D-Gluconate

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Principle

Gluconokinase and 6-phosphogluconic dehydrogenase convert gluconic acid to ribulose-5-phosphate and CO_2 with the simultaneous reduction of an equivalent amount of triphosphopyridine nucleotide (TPN)[1,2]:

(1) Gluconate + ATP ⟶ 6-phosphogluconate + ADP

(2) 6-Phosphogluconate + TPN^+ ⟶ TPNH + CO_2 + ribulose-5-phosphate + H^+

Reagents

1. Potassium hydroxide, A. R.
2. Perchloric acid, A. R.; sp. gr. 1.67; *ca.* 70% (w/w)
3. Glycylglycine
4. Magnesium chloride, $MgCl_2 \cdot 6H_2O$
5. Adenosine triphosphate, ATP
   crystalline disodium salt, ATP-$Na_2H_2 \cdot 3H_2O$; commercial preparation, see p. 1006.
6. Triphosphopyridine nucleotide, TPN
   sodium salt, TPN-NaH_2; commercial preparation, see p. 1029.
7. 6-Phosphogluconic dehydrogenase
   from brewer's yeast[3] or rat liver[4], see Appendix, p. 141. Commercial preparation, see p. 993.
8. Gluconokinase
   from pig kidney cortex[1] or yeast[5], see Appendix, p. 141.

Purity of the enzyme preparations

Both enzymes have been only partially purified. The preparations obtained from yeast may be contaminated with glucose-6-phosphate dehydrogenase, isocitric dehydrogenase and hexokinase*). The 6-phosphogluconic dehydrogenase should contain no gluconokinase and both enzymes must be essentially free of TPNH oxidase. Suitable preparations give a stable optical density at 340 mμ on the completion of the reaction. If TPNH oxidase is present the optical density will slowly decrease after reaching a maximum.

Preparation of Solutions

I. Potassium hydroxide (1 N):
Dissolve 5.6 g. KOH in distilled water and make up to 100 ml.

II. Perchloric acid (40% w/v):
Dilute 34 ml. $HClO_4$ (sp. gr. 1.67) to 100 ml. with distilled water.

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*) A simple preparation of 6-phosphogluconic dehydrogenase from rat liver has been used to determine 6-phosphogluconic acid[4]. This preparation contains no glucose-6-phosphate dehydrogenase and should be suitable for use in the method described here instead of the 6-phosphogluconic dehydrogenase from yeast.

1) *I. G. Leder*, J. biol. Chemistry *225*, 125 [1957].
2) *R. D. DeMoss* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1957, Vol. III, p. 232.
3) *B. L. Horecker* and *P. Z. Smyrniotis*, J. biol. Chemistry *193*, 371 [1951].
4) *G. E. Glock* and *P. McLean*, Biochem. J. *55*, 400 [1953].
5) *H. Z. Sable* and *A. J. Guarino*, J. biol. Chemistry *196*, 395 [1952].

III. Glycylglycine buffer (1 M; pH 7.4):
Dissolve 13.2 g. glycylglycine in *ca.* 70 ml. distilled water, adjust pH to 7.4 with *ca.* 4 ml. 2 N NaOH and dilute to 100 ml. with distilled water.

IV. Magnesium chloride (0.1 M):
Dissolve 203 mg. $MgCl_2 \cdot 6H_2O$ in distilled water and make up to 10 ml.

V. Adenosine triphosphate (0.1 M; pH 6.5):
Dissolve 60.5 mg. ATP-$Na_2H_2 \cdot 3H_2O$ in distilled water and make up to 1 ml.

VI. Triphosphopyridine nucleotide (0.15 M β-TPN; pH 6.5):
Dissolve 130 mg. TPN-NaH_2 in distilled water and make up to 1 ml.

VII. 6-Phosphogluconic dehydrogenase (ca. 10 units *)/ml.):
If necessary, dilute with buffer solution (III) diluted 1 : 4.

VIII. Gluconokinase (*ca.* 2.5 units *)/ml.):
If necessary, dilute with buffer solution (III) diluted: 1 : 40.

Stability of the solutions

The solutions V—VIII are stored at −15°C. In order to reduce loss of activity by repeated freezing and thawing divide the enzyme solutions into several small portions. Under these conditions the enzyme preparations should retain at least 50% of their original activity after six months storage.

Procedure

Deproteinization

To a solution of the experimental material add

$^1/_{10}$ volume of perchloric acid solution (II).

After 5—10 min. centrifuge and neutralize a portion of the clear supernatant with the previously determined amount of

1 N KOH (solution I).

Cool in an ice bath for 10 min. and centrifuge off the precipitated potassium perchlorate. Take a portion of the supernatant for analysis.

