

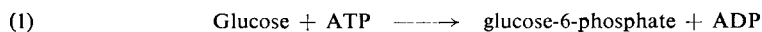
D-Glucose

Determination with Hexokinase and Glucose-6-phosphate Dehydrogenase^{*)}

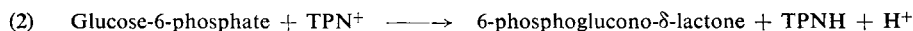
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Principle

The optical method for the determination of glucose-6-phosphate on which the following method is based was developed by *O. Warburg* and his collaborators. Hexokinase catalyses the phosphorylation of glucose by adenosine triphosphate (ATP):



Glucose-6-phosphate is oxidized in the presence of triphosphopyridine nucleotide (TPN) by glucose-6-phosphate dehydrogenase:



For all practical purposes, both reactions proceed stoichiometrically and quantitatively, although reversibility has been demonstrated under special conditions¹⁻³⁾. The TPNH arising in equation (2) is determined spectrophotometrically at 340 or 366 m μ and serves as a measure of the glucose-6-phosphate formed from glucose in equation (1).

Reagents

1. Zinc sulphate, ZnSO₄·7H₂O, A. R.
2. Barium hydroxide, Ba(OH)₂·8H₂O, A. R.
3. Tris-hydroxymethyl-aminomethane, tris
4. Magnesium chloride, MgCl₂·6H₂O, A. R.
5. Serum albumin
crystalline.
6. Adenosine triphosphate, ATP
disodium salt, ATP-Na₂H₂·3H₂O; commercial preparation, see p. 1006.
7. Triphosphopyridine nucleotide, TPN
monosodium salt, TPN-Na-H₂; commercial preparation, see p. 1029.
8. Hexokinase, HK
crystalline, from baker's yeast; commercial preparation, see p. 983.
9. Glucose-6-phosphate dehydrogenase, G6P-DH
purified from brewer's yeast; commercial preparation, see p. 975.

Purity of the enzyme preparations

Hexokinase: The preparation need not be crystalline, but must be relatively free from compounds which interfere with the glucose determination. Sometimes the preparations still contain glucose,

^{*)} For the determination with hexokinase, pyruvic kinase and lactic dehydrogenase, see "Glycogen", p. 59.

¹⁾ *J. L. Gamble jr.* and *V. A. Najjar*, *Science* [Washington] *120*, 1023 [1954]; *J. biol. Chemistry* *217*, 595 [1955].

²⁾ *S. Kaufman*, *J. biol. Chemistry* *216*, 153 [1955].

³⁾ *B. L. Horecker* and *P. Z. Smyrniotis*, *Biochim. biophysica Acta* *12*, 98 [1953].

which has been added as a stabilizing agent during purification^{4,5}). It can be removed from highly purified hexokinase by dialysis. The least interference from contaminating enzymes occurs, if the specific activity of the hexokinase preparation used approaches that of the crystalline enzyme (ca. 3000 BSCC⁴) units/mg. or 1400 KM⁵) units/mg. *). Hexokinase of this purity catalyses the phosphorylation of ca. 32 mg. glucose/min./mg. protein at 30°C and pH 8.0. An improved method for the purification of hexokinase which results in a yield of crystalline enzyme representing 15% of the activity of the crude extract (instead of 1% by other methods^{4,5}) is described by *Darrow and Colowick*⁶).

Glucose-6-phosphate dehydrogenase: The enzyme must be essentially free from 6-phosphogluconic dehydrogenase, otherwise with known glucose concentrations values for TPNH will be too high and reaching of the end-point will be delayed. On the other hand addition of a large amount of 6-phosphogluconic dehydrogenase would increase the sensitivity of the determination, as 2 moles of TPNH would be formed for each mole of glucose. To obtain a G6P-DH preparation sufficiently free from 6-phosphogluconic dehydrogenase activity, the purification should be carried through the step involving precipitation at pH 4.5 in the method of *Kornberg*⁷), or elution from a starch-celite column in the method of *Glaser and Brown*⁸).

The presence of enzymes which catalyse the oxidation or destruction of TPNH is indicated when the optical density reaches a maximum value and then decreases. Normally a stable end-point for TPN reduction according to equation (2) is obtained in 5–20 min.

