# D-Glucosamine

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## Principle

D-Glucosamine is phosphorylated by adenosine triphosphate (ATP) in the presence of yeast hexokinase to give D-glucosamine-6-phosphate[1], which can be quantitatively removed from the solution with $Ba(OH)_2$-$ZnSO_4$. The glucosamine content can be found from the difference in the amounts of amino sugar measured before and after successive treatment with hexokinase-ATP and $Ba(OH)_2$-$ZnSO_4$. Of the known free amino sugars and amino sugar phosphates, only D-mannosamine interferes with the reaction; it behaves exactly like D-glucosamine. The determination of D-glucosamine in the presence of D-galactosamine with the aid of hexokinase has been described by *Slein*[2].

## Reagents

1. Barium hydroxide, $Ba(OH)_2 \cdot 8\,H_2O$
2. Zinc sulphate, $ZnSO_4 \cdot 7\,H_2O$
3. Tris-hydroxymethyl-aminomethane, tris
4. Magnesium chloride, $MgCl_2 \cdot 6\,H_2O$
5. Adenosine triphosphate, ATP
   disodium salt, $ATP\text{-}Na_2H_2 \cdot 3\,H_2O$; commercial preparation, see p. 1006.
6. Bovine serum albumin
   crystalline.
7. Hydrochloric acid, 1 N and conc.
8. Potassium hydroxide
   at least 85% pure.
9. D-Glucosamine hydrochloride
   commercial preparation, see p. 1017.
10. Ethanol, 95%
11. Sodium carbonate, $Na_2CO_3$, anhydrous.
12. *p*-Dimethylaminobenzaldehyde *)
13. 2,4-Pentanedione (acetyl acetone) *)
14. Yeast hexokinase, HK
    preferably crystalline; commercial preparation, see p. 983.

### Purity of the enzyme preparation

The hexokinase should be at least 30% pure, *i. e.* the turnover number of the enzyme at pH 7.5 should be at least 4000 moles of glucose/$10^5$ g. protein/minute. Hexokinase can be prepared by the method of *Kunitz* and *McDonald*[3,4] or by the method of *Berger* et al.[5]. *Sols* et al.[6] have

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*) For example: Eastman Kodak Co., White Label.


1) *D. H. Brown*, Biochim. biophysica Acta *7*, 487 [1951].
2) *M. W. Slein*, Proc. Soc. exp. Biol. Med. *80*, 646 [1952].
3) *M. Kunitz* and *M. R. McDonald*, J. gen. Physiol. *29*, 393 [1946].
4) *M. R. McDonald* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1955, Vol. I, p. 269.
5) *L. Berger, M. W. Slein, S. P. Colowick* and *C. F. Cori*, J. gen. Physiol. *29*, 379 [1946].
6) *A. Sols, G. De La Fuente, C. Villar-Palasi* and *C. Asensio*, Biochim. biophysica Acta *30*, 92 [1958].

described a modification of the method of *Kunitz* and *McDonald* which yields a partly purified preparation suitable for the assay of D-glucosamine. Commercial preparations are frequently not sufficiently pure.

## Preparation of Solutions

I a) Barium hydroxide (*ca.* 0.15 M):
Dissolve 47 g. $Ba(OH)_2 \cdot 8H_2O$ in freshly boiled distilled water and make up to 1000 ml.

b) Zinc sulphate (5% w/v $ZnSO_4 \cdot 7H_2O$):
Dissolve 50 g. $ZnSO_4 \cdot 7H_2O$ in freshly boiled distilled water and make up to 1000 ml. Titrate 10 ml. of solution (b) with solution (a) using phenolphthalein as indicator. Adjust the concentrations of the solutions so that 10.0 ml. of (b) require 9.7 to 9.8 ml. of (a) to reach the phenolphthalein end-point. Store solutions (a) and (b) in bottles fitted with syphon tubes. Solution (a) should also be protected from atmospheric $CO_2$ with a soda lime tube.

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

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

IV. Adenosine triphosphate (0.10 M ATP; pH 6.9):
Dissolve 1.58 g. ATP-$Na_2H_2 \cdot 3H_2O$ (crystalline) in distilled water, add 4.0 ml. 1.0 N KOH and make up to 25 ml. If necessary, adjust pH to 6.8–6.9 (glass electrode).

