Diphosphopyridine nucleotide (*ca.* 2.5×10^{-2} M β-DPN):
   Dissolve 19.3 mg. DPN in 1 ml. distilled water.

---

[7] *E. E. B. Smith, G. T. Mills, H. P. Bernheimer* and *R. Austrian*, Biochim. biophysica Acta *28*, 211 [1958].
[8] *L. F. Leloir*, Arch. Biochem. Biophysics *33*, 186 [1951].
[9] *H. M. Kalckar* and *E. S. Maxwell*, Biochim. biophysica Acta *22*, 588 [1956].
[10] *E. S. Maxwell*, J. biol. Chemistry *229*, 139 [1957].

For methods A and B:

III. UDPGal-4-epimerase (2.5 mg. protein/ml.):
Dissolve 2.5 mg. enzyme in 1 ml. distilled water. Prepare the solution freshly each day.

Method A

Procedure

See p. 584. Prepare two experimental cuvettes (C1, C2) and a control cuvette. Set up the cuvettes as described on p. 584, but instead of the tris buffer (pH 7.8) use the tris buffer (pH 8.0) described on p. 586. After the addition of the uridyl transferase and before reading optical density E_1, add

0.01 ml. DPN solution (p. 586)

to both experimental cuvettes and

0.01 ml. UDPGal-4-epimerase solution (p. 586)

only to cuvette C2. Read the optical densities at 340 mμ as described on p. 584. ΔE_{C2} is a measure of UDPG and UDPGal content of the assay mixture. ΔE_{C1} gives the UDPG content.

Calculations

$$\frac{\Delta E_{C2} - \Delta E_{C1}}{6.22} = \mu\text{moles UDPGal/assay mixture}$$

where

$\Delta E_{C1} = E_2 - E_1$ for the experimental cuvette C1 (without UDPGal-4-epimerase)

$\Delta E_{C2} = E_2 - E_1$ for the experimental cuvette C2 (with UDPGal-4-epimerase)

6.22 = extinction coefficient of DPNH at 340 mμ [cm.²/μmole]

Method B

Procedure

See p. 585. Prepare two experimental cuvettes (C1, C2) and a control cuvette, and set up as described on p. 585. After the addition of the UDPG dehydrogenase and before reading the optical density E_1, add

0.01 ml. UDPGal-4-epimerase solution (p. 586)

to the experimental cuvette C2. Read the optical densities as described on p. 585. ΔE_{C2} is a measure of UDPG and UDPGal content of the assay mixture. ΔE_{C1} gives the UDPG content.

Calculations

2 μmoles of DPNH are formed for each μmole of UDPG and UDPGal (see Scheme 1 on p. 581). Therefore it follows:

$$\frac{\Delta E_{C2} - \Delta E_{C1}}{6.22 \times 2} = \frac{\Delta E_{C2} - \Delta E_{C1}}{12.44} = \mu\text{moles UDPGal/assay mixture}$$

where

$\Delta E_{C1} = E_2 - E_1$ for the experimental cuvette C1 (without UDPGal-4-epimerase)

$\Delta E_{C2} = E_2 - E_1$ for the experimental cuvette C2 (with UDPGal-4-epimerase)

6.22 = extinction coefficient of DPNH at 340 mμ [cm.²/μmole]

Uridine Triphosphate

There are two methods for the determination of uridine triphosphate (UTP). The first method estimates all the nucleoside triphosphates present in the sample. The nucleoside diphosphokinase of *Berg* and *Joklik*[11] is used for this method. In the second method the UTP is converted to UDPG (see reaction a in Scheme 1, p. 581) and the latter is determined with uridyl transferase and UDPG dehydrogenase [12,13].