Phosphoglucose isomerase and phosphomannose isomerase do not usually interfere with the determination of glucose except to delay the attaining of an end-point, since part of the glucose-6-phosphate is temporarily and reversibly converted to fructose and mannose-6-phosphate respectively. However if phosphofructokinase is also present, then low values for glucose are obtained owing to the irreversible formation of fructose diphosphate. Both isomerases interfere with the determination when the sample contains fructose-6-phosphate, mannose-6-phosphate, fructose or mannose, because these compounds are converted to glucose-6-phosphate. Similarly, invertase interferes when the sample contains sucrose. If the enzyme preparations are contaminated with phosphoglucomutase the presence of glucose-1-phosphate in the sample causes interference. However, phosphate esters are removed by the Ba(OH)₂ and ZnSO₄ used in the deproteinization procedure. It is possible to estimate both hexose monophosphates and glucose by use of a deproteinization procedure which does not remove the phosphorylated compounds (see "Other methods of deproteinization" and "Other Determinations").

Phosphorylase does not interfere with the glucose determination as long as, phosphoglucomutase, inorganic phosphate, arsenate or polysaccharides (starch, glycogen) are absent. Polysaccharides can be removed by deproteinization with a mercuric salt and H₂S (see "Other methods of deproteinization"). Phosphate buffers should be avoided or be diluted out, since phosphate ions inhibit G6P-DH^{8–10}). Small amounts of arsenate or phosphate are removed by deproteinization with Ba(OH)₂ and ZnSO₄.

In order to check the reliability of the glucose assay system it is tested for the possible presence of the following enzymes: 6-phosphogluconic dehydrogenase (with glucose); enzymes which oxidize

*) Conversion to other units, see p. 545.

4) *L. Berger, M. W. Stein, S. P. Colowick and C. F. Cori*, J. gen. Physiol. 29, 379 [1946].

5) *M. Kunitz and M. R. McDonald*, J. gen. Physiol. 29, 393 [1946]; in *S. P. Colowick and N. O. Kaplan: Methods in Enzymology*. Academic Press, New York 1955, Vol. I, p. 269.

6) *R. A. Darrow and S. P. Colowick* in *S. P. Colowick and N. O. Kaplan: Methods in Enzymology*. Academic Press, New York, 1962, Vol. V, p. 226.

7) *A. Kornberg*, J. biol. Chemistry 182, 805 [1950]; in *S. P. Colowick and N. O. Kaplan: Methods in Enzymology*. Academic Press, New York 1955, Vol. I, p. 323.

8) *L. Glaser and D. H. Brown*, J. biol. Chemistry 216, 67 [1955].

9) *H. Theorell*, Biochem. Z. 275, 416 [1935].

10) *E. Negelein and E. Haas*, Biochem. Z. 282, 206 [1935].

or destroy TPNH (with glucose or glucose-6-phosphate); phosphoglucose and phosphomannose isomerase (with fructose and mannose, which are phosphorylated by ATP and yeast hexokinase)¹¹); phosphoglucomutase (with glucose-1-phosphate); invertase (with sucrose).

