D-Glucosamine-6-phosphate

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Principle

D-Glucosamine-6-phosphate can be converted quantitatively to *N*-acetyl-D-glucosamine-6-phosphate by acetyl coenzyme A in the presence of a specific acetylase from baker's yeast[1]. The amount of *N*-acetyl derivative formed can be determined colorimetrically by means of the modification of the colour reaction of *Aminoff, Morgan* and *Watkins*[3], described by *Reissig et al.*[2]. The acetylase preparation used here (see p. 155) contains sufficient of the acetate activating enzyme[4], so that the necessary acetyl-CoA is formed from the components (acetate, ATP, CoA) of the reaction mixture. The rate of acetyl-CoA formation is three to four times faster than the rate of acetyl-CoA consumption in the acetylase reaction.

Reagents

1. Glutathione (SH-form)
   commercial preparation, see p. 1018.
2. Magnesium chloride, $MgCl_2 \cdot 6\,H_2O$
3. Potassium acetate, CH_3CO_2K
4. Coenzyme A (SH-form)
   purity at least 50%, commercial preparation, see p. 1007.
5. Tris-hydroxymethyl-aminomethane, tris
6. Adenosine triphosphate, ATP
   disodium salt, ATP-$Na_2H_2 \cdot 3\,H_2O$; commercial preparation, see p. 1006.
7. Perchloric acid, A. R.; sp. gr. 1.54; *ca.* 60% (w/w)
8. Potassium hydroxide
   purity at least 85%.
9. Boric acid, H_3BO_3
10. Acetic acid
11. Hydrochloric acid, conc.
12. *p*-Dimethylaminobenzaldehyde *)
13. *N*-Acetylglucosamine
    commercial preparation, see p. 1003.
14. Glucosamine-6-phosphate-*N*-acetylase
    from baker's yeast, isolation, see p. 155.

Purity of the enzyme preparation
The preparation described on p. 155 satisfies the requirements.

Preparation of Solutions

I. Magnesium chloride (0.25 M):
Dissolve 5.1 g. $MgCl_2 \cdot 6\,H_2O$ in distilled water and make up to 100 ml.

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*) *e.g.* from Eastman Kodak Co., White label.

1) *D. H. Brown*, Biochim. biophysica Acta *16*, 429 [1955].
2) *J. L. Reissig, J. L. Strominger* and *L. F. Leloir*, J. biol. Chemistry *217*, 959 [1955].
3) *D. Aminoff, W. T. J. Morgan* and *W. M. Watkins*, Biochem. J. *51*, 379 [1952].
4) *M. E. Jones, S. Black, R. M. Flynn* and *F. Lipmann*, Biochim. biophysica Acta *12*, 141 [1953].

II. Potassium acetate (0.1 M):
Dissolve 1 g. CH_3CO_2K in distilled water and make up to 100 ml.

III. Tris buffer (0.20 M; pH 8.0):
Dissolve 2.42 g. tris-hydroxymethyl-aminomethane in distilled water, add 1 ml. conc. HCl (*ca.* 12 N) and make up to 100 ml. with distilled water. If necessary, adjust to pH 8.0 (glass electrode).

IV. Adenosine triphosphate (0.10 M ATP; pH 6.9):
Dissolve 1.58 g. crystalline ATP-$Na_2H_2 \cdot 3\ H_2O$ in distilled water, add 4.0 ml. 1.0 N KOH and make up to 25 ml.

V. Potassium hydroxide (1.0 N):
Dissolve 6.2 g. KOH (minimum purity 85%) in distilled water and make up to 100 ml. Determine the concentration of the solution titrimetrically and, if necessary, adjust to 1.0 N.

VI. Perchloric acid (*ca.* 3 N; *ca.* 30% w/v):
Dilute 33 ml. $HClO_4$ (60% w/w; *ca.* 9 N) to 100 ml. with distilled water.

VII. Potassium borate buffer (0.8 M with respect to borate; pH 9.2):
Dissolve 5.0 g. H_3BO_3 and 2.4 g. KOH (purity at least 85%) in distilled water and make up to 100 ml. If necessary, adjust to pH 9.2–9.3 with 1.0 N KOH (glass electrode).

VIII. *p*-Dimethylaminobenzaldehyde:
a) Dissolve 10 g. *p*-dimethylaminobenzaldehyde in 90 ml. acetic acid. Add 10 ml. conc. HCl (*ca.* 12 N). The solution is stable for a month if stored in the dark at 2–5° C.
b) Immediately before use, mix 5 ml. solution a) with 45 ml. acetic acid. Store the mixture in the cold.