A) Determination with Nucleoside Diphosphokinase

Principle

Nucleoside diphosphokinase catalyses the reaction:

(1) $$\text{UTP} + \text{ADP} \longrightarrow \text{UDP} + \text{ATP}$$

The adenosine triphosphate (ATP) formed is determined with hexokinase (HK) and glucose-6-phosphate dehydrogenase (G6P-DH):

(2) $$\text{ATP} + \text{glucose} \xrightarrow{\text{HK}} \text{glucose-6-phosphate} + \text{ADP}$$

(3) $$\text{Glucose-6-phosphate} + \text{TPN}^+ \xrightarrow{\text{G6P-DH}} \text{6-phosphogluconic acid} + \text{TPNH} + \text{H}^+$$

$$\text{UTP} + \text{glucose} + \text{TPN}^+ \longrightarrow \text{UDP} + \text{6-PG} + \text{TPNH} + \text{H}^+$$

1 μmole of TPNH is formed for each μmole of UTP. The increase of optical density at 340 mμ due to the formation of TPNH is a measure of the over-all reaction. Only small amounts of ADP need be added to the assay mixture, because it is regenerated in reaction (2). Nucleoside diphosphokinase is activated by Mg^{2+} (5×10^{-3} M). It has an activity optimum between pH 6 and 8.

Reagents

1. Tris-hydroxymethyl-aminomethane, tris
2. Magnesium chloride, A. R., $MgCl_2 \cdot 6\,H_2O$
3. Glucose
4. Adenosine diphosphate, ADP
   sodium salt, $ADP\text{-}Na_3$; commercial preparation, see p. 1004.
5. Triphosphopyridine nucleotide, TPN
   sodium salt, $TPN\text{-}NaH_2$; commercial preparation, see p. 1029.
6. Nucleoside diphosphokinase
   prepared according to[11] from rabbit muscle (see p. 595). The preparation keeps for 2—3 weeks at 3°C.
7. Hexokinase, HK
   from yeast, dry powder or crystalline suspension in 3 M ammonium sulphate solution; commercial preparation, see p. 983.
8. Glucose-6-phosphate dehydrogenase, G6P-DH
   see p. 975.

Purity of the enzyme preparations

G6P-DH: see p. 583. — Nucleoside diphosphokinase: the preparation obtained according to[11] satisfies the requirements. — Hexokinase: a crude preparation is satisfactory (Type II of the Sigma Chemical Comp.).

[11] *P. Berg* and *W. Joklik*, J. biol. Chemistry *210*, 657 [1954].
[12] *E. P. Anderson*, *H. M. Kalckar* and *A. Munch-Petersen*, Publ. Staz. Zool. Napoli *29*, 119 [1957].
[13] *G. T. Mills*, *A. C. Lochhead* and *E. E. B. Smith*, Biochim. biophysica Acta *27*, 103 [1958].

Preparation of Solutions

I. Tris buffer (0.1 M; pH 7.8):
See p. 583.

II. Magnesium chloride (0.5 M):
See p. 583.

III. Glucose (10% w/v):
Dissolve 1 g. glucose in 10 ml. distilled water.

IV. Adenosine diphosphate (5×10^{-3} M ADP):
Dissolve 2.5 mg. ADP-Na_3 in 1 ml. distilled water.

V. Triphosphopyridine nucleotide (*ca.* 10^{-2} M β-TPN):
See p. 583.

VI. Nucleoside diphosphokinase (2 mg. protein/ml.):
Dilute the solution obtained according to[11] with distilled water.

VII. Hexokinase, HK (10 mg. protein/ml.):
Use distilled water to dissolve the dry preparation or dilute the crystalline suspension.

VIII. Glucose-6-phosphate dehydrogenase, G6P-DH (10 mg. protein/ml.):
See p. 583.

Stability of the solutions

See p. 583. The ADP solution keeps for 10 weeks at −15° C. Store the nucleoside diphosphokinase solution at 3° C. The solution is usable for 2−3 weeks.

Procedure

Experimental material

See p. 584.

Spectrophotometric measurements

Wavelength: 340 mμ; silica cuvettes (light path: 1 cm., width: 0.4 cm., volume: 1.3 ml.); final volume: 1.005 ml.. Measure against the control cuvette.

