D-Sorbitol

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Sorbitol can be determined spectrophotometrically with the soluble DPN-dependent sorbitol dehydrogenase from liver. This enzymatic method has several advantages over the chemical determination of sorbitol with ferricyanide[1] or periodate[2].

Principle

Sorbitol dehydrogenase (ketose reductase) from mammalian liver[3-6] catalyses the reversible oxidation of sorbitol to D-fructose with diphosphopyridine nucleotide (DPN) as the hydrogen acceptor:

(1) $$\text{Sorbitol} + \text{DPN}^+ \rightleftharpoons \text{D-fructose} + \text{DPNH} + \text{H}^+$$

At pH 0 the equilibrium constant

$$K_H = \frac{[\text{D-fructose}] \times [\text{DPNH}]}{[\text{sorbitol}] \times [\text{DPN}^+]} \text{ is } 3.3 \times 10^9.$$

With an excess of DPN at pH 9.5 sorbitol is quantitatively oxidized and a stoichiometric amount of reduced diphosphopyridine nucleotide (DPNH) is formed. The increase in optical density at 340 mμ due to the formation of DPNH is a measure of the reaction.

Reagents

1. Sodium pyrophosphate, $Na_4P_2O_7 \cdot 10H_2O$
2. Tris-hydroxymethyl-aminomethane, tris
3. Diphosphopyridine nucleotide, DPN
   free acid, DPN; commercial preparation, see p. 1010.
4. Sorbitol dehydrogenase
   from rat liver[7,8] or sheep liver[9] (see p. 208).

For other reagents see sections on "Deproteinization" and "Deionization".

Purity of the enzyme preparation

The sorbitol dehydrogenase (specific activity, determined according to[5], at least 2000 units*)/mg.) should be as free as possible from contaminating DPN-linked dehydrogenases. In particular, it must be free from glucose dehydrogenase and alcohol dehydrogenase. In the absence of substrate there should be no reduction of DPN. The preparations obtained from rat or sheep liver according to[7-9] fulfill these requirements. Such preparations[7,9] are also free from lactic dehydrogenase.

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*) According to[5] a unit is the amount of enzyme in a 1.5 ml. assay mixture, which changes the optical density at 340 mμ by 0.001 in 1 min. at pH 8.6 and 25° C.

1) *W. R. Todd, J. Vreeland, J. Myers* and *E. S. West*, J. biol. Chemistry *127*, 269 [1939].
2) *W. W. Smith, N. Finkelstein* and *H. Smith*, J. biol. Chemistry *135*, 231 [1940].
3) *R. L. Blakley*, Biochem. J. *49*, 257 [1951].
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5) *H. G. Williams-Ashman* and *J. Banks*, Arch. Biochem. Biophysics *50*, 513 [1954].
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9) *H. Holzer* and *H. W. Goedde*, Biochim. biophysica Acta *40*, 297 [1960].

Preparation of Solutions

Prepare all solutions with freshly deionized water which has been distilled in an all glass apparatus. Sterilize glassware in order to prevent the growth of bacteria.

I. Pyrophosphate buffer (0.1 M; pH 9.5):
Dissolve 44.6 g. $Na_4P_2O_7 \cdot 10H_2O$ in 800 ml. distilled water, adjust to pH 9.5 with *ca.* 10 N HCl (measure pH at 25°C with a glass electrode) and dilute to 1000 ml. with distilled water.

II. Tris buffer (0.01 M tris; pH 7.4):
Dissolve 0.121 g. tris-hydroxymethyl-aminomethane in 50 ml. distilled water, adjust to pH 7.4 with *ca.* 8.5 ml. 1 N HCl and dilute to 100 ml. with distilled water.

III. Diphosphopyridine nucleotide (*ca.* 2×10^{-2} M β-DPN):
Dissolve 150 mg. DPN in distilled water and make up to 10 ml.

IV. Sorbitol dehydrogenase (15000 units/ml.):
Dilute the enzyme preparation obtained according to[7–9] with tris buffer (solution II).

Stability of the solutions

The pyrophosphate buffer keeps for at least three weeks at 25°C. Store the DPN and enzyme solution frozen at −20°C. In this state the enzyme solution is stable for two months, but repeated freezing and thawing leads to considerable loss of activity.

Procedure

Experimental material

Freeze tissue samples in liquid nitrogen immediately after removal. The sorbitol content of ram seminal vesicle may increase considerably if allowed to stand at room temperature after removal from the animal[10]. Deproteinize blood and other extracellular fluids immediately.

Deproteinization

Homogenize tissue samples with 5 to 10 volumes of ice-cold 5% (w/v) trichloroacetic acid or 6% (w/v) perchloric acid. Centrifuge off protein. If perchloric acid is used, adjust the supernatant to pH *ca.* 3.5 with 0.5 M $KHCO_3$ solution in an ice bath and centrifuge off the precipitate of potassium perchlorate at 2°C.

The sample can also be deproteinized by homogenization with 10 volumes 66% (v/v) alcohol. Centrifuge and wash the precipitate with four volumes of 66% (v/v) ethanol. Remove the alcohol from the combined supernatants *in vacuo* and extract the aqueous residue three times with twice the volume of chloroform. Remove the residual chloroform from the aqueous phase by passing a stream of nitrogen through the solution.

