D-Fructose

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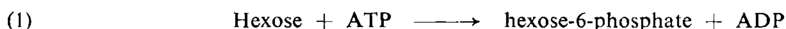
Chemical methods for the determination of fructose, for example, the colorimetric determination with resorcinol, are interfered with to some extent by glucose and related substances. The enzymatic method[1] provides a rapid and specific determination of fructose. With the method described here, fructose, glucose, glucose-6-phosphate and fructose-6-phosphate can be estimated in the same reaction mixture[1a].

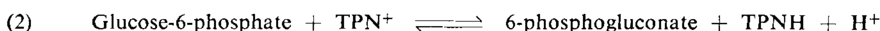
Principle

According to *M. R. McDonald*[2] hexoses such as glucose, fructose and mannose are phosphorylated to the corresponding hexose-6-phosphate by ATP and hexokinase (HK):



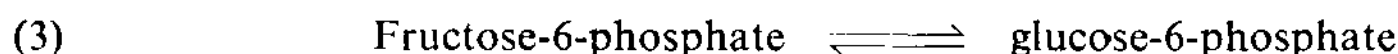
(1) Hexose + ATP ⟶ hexose-6-phosphate + ADP

Of the hexose phosphates formed, glucose-6-phosphate (G-6-P) is first oxidized with glucose-6-phosphate dehydrogenase (G6P-DH) and TPN to give 6-phosphogluconate and TPNH:



(2) Glucose-6-phosphate + TPN^+ ⇌ 6-phosphogluconate + TPNH + H^+

After completion of this reaction fructose-6-phosphate (F-6-P) is determined. The reaction mixture contains the F-6-P originally present in the sample and also the F-6-P formed according to equation (1) from fructose. By addition of phosphoglucose isomerase (PGI) the F-6-P is converted to G-6-P:



(3) Fructose-6-phosphate ⇌ glucose-6-phosphate

The G-6-P is estimated according to equation (2).

The increase of optical density at 366 or 340 mμ due to the formation of TPNH is measured.

The equilibria of reactions (1) and (2) lie far to the right[3,4]. The equilibrium of reaction (3) is not important, since the G-6-P formed immediately reacts according to equation (2). All three reactions proceed stoichiometrically.

Reagents

1. Triethanolamine hydrochloride
2. Sodium hydroxide, A. R., 1 N
3. Magnesium chloride, A. R., $MgCl_2 \cdot 6\ H_2O$
4. Adenosine triphosphate, ATP
   crystalline sodium salt, $ATP\text{-}Na_2H_2 \cdot 3\ H_2O$; commercial preparation, see p. 1006.
5. Triphosphopyridine nucleotide, TPN
   sodium salt, $TPN\text{-}NaH_2$; commercial preparation, see p. 1029.
6. Hexokinase, HK
   crystalline, from yeast, suspension in 3.0 M ammonium sulphate solution; specific activity at least 140 units*)/mg.; commercial preparation, see p. 983.
7. Glucose-6-phosphate dehydrogenase, G6P-DH
   from yeast, suspension in 3.2 M ammonium sulphate solution; specific activity at least 70 units*)/mg.; commercial preparation, see p. 975.

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*) A unit is the amount of enzyme which converts 1 μmole of substrate in 1 min. at 25° C.

[1] *F. H. Schmidt*, Klin. Wschr. *39*, 1244 [1961].
[1a] *J. Cooper, P. A. Srere, M. Tabachnick* and *E. Racker*, Arch. Biochem. Biophysics *74*, 306 [1958].
[2] *M. R. McDonald* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1955, Vol. I, p. 326.
[3] *J. L. Gamble* and *V. A. Najjar*, J. biol. Chemistry *217*, 595 [1955].
[4] *L. Glaser* and *D. H. Brown*, J. biol. Chemistry *216*, 67 [1955].

8. Phosphoglucose isomerase, PGI
crystalline, from yeast, suspension in 2.4 M ammonium sulphate solution; specific activity at least 390 units *)/mg.; commercial preparation, see p. 993.

Purity of the enzyme preparations

Relative to their own specific activities, the hexokinase, glucose-6-phosphate dehydrogenase and phosphoglucose isomerase must contain no more than 0.1% TPNH oxidase, 6-phosphogluconic dehydrogenase or phosphoglucomutase, and no more than 0.01% invertase. In the same terms, the HK and G6P-DH must contain no more than 0.5% hexose isomerases.

Preparation of Solutions

To prevent the growth of micro-organisms sterilize the containers.

I. Triethanolamine buffer (0.05 M; pH 7.6):
Dissolve 9.3 g. triethanolamine hydrochloride in 22 ml. N NaOH and dilute to 1000 ml. with doubly distilled water. Check the pH (glass electrode).

II. Magnesium chloride (0.1 M):
Dissolve 2.03 g. $MgCl_2 \cdot 6 H_2O$ in doubly distilled water and make up to 100 ml.

III. Adenosine triphosphate (*ca.* 0.017 M ATP):
Dissolve 10 mg. $ATP\text{-}Na_2H_2 \cdot 3 H_2O$ in 1 ml. doubly distilled water.

IV. Triphosphopyridine nucleotide (*ca.* 0.012 M β-TPN):
Dissolve 10 mg. $TPN\text{-}NaH_2$ in 1 ml. doubly distilled water.