Pipette into the experimental and control cuvette:

0.100 ml. sample
0.800 ml. buffer (solution I)
0.010 ml. $MgCl_2$ solution (II)
0.010 ml. glucose solution (III)
0.020 ml. nucleoside diphosphokinase solution (VI)
0.020 ml. HK solution (V)
0.010 ml. G6P-DH solution (VIII)
0.025 ml. TPN solution (V).

Read the optical density E_1. Mix into the experimental cuvette

0.010 ml. ADP solution (IV)

and read the optical density every minute until constant: E_2. The optical density difference $\Delta E = E_2 - E_1$ is used for the calculations.

Calculations

$$\frac{\Delta E}{6.22} = \mu\text{moles nucleoside triphosphate/assay mixture}$$

where

$\Delta E = E_2 - E_1$

6.22 = extinction coefficient of TPNH at 340 mμ [cm.²/μmole].

Specificity

All the nucleoside triphosphates contained in the sample react. If the optical density E_0 is read before the addition of the TPN solution, then the difference $E_1 - E_0$ gives the ATP content of the sample, and the difference $E_2 - E_1$ gives the amount of UTP + other nucleoside triphosphates (except for ATP).

B) Determination with Uridyl Transferase

Principle

Uridine triphosphate (UTP) reacts with glucose-1-phosphate (G-1-P) to give uridine diphosphoglucose (UDPG) and pyrophosphate in a reversible reaction catalysed by uridyl transferase. The pyrophosphate is hydrolysed by inorganic pyrophosphatase (see reactions a and i in Scheme 1, p. 581). The UDPG is oxidized by DPN and UDPG dehydrogenase to uridine diphosphoglucuronic acid (UDPGA) (see reaction c in Scheme 1, p. 581). 2 μmoles of DPNH are formed for each μmole of UTP. The increase of optical density at 340 mμ due to the formation DPNH is measured.

Reagents

1. Tris-hydroxymethyl-aminomethane, tris
2. Hydrochloric acid, A. R., 1 N
3. Magnesium chloride, A. R., $MgCl_2 \cdot 6\ H_2O$
4. Glucose-1-phosphate, G-1-P
   dipotassium salt. $2\ H_2O$.
5. Diphosphopyridine nucleotide, DPN
   free acid; commercial preparation, see p. 1010.
6. Uridyl transferase
   see p. 582.
7. UDPG dehydrogenase
   see p. 585.
8. Inorganic pyrophosphatase
   prepared according to[14] from yeast (see p. 595).

Purity of the enzyme preparations

Uridyl transferase: see p. 583. — UDPG dehydrogenase: see p. 585. — Inorganic pyrophosphatase: Carry out the purification according to[14] up to the fractionation with alcohol and stop before the adsorption on Al_2O_3 gel. The resulting solution in 0.1 M acetate buffer (pH 7) satisfies the requirements.

Preparation of Solutions

I. Tris buffer (0.1 M; pH 8.5):
   Dissolve 6.06 g. tris-hydroxymethyl-aminomethane in 200 ml. distilled water, add 15 ml. 1 N HCl and make up to 500 ml. with distilled water.

[14] *L. A. Heppel* and *R. J. Hilmoe*, Biochem. Prep. *4*, 34 [1955].

II. Magnesium chloride (0.5 M):
See p. 583.

III. Glucose-1-phosphate, G-1-P (0.02 M):
Dissolve 7.4 mg. G-1-P-dipotassium salt·2 H_2O in 1 ml. distilled water.

IV. Diphosphopyridine nucleotide (*ca.* 2.5×10^{-2} M β-DPN):
See p. 585.

V. Uridyl transferase:
See p. 583.

VI. UDPG dehydrogenase (25000 units[6]/ml.):
See p. 585.

VII. Inorganic pyrophosphatase:
Use the enzyme solution in 0.1 M acetate buffer (pH 7) (see "Purity of the enzyme preparations") prepared according to[14] undiluted.