Spectrophotometric measurements

Wavelength: 340 mμ; light path: 1 cm.; final volume: 1 ml.
Read the experimental against the control cuvette. Pipette the solutions in the stated order:

	Experimental cuvette	*Control cuvette*
buffer (solution III)	0.05 ml.	0.05 ml.
ATP solution (V)	0.05 ml.	0.05 ml.
$MgCl_2$ solution (IV)	0.025 ml.	0.025 ml.
TPN solution (VI)	0.02 ml.	0.02 ml.
deproteinized, neutralized supernatant (containing 0.01 to 0.05 μmoles gluconate)	0.10 ml.	—
distilled water	0.63 ml.	0.73 ml.

*) A unit is the amount of enzyme which will catalyse the formation of 1 μmole of product per minute under the test conditions described in the purification of the respective enzymes[1,3].

Mix, read optical density and then into both cuvettes mix

0.025 ml. 6-phosphogluconic dehydrogenase solution (VII).

An increase in optical density indicates the presence of 6-phosphogluconate or substrates of the contaminating TPN-linked dehydrogenases, which are still present in enzyme solution (VII). Wait until a stable optical density is obtained, then add

0.1 ml. gluconokinase solution (VIII)

to both cuvettes, mix and follow the change in optical density until a maximum value is reached.

Calculations

The extinction coefficient ε for TPNH at 340 mμ is 6.22 cm.^{2}/μmole. The gluconic acid content of the assay mixture is calculated from the change in optical density ΔE on addition of gluconokinase.

$$\frac{\Delta E}{6.22} = \mu\text{moles gluconate/assay mixture.}$$

Specificity

Gluconokinase appears to be specific for D-gluconic acid. Neither L-gluconate nor any related sugar acids react with kidney gluconokinase. The specificity of the assay method is limited by the possible presence of TPN-dependent dehydrogenases as contaminants in the test enzyme preparations Therefore the method should be applied with caution to crude experimental material.

Other Methods of Determination

Cohen and *Raff*[6] have described a manometric method based on the fermentation of gluconate by gluconate-adapted *Escherichia coli*, with a lower limit of sensitivity of about 1 μmole.

Appendix

6-Phosphogluconic dehydrogenase [4]

Homogenize livers from rats which have been starved overnight with 8 volumes of ice-cold solution containing 0.15 M KCl and 1.6×10^{-4} M $NaHCO_3$ (pH 7.0) in a Potter-Elvehjem homogenizer. Centrifuge for 60 min. at 2 to 4°C and 4000 g. Fractionate the supernatant with $(NH_4)_2SO_4$ at pH 7.3, collect the fraction between 60 and 70% saturation and dialyse overnight at 4°C against distilled water. Dilute the contents of the dialysis sac to $^1/_{10}$ of the volume of the original supernatant and store at −15°C. The protein content is about 10 mg./ml.

Gluconokinase [1]

Homogenize the cortex (*ca.* 450 g.) from 4 to 5 pig kidneys with 700 ml. 0.1 M phosphate buffer (pH 7.4) in a blendor, centrifuge for 40 min. at 2°C and 4700 g. Extract the residue with distilled water and dilute the combined supernatants to 1700 ml. with distilled water. Adjust to pH 5.0 with 2 N acetic acid, cool to 3—5°C and after 5 min. adjust to pH 5.5 with 5 N KOH. Cool the turbid solution to 0°C and slowly add 800 ml. of acetone at −15°C over a period of 30 min. The temperature must not rise above −8°C. Allow to stand for 5 min. and then centrifuge off the precipitate. To the supernatant add 172 ml. acetone at −10°C and centrifuge off the precipitate. Repeat this process. To the supernatant (*ca.* 2500 ml.) add 1340 ml. acetone at −12°C over a period of 10 min., stir for 5 min. and centrifuge. Dissolve the precipitate in 150 ml. 0.02 M phosphate buffer (pH 7.4), dialyse for two 2 hour periods against 2000 ml. portions of the buffer each time. The contents of the dialysis sac are stable overnight in the frozen state.

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[6] *S. S. Cohen* and *R. Raff*, J. biol. Chemistry *188*, 501 [1951].

Dilute with water to 10 mg. protein/ml. and adjust to pH 7.5 with NH_4OH. If protein precipitates out centrifuge the solution. Add solid $(NH_4)_2SO_4$ to the supernatant (304 ml.) over a period of 40 min. to give 48% saturation. Centrifuge and discard the precipitate. Adjust the supernatant to 56% saturation with $(NH_4)_2SO_4$, centrifuge and dissolve the precipitate in 23 ml. 0.02 M phosphate buffer (pH 7.4).

Dialyse the enzyme solution overnight against 10 litres distilled water. Dilute the contents of the dialysis sac to 3—5 mg. protein/ml. To this solution at pH 5.4 and −10°C add 62.5 ml. acetone over 10 min. Allow to stand for 10 min. in the cold, then centrifuge and discard the precipitate. To the supernatant (165 ml.) add 39.5 ml. acetone at −10°C over a period of 15 min., stir for a further 5 min. and centrifuge. Dissolve the precipitate in 15.5 ml. 0.025 M glycine buffer (pH 7.4). Store at −15°C.