V. Hexokinase, HK (1 mg. protein/ml.):
Dilute the stock suspension with 3.0 M ammonium sulphate solution.

VI. Glucose-6-phosphate dehydrogenase, G6P-DH (1 mg. protein/ml.):
Dilute the stock suspension with 3.2 M ammonium sulphate solution.

VII. Phosphoglucose isomerase, PGI (1 mg. protein/ml.):
Dilute the stock suspension with 2.4 M ammonium sulphate solution.

Stability of the solutions

Store all solutions, stoppered, in a refrigerator at 0 to 4° C. In this state they keep for several weeks.

Procedure

Preliminary treatment of the experimental material

Biological material, for example, blood, tissue homogenates and plant extracts must be deproteinized before the determination[1,5]. Dissolve soluble samples in distilled water, adjust to a suitable volume and use directly (*e.g.* a 0.2% solution of honey or artificial honey). Shake material which is only partially soluble (milk powder or ice-cream powder) with doubly distilled water for *ca.* 10 min. Filter off the insoluble residue, wash the filter with doubly distilled water and adjust the filtrate to a suitable volume.

Spectrophotometric measurements

Wavelength: 366 mμ or 340 mμ; light path: 1 cm.; final volume: 3.0 ml.; room temperature. Measure against the blank.

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*) A unit is the amount of enzyme which converts 1 μmole of substrate in 1 min. at 25°C.

5) *M. W. Slein*, chapter on "Glucose", p. 117.

Pipette successively into the cuvettes:

Blank:	2.88 ml. buffer (solution I)
	0.02 ml. sample
Experimental:	2.65 ml. buffer (solution I)
	0.10 ml. $MgCl_2$ solution (II)
	0.10 ml. ATP solution (III)
	0.10 ml. TPN solution (IV)
	0.02 ml. sample
	0.01 ml. HK suspension (V).

Mix thoroughly with a small glass or plastic rod flattened at one end and read the optical density E_1.

Mix in

0.01 ml. G6P-DH suspension (VI).

Read the optical density after 10, 12, 14 and 16 min., and extrapolate to the time of the G6P-DH addition (E_2). Mix in

0.01 ml. PHI suspension (VII),

wait for the end of the reaction and then read the optical density E_3.

$$E_3 - E_1 = \Delta E_{\text{glucose + fructose + G-6-P + F-6-P}}$$

$$E_2 - E_1 = \Delta E_{\text{glucose + G-6-P}}$$

$$E_3 - E_2 = \Delta E_{\text{fructose + F-6-P}}$$

Even highly purified HK and G6P-DH preparations contain traces of PGI. Consequently, there is a slow conversion of F-6-P before the addition of PGI to the assay system and for this reason E_2 must be obtained exactly by extrapolation (E_2 values not obtained by extrapolation give erroneous results).

If the sample contains fructose-6-phosphate (and glucose-6-phosphate), prepare a second cuvette containing doubly distilled water instead of hexokinase and measure as described above. The measured values are indicated by E_1', E_2' and E_3'.

$$E_3' - E_1' = \Delta E_{\text{G-6-P + F-6-P}}$$

$$E_2' - E_1' = \Delta E_{\text{G-6-P}}$$

$$E_3' - E_2' = \Delta E_{\text{F-6-P}}$$

$\Delta E_{\text{fructose + F-6-P}} - \Delta E_{\text{F-6-P}} = \Delta E_{\text{fructose}}$; this value is used for the calculations.

Calculations

For a final volume in the cuvette of 3.0 ml. (refer to p. 37),

$$\text{at 340 m}\mu\text{:} \quad \frac{\Delta E_{\text{fructose}} \times 3.0}{6.22} = \mu\text{moles fructose/reaction mixture}$$

$$\text{at 366 m}\mu\text{:} \quad \frac{\Delta E_{\text{fructose}} \times 3.0}{3.3} = \mu\text{moles fructose/reaction mixture}$$

Similar formulae are valid for the calculation of the values for glucose, G-6-P and F-6-P. To convert from μmoles to μg. it is necessary to multiply by the respective molecular weights (molecular weight of fructose = 180.2).

To obtain the fructose content of the substance being analysed, the weight taken and the dilution must be allowed for.

Example

0.2 g. artificial honey were dissolved in doubly distilled water, made up to 100 ml. and 0.02 ml. of this was taken for the assay. The following optical densities were measured at 366 mμ: $E_1 = 0.020$, $E_2 = 0.124$, $E_3 = 0.220$. The sample was free from G-6-P and F-6-P. $\Delta E_{glucose + fructose} = 0.200$; $\Delta E_{glucose} = 0.104$ and $\Delta E_{fructose} = 0.096$. Therefore 100 ml. of the sample (= 0.2 g. artificial honey) contained

$$\frac{0.096 \times 3.0 \times 100 \times 180.2}{3.3 \times 0.02} = 78\,700 \ \mu g. \text{ fructose}$$

and by a similar calculation it also contained 85 200 μg. glucose.

Sources of Error

Insufficient purity of the reagents used, especially the enzymes, leads to erroneous results. For example, if one of the enzymes contains too much 6-phosphogluconic dehydrogenase, then the fructose values will be too high. On the other hand, if one of the enzymes contains too much TPNH oxidase, then the fructose results will be too low. Other phosphorylated or free sugars do not interfere.

Specificity

Glucose-6-phosphate dehydrogenase and phosphoglucose isomerase are specific for G-6-P or F-6-P. Mannose, which can be phosphorylated according to equation (1), does not react further in reactions (2) and (3).