Stability of the solutions

See p. 583 and 585. The solution of the inorganic pyrophosphatase is stable for 6 months at −15° C.

Procedure

Experimental material

See p. 584.

Spectrophotometric measurements

Wavelength: 340 mμ; silica cuvettes, light path: 1 cm.; final volume: 1.01 ml. Measure against the control cuvette.

Pipette into the experimental and control cuvette:

0.10 ml. sample
0.80 ml. tris buffer (solution I)
0.01 ml. $MgCl_2$ solution (II)
0.01 ml. DPN solution (III)
0.01 ml. uridyl transferase solution (V)
0.02 ml. pyrophosphatase solution (VII)
0.05 ml. UDPG dehydrogenase solution (VI).

Read the optical density E_1. Mix into the experimental cuvette

0.01 ml. G-1-P solution (III).

Follow the optical density until constant: E_2. The difference $\Delta E = E_2 - E_1$ is used for the calculations.

Calculations

2 μmoles of DPNH are formed for each μmole of UTP. Therefore it follows:

$$\frac{\Delta E}{6.22 \times 2} = \frac{\Delta E}{12.44} = \text{μmoles UTP/assay mixture}$$

where

$\Delta E = E_2 - E_1$

6.22 = extinction coefficient of DPNH at 340 mμ [cm.²/μmole].

Specificity

The method is completely specific for UTP. If the sample contains UDPG, this leads to additional reduction of DPN, but this source of error is eliminated by the control cuvette.

Uridine Diphosphoglucuronic Acid

Principle

Glucuronosyl transferase from liver microsomes catalyses the reaction:

(1) $$\text{UDPGA} + \text{ROH} \longrightarrow \text{R-glucuronide} + \text{UDP}$$

(UDPGA = uridine diphosphoglucuronic acid). ROH is a phenol. The phenol consumed or the glucuronide formed is estimated. Numerous glucuronic acid acceptors can be used. *o*-Aminophenol and phenolphthalein provide the simplest methods of assay, since *o*-aminophenyl glucuronide can be estimated in micro-quantities in the presence of free *o*-aminophenol [15] and similarly, phenolphthalein can be easily determined in the presence of phenolphthalein glucuronide[6]. If the sample contains only small amounts of UDPGA, the method employing *o*-aminophenol is the one of choice. After the enzymatic reaction between UDPGA and *o*-aminophenol, the protein is removed from the reaction mixture and the *o*-aminophenyl glucuronide formed is estimated according to the method developed by *Bratton* and *Marshall*[16] for the determination of sulphonamides. If this reaction is carried out at pH 2.2, then the contribution of free *o*-aminophenol to the colour production is negligible[15]. This method has been widely used in studies on UDPGA and glucuronide formation [13,17,18].

Reagents

1. Glycylglycine
2. Potassium hydroxide, 1 N
3. Potassium chloride
4. Magnesium chloride, $MgCl_2 \cdot 6\ H_2O$
5. *o*-Aminophenol, sublimed
6. Glycine
7. Trichloroacetic acid
8. Sodium nitrite, $NaNO_2$
9. Ammonium sulphamate, $NH_4SO_3NH_2$
10. *N*-(1-Naphthyl)-ethylenediamine dihydrochloride
11. Uridine diphosphoglucuronic acid
    ammonium salt; commercial preparation, see p. 1032.
12. *o*-Aminophenyl glucuronide
    prepared according to [19].
13. Glucuronosyl transferase
    microsomal fraction from guinea pig liver. For preparation, see Appendix, p. 595.