IX. *N*-Acetylglucosamine standard solution (10^{-3} M):
Dissolve 22.1 mg. pure *N*-acetylglucosamine in freshly boiled, cold distilled water and make up to 100 ml. Store the solution at 2–5° C under toluene.

X. Glucosamine-6-phosphate-*N*-acetylase (25 mg. protein/ml.; specific activity: 0.04 μmoles *N*-acetylglucosamine-6-phosphate/min./mg. protein):
Use the stock solution (see p. 155) undiluted. Store frozen and only thaw just before use. The thawed solution may be kept in ice for 6–8 hours. Assay of activity, see p. 153.

Procedure

D-Glucosamine-6-phosphate can be determined in any solution or tissue extract, providing that protein has been removed. The presence of *N*-acetylglucosamine or *N*-acetylglucosamine-6-phosphate in the sample does not interfere. The presence of nitrogen-free hexoses, pentoses and their phosphate esters has no effect on the assay. For further details, see the section "Sources of Error and Specificity".

Deproteinization

Preliminary remarks: If the sample contains protein (*e.g.* tissue extracts, enzymatic reaction mixtures), it should be removed with perchloric acid so that the determination of glucosamine-6-phosphate is more satisfactory. The perchlorate ion is then largely removed from the supernatant by addition of KOH to form the insoluble $KClO_4$.

Method: Prepare a 12 ml. conical centrifuge tube with

1.0 ml. sample
0.20 ml. perchloric acid solution (VI)

or with multiples of these volumes. Allow to stand for 10 min. in an ice bath. Centrifuge for 10 min. at 3000 g and carefully transfer the clear supernatant to a conical 12 ml. graduated centrifuge tube with a pipette having a small piece of glass wool in the tip (to prevent the transfer of protein). The solution is approximately 0.5 N with respect to $HClO_4$. To every 1.0 ml. supernatant add

0.40 ml. KOH (solution V).

Determine the pH with indicator paper or, if the volume is sufficiently large, with a pH meter, and adjust to between pH 6 and 7 by the careful addition of

KOH (solution V) (up to 0.1 ml. may be required).

Allow the mixture to stand for 15 min. in an ice bath to permit the complete crystallization of $KClO_4$. Then centrifuge at 3000 g (5° C) and decant off the supernatant fluid.

Enzymatic acetylation of glucosamine-6-phosphate

Add to a 10 ml. beaker not more than

2.0 ml. of the neutralized supernatant (containing up to 2.5 μmoles glucosamine-6-phosphate).

If necessary, make up to 2.0 ml. with distilled water. Then add in the following order:

15 mg. glutathione (SH-form)
0.05 ml. potassium acetate solution (II)
0.15 ml. distilled water
2.00 ml. tris buffer (solution III)
0.10 ml. ATP solution (IV)
0.20 ml. $MgCl_2$ solution (I)
2 mg. coenzyme A (calculated as pure CoA-SH).

Warm the mixture to 37° C in a water bath and keep at this temperature until the end of the experiment. Measure the pH with a glass electrode and, if necessary, adjust to pH 7.8 (7.7—7.9) with

KOH (solution V).

Start the enzymatic reaction by the addition of

0.50 ml. glucosamine-6-phosphate-*N*-acetylase solution (X)

and record the time. Immediately after the addition of the enzyme, remove 1 ml. of the reaction mixture with a pipette and transfer to a test tube, cover this with a marble, heat for 4 min. in a boiling water bath, cool and stopper. After 45 min. incubation, take another 1 ml. of the reaction mixture and heat as described above. Incubate the remainder of the incubation mixture for a further 45 min. at 37° C, then treat all the solution as above.

During the 90 min. incubation period with enzyme the pH of the reaction mixture falls slightly, but usually not below 7.3 (measured at 37° C).

Colorimetric determination of N-acetylglucosamine-6-phosphate

Pipette into three test tubes (13 mm. diameter, 100 mm. long):

0.5 ml. of the 0, 45 and 90 min. samples of the boiled reaction mixture.

Prepare another test tube (control) with 0.5 ml. distilled water
and three other test tubes (standards) with

a) 0.05 ml. solution IX (= 0.05 μmoles *N*-acetylglucosamine) and 0.45 ml. distilled water
b) 0.10 ml. solution IX (= 0.1 μmoles *N*-acetylglucosamine) and 0.40 ml. distilled water
c) 0.20 ml. solution IX (= 0.2 μmoles *N*-acetylglucosamine) and 0.30 ml. distilled water.

To the seven tubes add

0.10 ml. potassium borate buffer (solution VII),

stopper the tubes with marbles and heat for exactly 3 min. in a boiling water bath. Cool with tap water and add

3.0 ml. *p*-dimethylaminobenzaldehyde solution (VIII)

to each tube. Mix and incubate for exactly 20 min. at 37° C. Cool with tap water to 15—20° C. Set the optical density of the control solution to zero at 544 mμ and read the optical densities of the other six solutions against the control. Use cuvettes with a light path of 1 cm.