Preparation of Solutions

- I. Zinc sulphate (*ca.* 4.5% w/v ZnSO₄·7H₂O):
Dissolve *ca.* 4.5 g. ZnSO₄·7H₂O in distilled water and make up to 100 ml.
- II. Barium hydroxide (*ca.* 0.3 N):
Dissolve *ca.* 4.7 g. Ba(OH)₂·8H₂O in distilled water and make up to 100 ml. Store in a bottle protected from atmospheric CO₂ by means of a soda-lime tube attached to the stopper. For convenience in drawing off small quantities of solution it is best stored in a Mariotte bottle.
- III. Tris buffer (*ca.* 0.1 M; pH 8.0):
Dissolve 1.21 g. tris-hydroxymethyl-aminomethane (buffer or A. R. quality, or else recrystallized^{12, 13}) in distilled water, add 5.36 ml. 1.0 N HCl and make up to 100 ml.
- IV. Magnesium chloride (*ca.* 0.1 M):
Dissolve *ca.* 0.5 g. MgCl₂·6H₂O in distilled water and make up to 25 ml.
- V. Serum albumin:
 - a) Dissolve *ca.* 20 mg. crystalline serum albumin in 5 ml. distilled water. This serves as a protective protein for hexokinase at high dilutions⁴).
 - b) Dilute solution (a) 40-fold with distilled water.
- VI. Adenosine triphosphate (*ca.* 0.01 M ATP):
Dissolve *ca.* 60 mg. ATP-Na₂H₂·3H₂O in distilled water and make up to 10 ml.
- VII. Triphosphopyridine nucleotide (*ca.* 3 × 10⁻³ M β-TPN):
Dissolve *ca.* 13 mg. TPN-NaH₂ in distilled water and make up to 5 ml.
- VIII. Hexokinase, HK (*ca.* 300 BSCC⁴) units or 140 KM⁵) units/ml.):
1 BSCC unit catalyses the phosphorylation of *ca.* 10 μg. glucose in 1 min. at 30°C, and pH 8.0. Dilute 1 mg. of crystalline protein (3000 BSCC units) or its equivalent with 10 ml. serum albumin solution (Vb). The presence of serum albumin as a protective protein⁴) may not be essential, especially with less highly purified enzyme preparations. However, its presence is advisable at the high dilution of the test system.
- IX. Glucose-6-phosphate dehydrogenase, G6P-DH (*ca.* 2 K⁷) units or 1 GB⁸) unit/ml.):
1 GB unit catalyses the oxidation of 1 μmole glucose-6-phosphate in 1 min. at 25°C and pH 8.0. Dilute 0.1 mg. of the purified enzyme (= 7 GB units) or its equivalent in distilled water and make up to 7 ml.

Stability of the solutions

The solutions of the inorganic reagents may be stored at room temperature if well stoppered. Store buffer at 5°C, it is usable as long as no bacterial growth is evident. Lyophilized preparations of hexokinase and G6P-DH retain their activity for long periods when kept in a desiccator at 5°C. The enzyme solutions may be stored for several weeks at -15°C without much loss of activity. They should be carefully thawed without excessive shaking and be kept in an ice bath while in use. The remaining solutions are stable for long periods at -15°C.

¹¹) M. W. Slein, G. T. Cori and C. F. Cori, J. biol. Chemistry 186, 763 [1950].

¹²) T. P. Wang, H. Z. Sable and J. O. Lampen, J. biol. Chemistry 184, 17 [1950].

¹³) J. H. Fossum, P. C. Markunas and J. A. Riddick, Analytic. Chem. 23, 491 [1951].

Procedure

Experimental material

The method may be used for the determination of glucose in blood, plasma, serum, tissue extracts, or enzyme systems which form or utilize glucose. In the following description the determination of glucose in blood will be used as an example.

Deproteinization

Preliminary remarks: Deproteinization with $\text{Ba}(\text{OH})_2$ and ZnSO_4 was developed for the preparation of blood samples for the determination of reducing sugar^{14, 15}. Careful use of these reagents allows the removal of protein and phosphate esters from the sample to be analysed. Excess ZnSO_4 inhibits the enzymatic assay system, excess $\text{Ba}(\text{OH})_2$ leads to incomplete deproteinization. With blood and serum this is indicated by foaming when the sample is shaken, and by the presence of haemoglobin or an opalescence in the supernatant after centrifuging the sample. If the experimental material contains ions which precipitate with ZnSO_4 or $\text{Ba}(\text{OH})_2$, then it is necessary to choose ratios of both reagents which will compensate for the loss. The concentration of the $\text{Ba}(\text{OH})_2$ and ZnSO_4 solutions must be so adjusted that, when a mixture of equal volumes of both reagents is diluted with 10 volumes of water, the supernatant fluid is neutral (about pH 7). The ratio of $\text{ZnSO}_4 : \text{Ba}(\text{OH})_2$ used in the deproteinization may then be varied from about 1 : 1 to 1 : 1.1, so that the supernatant fluid is about pH 7.0—7.5 (glass electrode) and therefore does not interfere with the enzymatic assay method. It is advisable to check whether the deproteinized sample inhibits the enzyme system by preparing a control containing added glucose. There must be no marked delay in reaching an end-point. Blood and serum samples appear to contain about 10% less glucose when analysed by the specific enzymatic determination method than when the same deproteinized samples are analysed by the copper reduction method of *Nelson*¹⁴. This may be due to the presence of other reducing substances in the blood sample, since both methods give the same results with a standard glucose solution.