---

[15] *G. A. Levvy* and *I. D. E. Storey*, Biochem. J. *44*, 295 [1949].
[16] *A. C. Bratton* and *E. K. Marshall*, J. biol. Chemistry *128*, 537 [1939].
[17] *E. E. B. Smith* and *G. T. Mills*, Biochim. biophysica Acta *13*, 386 [1954].
[18] *I. D. E. Storey* and *G. J. Dutton*, Biochem. J. *59*, 279 [1955].
[19] *R. T. Williams*, Biochem. J. *37*, 329 [1943].

Preparation of Solutions

I. Glycylglycine buffer (0.2 M; pH 7.7):
Dissolve 2.65 g. glycylglycine in 70 ml. distilled water, adjust to pH 7.7 with 1 N KOH (glass electrode) and dilute to 100 ml. with distilled water.

II. Potassium chloride (0.15 M):
Dissolve 1.12 g. KCl in 100 ml. distilled water.

III. Magnesium chloride (0.5 M):
Dissolve 10.2 g. $MgCl_2 \cdot 6\ H_2O$ in 100 ml. distilled water.

IV. *o*-Aminophenol:
Dissolve 8 mg. *o*-aminophenol in 50 ml. distilled water. Prepare the solution just before use.

V. Glycine-trichloroacetic acid buffer (0.6 M glycine; 0.4 M trichloroacetic acid; pH 2.2):
Dissolve 4.5 g. glycine in distilled water and make up to 60 ml. Dissolve 6.5 g. trichloroacetic acid in distilled water and make up to 40 ml. Mix the solutions. Check the pH (glass electrode) and, if necessary, adjust to pH 2.2 with glycine or trichloroacetic acid solution.

VI. Sodium nitrite (0.1%):
Dissolve 10 mg. $NaNO_2$ in 10 ml. distilled water.

VII. Ammonium sulphamate (0.5%):
Dissolve 50 mg. $NH_4SO_3NH_2$ in 10 ml. distilled water.

VIII. *N*-(1-Naphthyl)-ethylenediamine (0.1%):
Dissolve 10 mg. *N*-(1-naphthyl)-ethylenediamine dihydrochloride in 10 ml. distilled water.

IX. Uridine diphosphoglucuronic acid standard solution (10^{-4} M):
Dissolve 1.0 mg. UDPGA-NH_4^+ salt in 13.6 ml. distilled water.

X. *o*-Aminophenyl glucuronide standard solution (2×10^{-4} M):
Dissolve 3.74 mg. *o*-aminophenyl glucuronide in 50 ml. distilled water.

XI. Glucuronosyl transferase:
Use the suspension of microsomes in 0.15 M KCl solution described in the Appendix, p. 595.

Procedure

Experimental material

See p. 584.

Standard curve

Pipette into centrifuge tubes:

0–1.0 ml. *o*-aminophenyl glucuronide standard solution (X)
0.5 ml. glycylglycine buffer (solution I)
distilled water to 2.0 ml.

Proceed as for the unknown samples (see "Colorimetric measurements"). Plot the optical densities measured at 535 mμ (against the control) (ordinate) against μmoles *o*-aminophenyl glucuronide/tube (= μmoles UDPGA/tube) (abscissa).

Colorimetric measurements

Wavelength: 535 mμ; light path: 1 cm. Prepare the control, standard and experimental tubes in duplicate.

Pipette into centrifuge tubes:

0.50 ml. sample or distilled water for control tube or UDPGA standard solution (IX) for standard tube
0.50 ml. glycylglycine buffer (solution I)
0.02 ml. $MgCl_2$ solution (III)
0.30 ml. *o*-aminophenol solution (IV)
0.10 ml. microsome suspension (XI)
distilled water to 2.00 ml.

Incubate all the tubes for 30 min. at 37°C. Add to each tube

2.00 ml. glycine-trichloroacetic acid buffer (solution V).

Centrifuge for 5 min. at 2000 g.

Pipette into clean test tubes:

3.0 ml. supernatant
1.0 ml. $NaNO_2$ solution (VI).

Mix, allow to stand for 3 min. and then add

1.0 ml. $NH_4SO_3NH_2$ solution (VII).