Calculations

The optical densities of the standards must be proportional to their *N*-acetylglucosamine content. The optical density of a standard containing 0.1 ml. solution IX is *ca.* 0.550. From the optical densities of the standard solutions is calculated the mean absorption ($E_{standard}$) for 0.10 μmoles *N*-acetylglucosamine in 3.6 ml. final reaction mixture. The optical densities of the three experimental solutions are designated

$E^{0\ min.}_{sample}$, $E^{45\ min.}_{sample}$ and $E^{90\ min.}_{sample}$. The differences

$E^{45\ min.}_{sample} - E^{0\ min.}_{sample}$ and $E^{90\ min.}_{sample} - E^{0\ min.}_{sample}$, which are usually nearly the same, are averaged to obtain ΔE_{sample}. This value is related to $E_{standard}$. Hence:

$\frac{\Delta E_{sample}}{E_{standard}} \times 0.1 =$ μmoles *N*-acetylglucosamine-6-phosphate/reaction mixture of the colorimetric estimation.

The value for 1 ml. of the original sample is obtained by multiplying by the dilution factors (see p. 37) for deproteinization and neutralization, and by the factor 0.5 (0.5 ml. of the enzymatic reaction mixture was used for the colorimetric determination).

Example

A tissue homogenate (2.00 ml.) was deproteinized with 0.4 ml. $HClO_4$. 1.90 ml. of the filtrate was added to a centrifuge tube and neutralized with 0.95 ml. KOH. 2.00 ml. of the neutralized solution was taken for the enzymatic reaction. 0.50 ml. of the enzymatic reaction mixture was found to contain 0.04 μmoles *N*-acetylglucosamine-6-phosphate. The *N*-acetylglucosamine-6-phosphate content of the sample was therefore:

$$0.04 \times 10 \frac{1.90 + 0.95}{2.00} \times \frac{2.00 + 0.40}{1.90} \times \frac{1}{2} = 0.36 \text{ μmoles/ml.}$$

Sources of Error and Specificity

If the sample contains D-glucosamine, this will be converted to D-glucosamine-6-phosphate by the hexokinase present as an impurity in the yeast glucosamine-6-phosphate-*N*-acetylase. In this case, the sum of the glucosamine and glucosamine-6-phosphate is obtained. Glucosamine-1-phosphate does not usually interfere, since it is not a substrate for the acetylase and is only converted very slowly to glucosamine-6-phosphate by the phosphoglucomutase which contaminates the yeast enzyme preparation. This conversion is further retarded by the very low concentration of α-glucose-1,6-diphosphate in the well-dialysed yeast enzyme.

Appendix

Preparation of D-glucosamine-6-phosphate-N-acetylase

Solutions

I. Dipotassium hydrogen phosphate (0.1 M): dissolve 17.4 g. K_2HPO_4 in distilled water and make up to 1000 ml.

II. Protamine sulphate (2%): dissolve 0.4 g. protamine sulphate in distilled water and make up to 20 ml. Filter off any insoluble material at room temperature.

III. EDTA (0.002 M): Dissolve 0.75 g. disodium ethylene-diamine-tetra-acetate · 2 H_2O in distilled water and make up to 1000 ml. If necessary, adjust to between pH 6.8 and 7.0 with 1 N NaOH.

Procedure

Suspend 70 g. fresh baker's yeast (Fleischmann) in 70 ml. cold K_2HPO_4 solution (I). Sonicate the suspension for 40 min. in two portions (10 kc Raytheon*) sonic oscillator, current: 0.85 amp.), cooling the oscillation chamber with ice-water. Centrifuge for 30 min. in the cold at about 10000 g in an angle-head centrifuge (Servall) and then centrifuge the supernatant for 70 min. at 100000 g (Spinco centrifuge, Model L). Measure the volume of the clear supernatant (about 78 ml.).

Add protamine sulphate solution (II) to the cold supernatant (0.8 ml./10 ml.). Allow the mixture to stand for 30 min. in the cold and then centrifuge for 30 min. at 10000 g in an angle-head centrifuge (Servall). Dialyse the supernatant in the cold for 18 hours against two 2000 ml. portions of EDTA solution (III). If the contents of the dialysis sac are turbid, centrifuge again at 10000 g in an angle-head centrifuge. Freeze the supernatant in small portions. The enzyme is stable for a long period in this state.

The solution contains 22–27 mg. protein/ml. and catalyses the formation of 0.04 μmoles *N*-acetyl-glucosamine-6-phosphate/min./mg. protein.

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