V. Potassium hydroxide (1.0 N):
Dissolve 6.2 g. KOH (purity at least 85%) in distilled water and make up to 100 ml.

VI. Serum albumin (1.25 mg. protein/ml.):
Dissolve 250 mg. crystalline bovine serum albumin in distilled water and make up to 200 ml. Store solution at 2–5°C.

VII. D-Glucosamine hydrochloride, standard solution ($10^{-3}$ M):
Dissolve 21.5 mg. pure D-glucosamine hydrochloride in freshly boiled distilled water and make up to 100 ml. Store solution at 2–5°C under toluene.

VIII. Sodium carbonate (0.25 M):
Dissolve 26.5 g. anhydrous $Na_2CO_3$ and make up to 1000 ml.

IX. *p*-Dimethylaminobenzaldehyde:
Dissolve 3.2 g. *p*-dimethylaminobenzaldehyde in 120 ml. 95% ethanol, add 120 ml. of conc. HCl (*ca.* 12 N) and store in a brown bottle at 2–5°C.

X. 2,4-Pentanedione (acetyl acetone):
Mix 0.5 ml. 2,4-pentanedione with 25 ml. 0.25 M $Na_2CO_3$ solution (VIII). Prepare immediately before use, do not store!

XI. Yeast hexokinase, HK (0.5 mg. enzyme protein/ml.):
Immediately before use dilute the purest enzyme preparation available with ice-cold serum albumin solution to give a final concentration of pure hexokinase of 0.5 mg./ml. If the hexokinase preparation is only 50% pure, the final dilution should contain 1 mg. protein/ml. Keep the enzyme dilution at 0°C until required.

**Procedure**

D-Glucosamine can be determined in any solution or tissue extract from which protein and amino sugar phosphates have been removed (see "Barium-Zinc filtrate"). For each sample, three replicates are prepared for the enzymic phosphorylation (for measurement after 0, 15 and 30 min. incubation). In the colorimetric analysis a reagent blank and the three standards in duplicate are required.

*Barium-Zinc filtrate*[7]: Into conical 12 ml. centrifuge tubes pipette:

2.00 ml. sample (containing 1 – 20 μmoles glucosamine)
2.00 ml. barium hydroxide solution (Ia),

mix and add

2.00 ml. zinc sulphate solution (Ib).

Shake for 1 min., allow to stand 15 min. in a refrigerator (5° C) and then centrifuge for 5 min. at 3000 g. Filter supernatant using a small paper (Whatman No. 1) in a small funnel. Not more than 2 ml. of solution should be retained by the filter paper. The filtrate must be clear.

*Enzymatic phosphorylation:* To a 10 ml. beaker add

3.0 ml. barium-zinc filtrate

and then in the stated order add:

1.30 ml. tris buffer (solution II)
0.20 ml. albumin solution (VI)
0.30 ml. ATP solution (IV)
0.20 ml. $MgCl_2$ solution (III).

Measure the pH of the mixture and, if necessary, adjust with

1 N KOH or 1 N HCl

to 7.8 – 8.0. Note the volume of KOH or HCl added! Then treat the solutions as described under (1) and (2) below:

(1) *Zero time value* for substances in the barium-zinc filtrate which react in the modified *Elson-Morgan* method[8,9] for amino sugars: Into a 12 ml. centrifuge tube pipette:

2.00 ml. distilled water
1.00 ml. $Ba(OH)_2$ solution (Ia)
1.00 ml. of the phosphorylation reaction mixture
1.00 ml. $ZnSO_4$ solution (Ib).

Shake for 1 min. and filter as described under "Barium-Zinc filtrate".

(2) *15 and 30 minute values* after enzymatic phosphorylation of the glucosamine. The 30 minute value indicates whether the reaction has gone to completion. Into a test tube pipette:

3.00 ml. of the phosphorylation reaction mixture prepared above.