Mix, allow to stand for 2 min. and add

1.0 ml. *N*-(1-naphthyl)-ethylenediamine solution (III).

Mix and allow to stand for 2 hours in the dark at 25°C. Pour into cuvettes and read the optical density against the control tube.

Calculations

Obtain the μmoles UDPGA/tube corresponding to the measured optical densities from the standard curve.

Specificity

The method is specific for UDPGA, providing the microsomal suspension used as the enzyme preparation is free from cell constituents which are normally found in the supernatant. The enzymatic activity of the preparations varies from animal to animal and this can be checked by means of the standard containing pure UDPGA.

Appendix

Isolation of uridyl transferase[2])

The method includes the following steps: autolysis of dry baker's yeast and extraction of the autolysate by shaking for 18 hours at 20°C with double the volume of 0.07 M $(NH_4)_2HPO_4$ solution. Ammonium sulphate fractionation. Dissolve the active protein which precipitates between 40 and 60% saturation with $(NH_4)_2SO_4$ in 0.015 M acetate buffer (pH 6.3) and dialyse for 30 min. against running tap water. Protamine sulphate precipitation of inactive protein. Ethanol fractionation at −8°C. The most active fraction precipitates between 20 and 24% ethanol. Precipitation with $(NH_4)_2SO_4$ at 60% saturation and extraction of the precipitate with $(NH_4)_2SO_4$ solutions (pH 7.5)

of decreasing concentration. The most active fraction is extracted with 50 to 56% saturated $(NH_4)_2SO_4$ solution. The enzyme is then completely precipitated and stored as a paste at $-20°C$.

The preparation is free from glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, hexokinase and phosphoglucomutase.

Isolation of uridine diphosphogalactose-4-epimerase[10]

The method includes the following steps: extraction of acetone-dried powder of calf liver with water; fractionation of the extract at $-2°C$ with acetone; fractionation of the active precipitate at pH 8.0 with $(NH_4)_2SO_4$ (45 to 65% saturation). Precipitation of this fraction with acetate buffer (pH 4.6) from 50% saturated $(NH_4)_2SO_4$; adsorption on calcium phosphate gel. Precipitation from the eluate with $(NH_4)_2SO_4$ (45 to 65% saturation). Solution of this precipitate in water and lyophilization.

Isolation of nucleoside diphosphokinase[11]

The method includes the following steps: extraction of rabbit skeletal muscle with water; dialysis for 4 hours against running tap water; fractionation with saturated $(NH_4)_2SO_4$ solution.

A more rapid procedure, which only gives a preparation $^1/_4$ to $^1/_2$ as pure as that described above, consists of: dilution of the original extract with an equal volume of water, addition of 0.05 volumes 1 M acetic acid, heating to 55°C for 1 min. and precipitation of the active protein at pH 6.8 (1 N NaOH).

Isolation of inorganic pyrophosphatase[14]

The method includes the following steps: maceration of dried baker's yeast (Fleischmann) for 13 hours in 0.1 M $NaHCO_3$ solution at 34°C. Ammonium sulphate fractionation of the juice; dialysis of the aqueous solution of the active precipitate. Ethanol fractionation at pH 6.0 and $-10°C$.

Isolation of glucuronosyl transferase

Kill guinea pigs by stunning and drain off blood by decapitation. Remove the liver immediately. Homogenize 2 g. liver in a *Potter-Elvehjem* homogenizer for 1 min. with 12 ml. ice-cold 0.15 M KCl solution (1.12 g. KCl/100 ml. distilled water). Centrifuge the homogenate for 15 min. at 0°C and 10000 g, and discard the precipitate. Centrifuge the supernatant for 60 min. at 0°C and 35000 g. Discard the supernatant, suspend the microsomal sediment in 4 ml. ice-cold 0.15 M KCl solution and store the solution at 0°C until ready for use. Prepare the microsomal suspension freshly for each series of measurements.