L-Ribulose and L-Arabinose

Bernard L. Horecker

The cysteine-carbazole reaction developed by *Dische* and *Borenfreund*[1] was used by *Axelrod* and *Jang*[2] to assay pentose isomerases, taking advantage of the fact that ketopentoses are far more reactive in this test than aldopentoses. The cysteine-carbazole reaction also serves to differentiate between ribulose and xylulose, since with ribulose the full colour is developed in 10 minutes, compared with 100 minutes required for xylulose[3]. This difference permits the separate determination of the two sugars in a mixture[4], but the method is not very accurate or completely specific. For example, the D- and L-isomers react identically. A more specific and precise method is the determination of L-ribulose with L-arabinose isomerase and the cysteine-carbazole reaction. The enzyme does not react with D-ribulose, L-xylulose or D-xylulose.

Principle

L-Arabinose isomerase catalyses the reaction:

(1) L-Ribulose $\rightleftharpoons$ L-arabinose

The equilibrium of the reaction lies in favour of L-arabinose, with an equilibrium constant of 7.33 at 34°C. In the presence of excess isomerase 88% of the L-ribulose is converted to L-arabinose. L-Arabinose does not react in the cysteine-carbazole test. Therefore if the colour test is carried out before and after incubation with L-arabinose isomerase the difference in colour intensities is equivalent to 88% of the L-ribulose present in the sample. The reaction is standardized with L-ribulose-*o*-nitrophenylhydrazone.

The same method can be used for the determination of L-arabinose, if the enzymatic reaction is carried out in borate buffer at pH 8.2 instead of tris buffer pH 7.5. At pH 8.2 the equilibrium of reaction (1) is in favour of the ketopentose. Crystalline L-arabinose is used as a standard.

Reagents

See determination of D-xylulose and D-xylose (p. 196), but substituting:

10. L-Arabinose
    crystalline $[\alpha]_D^{20} = +104°$; commercial preparation, see p. 1007.
13. L-Arabinose isomerase
    from *Lactobacillus plantarum*, preparation, see p. 180.

Preparation of Solutions

See determination of D-xylulose and D-xylose (p. 197), but substituting:

VI. L-Arabinose, standard solution (2×10^{-3} M):
    Dissolve 0.3 mg. L-arabinose in distilled water and make up to 1 ml.

VIII. L-Arabinose isomerase (6 mg. protein/ml.):
    Dilute the enzyme solution prepared according to p. 180 with 0.05 M tris buffer (pH 7.5).

Stability of the solutions

See determination of D-xylulose and D-xylose (p. 197).

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1) *Z. Dische* and *E. Borenfreund*, J. biol. Chemistry *192*, 583 [1951].
2) *B. Axelrod* and *R. Jang*, J. biol. Chemistry *209*, 847 [1954].
3) *G. Ashwell* and *J. Hickman*, J. Amer. chem. Soc. *76*, 5889 [1954].
4) *P. K. Stumpf*, *B. L. Horecker*, *P. A. Smyrniotis* and *Y. Takagi*, J. biol. Chemistry *231*, 1031 [1958].

Procedure

Experimental material

See determination of D-xylulose and D-xylose (p. 197).

Standardization of the cysteine-carbazole reaction

See determination of D-xylulose and D-xylose (p. 197).

Determination of L-ribulose

Pipette into a small test tube with a conical tip:

0.30 ml. tris buffer solution (I)
0.04 ml. sample (containing about 2 μmoles L-ribulose).

Mix, remove 0.05 ml. and add

0.01 ml. L-arabinose isomerase solution (VIII)

to the residual mixture. Incubate at 23°C and remove 0.05 ml. samples at 20 min. intervals. Add to these 0.05 ml. samples (S_0, S_1, S_2 . . . . . .)

0.95 ml. distilled water
6 ml. H_2SO_4 (solution IV)
0.2 ml. cysteine solution (II)
0.2 ml. carbazole solution (III),

mix thoroughly after each addition. Allow the mixtures to stand 1 hour at room temperature, then pour into 1 cm. cuvettes and read the optical densities (E_0, E_1, E_2 . . . . . E_{Final}) at 540 mμ. All samples taken after 40 min. should have the same optical density (E_{Final}).

Calculations

The L-ribulose content is calculated according to the formula:

$$\frac{E_0 - E_{Final}}{E_{Standard}} \times 0.1 \times \frac{0.35}{0.05} \times 1.14 = \mu\text{moles L-ribulose/enzymatic incubation mixture}$$

The factor 0.1 allows for the cysteine-carbazole reaction being standardized with 0.1 μmole L-ribulose-*o*-nitrophenylhydrazone. The factor $\frac{0.35}{0.05}$ is to correct for the portion of the enzymatic incubation mixture taken for the colour test, while the factor 1.14 is to correct for the fact that only 88% of the L-ribulose is converted to L-arabinose.

The cysteine-carbazole reaction can also be standardized with L-arabinose instead of L-ribulose-*o*-nitrophenylhydrazone. In this case the procedure is as described above under "Determination of L-ribulose", but instead of the unknown sample take 0.04 ml. 0.25 M arabinose solution (corresponding to 10 μmoles L-arabinose) and only remove a 0.05 ml. sample at the end of the reaction (about 40 min. after addition of the enzyme). At equilibrium the 0.05 ml. sample contains 0.15 μmoles L-ribulose.