Bring to 30° C in a thermostatically controlled bath, add

0.10 ml. hexokinase solution (XI)

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7) *M. Somogyi*, J. biol. Chemistry *160*, 69 [1945].
8) *L. A. Elson* and *W. T. J. Morgan*, Biochem. J. *27*, 1824 [1933].
9) *D. H. Brown* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1957, Vol. III, p. 158.

start timing and continue incubation at 30°C. After exactly 15 and 30 min., add to a 1.0 ml. portion of the incubation mixture:

2.00 ml. distilled water
1.00 ml. $Ba(OH)_2$ solution (I a)
1.00 ml. $ZnSO_4$ solution (I b),

and proceed as described under "Zero time value". Both filtrates, together with the filtrate from (1) are analysed colorimetrically.

*Colorimetric analysis* (duplicate estimations):

Into 14 test tubes graduated at 10 ml., pipette the following solutions:

| | | |
|---|---|---|
| *1* and *2* | | 2.00 ml. $H_2O$ |
| *3* and *4* | 0.10 ml. solution VII | + 1.90 ml. $H_2O$ |
| *5* and *6* | 0.20 ml. solution VII | + 1.80 ml. $H_2O$ |
| *7* and *8* | 0.40 ml. solution VII | + 1.60 ml. $H_2O$ |
| *9* and *10* | 1.00 ml. zero time filtrate | + 1.00 ml. $H_2O$ |
| *11* and *12* | 1.00 ml. 15 min. filtrate | + 1.00 ml. $H_2O$ |
| *13* and *14* | 1.00 ml. 30 min. filtrate | + 1.00 ml. $H_2O$ |

Also to each tube add

1.00 ml. 2,4-pentanedione solution (X),

mix, cover test tubes with glass marbles and place in a boiling water bath for 30 min. Cool in water to 20–25°C. Then quickly add to each tube *without mixing*

5.00 ml. 95% ethanol.

Add to the first tube

1.00 ml. *p*-dimethylaminobenzaldehyde solution (IX)

and make up to 10 ml. with

95% ethanol.

Cover tube with Parafilm and mix by inversion. Remove Parafilm, cover tube with a glass marble and place in a water bath at 37°C, noting the time. Treat the remaining 13 tubes, one after another, in the same manner, commence incubation at 37°C and record time. Exactly 30 min. later read each tube against water in a photoelectric colorimeter at about 540 mμ (*e. g.* Klett colorimeter, filter No. 54).

**Calculations**

The colorimeter readings for each pair of duplicate tubes are averaged, the average of the reagent blank values (tube 1 and 2, *ca.* 16 Klett units) is subtracted from the other averages. The following values are obtained:

$$E^{0\text{ min.}}_{\text{sample}} \; ; \; E^{15\text{ min.}}_{\text{sample}} \; ; \; E^{30\text{ min.}}_{\text{sample}} \; ; \text{ and } E_{\text{standard}}$$

(the latter for 0.10, 0.20 and 0.40 μmoles glucosamine). From the $E_{\text{standard}}$ values the mean value for 0.10 μmole of glucosamine is calculated, assuming linear proportionality between amounts added and colour obtained. This assumption is usually valid, 0.10 μmole of glucosamine gives a value of about 45 Klett units.

To calculate the glucosamine content of the sample the difference

$$E^{0\text{ min.}}_{\text{sample}} - E^{15\text{ min.}}_{\text{sample}} = \Delta E_{\text{sample}}$$

is taken as the basis and this is divided by $E_{standard}$ for 0.10 μmole glucosamine. Usually the hexokinase reaction is completed after 15 min. $E_{sample}^{15\,min.}$ and $E_{sample}^{30\,min.}$ are averaged. If $E_{sample}^{30\,min.}$ is significantly smaller than $E_{sample}^{15\,min.}$ , then only $E_{sample}^{30\,min.}$ is used.

Therefore: $\frac{\Delta E_{sample}}{E_{standard}} \times 0.1 =$ μmoles glucosamine in the 1.0 ml. of filtrate taken for colorimetric estimation.

1 ml. of the original untreated sample contains 25 times this value.

## Sources of Error and Specificity

D-Mannosamine (2-amino-2-deoxy-D-mannose) reacts like D-glucosamine, because in the presence of hexokinase it is phosphorylated by ATP[10]. Although enzymic mechanisms are known in tissues, which can give rise to *N*-acetylmannosamine, the formation of the free amino sugar has not yet been observed. Nevertheless it is advisable to confirm the identity of any sugar giving a positive reaction in the D-glucosamine determination by paper chromatography.

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[10] *L. Glaser*, personal communication.