Deproteinize extracellular fluids (blood, semen) by addition of equimolar amounts of $ZnSO_4$ and $Ba(OH)_2$[11]. Another method used for the deproteinization of semen is to dialyse 5 ml. seminal plasma for 48 hours at 4°C against 100 ml. distilled water with stirring, lyophilize the dialysate and take up the residue in a small amount of water[8].

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[10] *H. G. Britton*, Biochem. J. *69*, 5P [1958].
[11] *T. E. Weichselbaum* and *M. Somogyi*, J. biol. Chemistry *140*, 5 [1941].

Deionization

Deionize deproteinized samples with a mixed-bed ion exchange resin (use a mixture of a strong acid and a strong base resin, *e.g.* Amberlite MB-1 *,[7] or AG 501-X8, 20–50 mesh **). Make a column of the resin and wash thoroughly with distilled water. Allow the deproteinized solution to run through the column and wash with at least two bed volumes of distilled water. The eluate must be completely deionized (check conductivity!). Concentrate the eluate *in vacuo*.

Spectrophotometric measurements

Preliminary remarks: To obtain complete oxidation of the sorbitol the initial sorbitol concentration of the assay mixture must be small in comparison to the initial DPN concentration (DPN: sorbitol >10 and between 0.02 and 0.20 μmoles sorbitol/assay mixture). In preliminary experiments determine the extent to which the deproteinized and deionized sample must be diluted so that 1.00 ml. contains 0.02 to 0.20 μmoles sorbitol.

Method: Wavelength: 340 mμ; light path: 1 cm.; final volume: 3.00 ml.; measure against the control cuvette.

Pipette successively into the cuvettes:

Experimental cuvette	*Control cuvette*
1.00 ml. buffer (solution I)	1.00 ml. buffer (solution I)
0.20 ml. DPN solution (III)	0.20 ml. DPN solution (III)
0.75 ml. distilled water	1.75 ml. distilled water
1.00 ml. deproteinized and deionized sample (containing 0.02 to 0.20 μmoles sorbitol).	

Read the optical density E_1 twice with an interval of 3 min. Mix into both cuvettes

0.05 ml. sorbitol dehydrogenase solution (IV)

and read the optical density every 3 min. until constant: E_2

Calculations

The deproteinized and deionized sample may contain compounds which absorb at 340 mμ. In this case E_1 should be corrected for the dilution on addition of the enzyme solution.

$$\Delta E = E_2 - 0.983 \times E_1$$

The molar extinction coefficient of DPNH at 340 mμ is 6.22 cm.²/μmole and therefore for an assay volume of 3 ml. it follows that:

$$\frac{\Delta E \times 3}{6.22} = \text{μmoles sorbitol/assay mixture.}$$

To obtain the sorbitol content of the sample it is necessary to multiply by the dilution factors on deproteinization and deionization.

Example

A tissue sample was diluted by the deproteinization and deionization procedure, so that 1.00 ml. of extract was equivalent to 0.2 g. tissue (fresh weight) (dilution factor: 5).

*) Rohm & Haas, Washington Square, Philadelphia 5, Pa., USA
**) Bio-Rad Laboratories, 32nd and Griffin Avenue, Richmond, Calif., USA

Experimental protocol:

0 min $E_1 = 0.082$
3 min $E_1 = 0.082$

Addition of sorbitol dehydrogenase

0 min $E_2 = 0.120$
6 min $E_2 = 0.201$
48 min $E_2 = 0.275$
73 min $E_2 = 0.274$

$\Delta E = 0.275 - 0.983 \times 0.082 = 0.194$

$\frac{0.194 \times 3}{6.22} = 0.0936$ μmoles sorbitol/assay mixture or $0.0936 \times 5 \times 100 = 46.8$ μmoles sorbitol/100 g. tissue.

Sources of Error

1. If the deproteinized and deionized sample is turbid, then a further control cuvette containing all the constituents of the assay axcept DPN is necessary.

2. If the extract contains large amounts of free ketoses (*e.g.* D-allulose, D-sedoheptulose, D-fructose, L-sorbose, D-xylulose, D-ribulose, L-erythrulose[3,4,6,7,12]) this can lead to reoxidation of DPNH. In animal tissues large amounts of most of these free ketoses do not occur (exceptions: certain male accessory glands[7,10], seminal plasma[8] and the foetal blood of ungulates[13] contain D-fructose). The extract of the sample should be examined for free ketoses by a colorimetric procedure[14,15]. D-Fructose can be removed by incubation with fermenting yeast[16]. The extract must then be deproteinized and deionized once again.

Specificity

Sorbitol dehydrogenase also catalyses the oxidation of L-iditol, xylitol, D-glycero-D-glucoheptitol, ribitol, allitol and L-threitol (see p. 210). The equilibrium constants for the oxidation of these compounds vary with the chain length[7,17]. Cyclic polyols, for example, *meso*-inositol, are not oxidized. Sorbitol can be identified by chromatographic isolation and conversion to its hexa-acetate[18].

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12) *S. Hollmann*, Hoppe-Seylers Z. physiol. Chem. *317*, 193 [1959].
13) *J. S. D. Bacon* and *D. J. Bell*, Biochem. J. *42*, 397 [1948].
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18) *T. E. King*, *F. A. Isherwood*, *T. Mann:* Abstracts IV. Internat. Congr. Biochem., Vienna 1958. Pergamon Press, London 1958, p. 77.