To calculate the results with this method the following formula is used:

$$\frac{E_0 - E_{Final}}{E_{Standard}} \times 0.15 \times \frac{0.35}{0.05} \times 1.14 = \mu\text{moles L-ribulose/enzymatic incubation mixture}$$

Determination of L-arabinose

Pipette the following solutions into three test tubes:

	Experimental	*Control*	*Standard*
borate buffer (solution V)	0.15 ml.	0.15 ml.	0.15 ml.
sample (containing about 0.1 μmole L-arabinose)	0.05 ml.	0.05 ml.	—
arabinose solution (VI, corresponding to 0.1 μmole L-arabinose)	—	—	0.05 ml.
enzyme solution (VIII)	0.01 ml.	—	0.01 ml.
distilled water	—	0.01 ml.	—

Mix and incubate for 1 hour at 37°C. To all three tubes add

0.8 ml. distilled water
6 ml. H_2SO_4 (solution IV)
0.2 ml. cysteine solution (II)
0.2 ml. carbazole solution (III),

mix thoroughly after the addition of each reagent. Allow the tubes to stand for 20 min. at room temperature and then read the optical density at 540 mμ.

Calculations

The L-arabinose content of the experimental tube is calculated from the formula:

$$\frac{E_E - E_C}{E_S - E_C} \times 0.1 = \text{μmoles L-arabinose/experimental tube}$$

where

E_E = optical density of experimental tube
E_C = optical density of control tube
E_S = optical density of standard tube

Sources of Error

The values found for L-ribulose are too low if substantial amounts of L-arabinose are present (−14% if L-ribulose : L-arabinose is 1 : 1). Interference with the determination of L-arabinose by L-ribulose and other ketoses is corrected for by the control.

Specificity

Apart from L-ribulose and L-arabinose no other compounds react with L-arabinose isomerase, therefore the method can serve for the determination and identification of the two sugars.

Appendix

Preparation of L-Arabinose Isomerase

Reagents

Difco yeast extract *)
Sodium acetate
Glucose
Magnesium sulphate, $MgSO_4 \cdot 7H_2O$
Ferrous sulphate, $FeSO_4 \cdot 7H_2O$
Manganous sulphate, $MnSO_4 \cdot 4H_2O$
Sodium hydrogen carbonate, $NaHCO_3$
Difco nutrient broth *)
L-Arabinose
Potassium dihydrogen phosphate, KH_2PO_4
Disodium hydrogen phosphate, $Na_2HPO_4 \cdot 2H_2O$
Ammonium sulphate, $(NH_4)_2SO_4$
Tris-hydroxymethyl-aminomethane, tris
Acetone

*) Obtainable from Difco Laboratories, Inc., Detroit 1, Mich., USA.

Preparation of solutions

I. Sodium hydrogen carbonate (0.02 M): Dissolve 1.68 g. $NaHCO_3$ in distilled water and make up to 1000 ml.

II. Phosphate buffer (0.05 M; pH 7.5): Dissolve 0.45 g. KH_2PO_4 and 3.0 g. $Na_2HPO_4 \cdot 2H_2O$ in distilled water and make up to 400 ml.

III. Tris buffer (0.05 M; pH 7.5): Dissolve 2.42 g. tris-hydroxymethyl-aminomethane in 100 ml. distilled water, adjust to pH 7.5 with *ca.* 80 ml. 0.2 N HCl and dilute to 400 ml. with distilled water.

Procedure

Strain of bacteria: Lactobacillus plantarum, strain 124—2 (ATCC 8041).

Growth medium: Contains 0.4% yeast extract; 1% nutrient broth; 1% sodium acetate; 1% L-arabinose; 0.1% glucose; 0.02% $MgSO_4 \cdot 7H_2O$; 0.001% $FeSO_4 \cdot 7H_2O$ and 0.001% $MnSO_4 \cdot 4H_2O$. For stab cultures use the same medium containing 2% agar. Sterilize the sugars separately as a 20 times more concentrated solution and add aseptically to the rest of the sterile medium.

Culture of bacteria: Maintain the bacteria in stab culture and transfer frequently. Prepare successive subcultures of 2, 10 and 100 ml. of medium, incubate for 24 hours at 37°C each time and inoculate each successive subculture with the whole of the previous one. Add the final subculture to 3 l. of medium in a 3 l. flask. Incubate for 18—24 hours at 37°C without aeration until a fine sediment of cells settles out. Harvest the cells at 2°C with a Sharples supercentrifuge and wash with about 100 ml. $NaHCO_3$ solution (I). The cell paste can be stored for 6 months at −16°C.

Preparation of the extract: Suspend 6 g. of cells in 60 ml. phosphate buffer (solution II) and sonicate for six, 15 min. periods at 10 kc. (Raytheon sonic oscillator*), while cooling in ice water. After each sonication allow the suspension to cool to 2°C (3—5 min.).

Fractionation with ammonium sulphate: Centrifuge the sonicated suspension and discard the precipitate. Add 17.1 g. $(NH_4)_2SO_4$ to the supernatant (59 ml.), centrifuge and discard the precipitate. To the supernatant add 7.4 g. $(NH_4)_2SO_4$, centrifuge and discard the precipitate. Add 10.7 g. $(NH_4)_2SO_4$ to the supernatant, centrifuge at 15000 g for 10 min. and dissolve the precipitate in 5 ml. tris buffer (solution III).

Fractionation with acetone: Dilute the solution of the last ammonium sulphate precipitate (9.3 ml.) with 84 ml. tris buffer (solution III) so that the protein content is 1.4 mg./ml. Stir gently in a freezing bath and add 46 ml. cold acetone dropwise. Make the addition over 5 min. and keep temperature at about −10°C. Centrifuge at 3000 g for 2 min. To the supernatant add 19 ml. acetone at −10°C and centrifuge. To the supernatant at −10°C add a further 18 ml. acetone and centrifuge. Dissolve the last precipitate in 4 ml. tris buffer (solution III) and store at −16°C. The preparation can be kept frozen for several months with little loss of activity. The inactive precipitate which forms on thawing should be discarded.

*) Raytheon Mfg. Comp., Waltham, Mass., USA.