Method: Mix

0.1 ml. blood and

1.5 ml. distilled water

in a centrifuge tube. To this add

0.2 ml. $\text{Ba}(\text{OH})_2$ solution (II),

mix well and add

0.2 ml. ZnSO_4 solution (I).

After thorough mixing, centrifuge for *ca.* 5 minutes at *ca.* 500 g. The supernatant must be clear and colourless. If particles should be floating on the surface of the liquid, filter solution, or withdraw a sample using a pipette, the tip of which carries a very small amount of twisted cotton wool.

Other methods of deproteinization: Biological samples or enzymatic reaction mixtures which are to be analysed for sugar phosphates and free sugars can be deproteinized by the addition of sufficient HClO_4 to give a final concentration of 3—5% (w/v). The supernatant is neutralized with KOH to remove most of the perchloric acid as the insoluble KClO_4 .

¹⁴ *N. Nelson*, *J. biol. Chemistry* 153, 375 [1944].

¹⁵ *M. Somogyi*, *J. biol. Chemistry* 160, 69 [1945].

Since the trichloroacetate ion inhibits G6P-DH it is not advisable to use this acid for deproteinization. However, in certain cases it is possible to remove most of the trichloroacetic acid from the deproteinized sample by ether extraction or decomposition by boiling, as long as the products of the decomposition of acid or heat labile substances do not interfere with the glucose assay.

Deproteinization by addition of an equal volume of 2.5% (w/v) HgCl_2 in 0.5 N HCl (*Schenck's* reagent¹⁶⁾), followed by filtration, treatment with H_2S , filtration, aeration and neutralization, does not remove sugar phosphates, but the precipitated HgS adsorbs polysaccharides like glycogen¹⁷⁾.

The use of *ca.* 0.2 volumes of another mercury reagent (*West's* reagents¹⁸⁾: 28% w/v $\text{Fe}_2(\text{SO}_4)_3 \cdot \text{H}_2\text{O} + 34\%$ w/v HgSO_4 in 1.5 N H_2SO_4) removes protein, polysaccharides and hexosephosphates, when solid BaCO_3 (A. R.) is added with vigorous shaking to a mixture of the reagent and the sample, until the solution gives a neutral reaction with bromothymol-blue paper. The filtrate is acidified with a trace of H_2SO_4 , treated with H_2S , filtered, aerated and neutralized.

Spectrophotometric measurements

Preliminary remarks: To increase the accuracy of the reagent additions use pipettes with drawn-out, ground tips. The solutions are mixed with a thin glass rod which has been flattened at one end to form a disc. If it is necessary for any reason to take more than 1.2 ml. of the sample, then the concentrations of the reagents may be correspondingly increased. A mixture of solutions III—VI may be prepared if several samples are to be analysed. Veronal or glycyglycine buffer may be used instead of tris buffer, but phosphate ions inhibit G6P-DH⁸⁻¹⁰⁾.

Method: Wavelength: 340 m μ ; light path: 1 cm.; final volume: 3.0 ml.

Pipette solutions into the cuvettes in the given order and before addition of the enzymes mix well:

- 1.2 ml. deproteinized sample or distilled water
- 1.0 ml. buffer (solution III)
- 0.2 ml. MgCl_2 solution (IV)
- 0.1 ml. albumin solution (Va)
- 0.1 ml. ATP solution (VI)
- 0.1 ml. hexokinase solution (VIII)
- 0.1 ml. G6P-DH solution (IX).

After addition of the enzymes mix again and read initial optical density E_1 against water. Add

- 0.2 ml. TPN solution (VII)

mix, read optical density at *ca.* 1 min. intervals until stable end-point E_2 is reached. Once it has been established in a control experiment that after reaching a maximum value, no decrease in the optical density occurs within a reasonable period, several determinations may be set up at the same time. Their final optical densities are then read after a fixed time has elapsed.

¹⁶⁾ *F. Schenck*, Pflügers Arch. ges. Physiol. Menschen Tiere 55, 203 [1894].

¹⁷⁾ *G. T. Cori* and *J. Larnner*, J. biol. Chemistry 188, 17 [1951].

¹⁸⁾ *A. Steiner*, Proc. Soc. exp. Biol. Med. 32, 968 [1935].

Calculations

The initial optical density E_1 is corrected for the volume change caused by the addition of TPN solution, by multiplying by 2.8/3.0. If an increase in optical density on addition of TPN solution has been observed in a control cuvette containing distilled water instead of the sample, then this value is added to the corrected E_1 . The resulting value E_1' is subtracted from the final optical density E_2 . The difference ΔE is divided by 0.0115 to obtain the $\mu\text{g. glucose}/3 \text{ ml. reaction mixture}$. The factor 0.0115 is calculated from the molecular weight of glucose and the extinction coefficient for TPNH ($\epsilon = 6.22 \times 10^6 \text{ cm}^2./\text{mole}$ with 1 cm. light path and 340 $\text{m}\mu$)¹⁹⁾. This corresponds to an optical density of 2.07 for each $\mu\text{mole TPN reduced or glucose phosphorylated}$ (also refer to p. 36).

Example

Rabbit blood was deproteinized as described above to give a 1 : 20 dilution of blood. 0.2 ml. (+ 1.0 ml. distilled water) of this was taken for analysis. E_1 before addition of TPN solution: 0.018. Corrected for 0.2 ml. TPN solution:

$$\frac{2.8}{3.0} \times 0.018 = 0.017$$

The increase in optical density on addition of TPN solution to a control containing water instead of sample: 0.010.

$E_1' = 0.017 + 0.010 = 0.027$. Final optical density $E_2 = 0.142$.

$\Delta E = E_2 - E_1' = 0.142 - 0.027 = 0.115$

$$\frac{0.115}{0.0115} = 10 \mu\text{g. glucose}/0.2 \text{ ml. blood (1:20 dilution)}$$

This is equivalent to 1 mg. glucose/ml. whole blood or 100 mg. %.

Range of the Method

Under the stated conditions the amount of glucose which can be measured depends on the TPN concentration. This is sufficient for estimation of 0.6 μmoles (108 $\mu\text{g.}$) glucose. However, with this amount of glucose the time for reaching a final optical density would be unduly delayed, as towards the end of the reaction the enzymes would no longer be saturated with respect to TPN²⁰⁾ and ATP²¹⁾. Furthermore, the final optical density E_2 (ca. 1.242) would lie on the portion of the spectrophotometer scale from which it is not possible to obtain accurate readings. Suitable E_2 values are obtained over the range of 5 $\mu\text{g.}$ ($\Delta E = 0.057$ in ca. 5 min.) to 60 $\mu\text{g.}$ ($\Delta E = 0.690$ in ca. 20 min.) glucose. Of course the range of the method can be extended by use of more TPN, ATP and enzymes. The time for reaching an end-point may also be shortened by use of larger quantities of the enzymes.

Other Determinations

The same system without hexokinase or ATP can be used for the determination of glucose-6-phosphate. Other hexose monophosphates (glucose-1-phosphate, fructose-6-phosphate (see p. 134), mannose-6-phosphate) can be assayed if sufficient amounts of the corresponding auxiliary enzymes are added, namely, phosphoglucomutase²²⁾, phosphoglucose isomerase^{23, 24)} and phosphomannose

19) B. L. Horecker and A. Kornberg, J. biol. Chemistry 175, 385 [1948].

20) The dissociation constant for TPN with G6P-DH is ca. $2 \times 10^{-5} \text{ M}^8$.

21) The dissociation constant for ATP with hexokinase and glucose as substrate is ca. $1.5 \times 10^{-4} \text{ M}^{11}$.

22) V. A. Najjar, J. biol. Chemistry 175, 281 [1948]; in S. P. Colowick and N. O. Kaplan: Methods in Enzymology. Academic Press, New York 1955, Vol. I, p. 294.

23) (from muscle) M. W. Slein in S. P. Colowick and N. O. Kaplan: Methods in Enzymology. Academic Press, New York 1955, Vol. I, p. 304.

24) (from yeast) E. Noltmann and F. H. Bruns, Biochem. Z. 331, 436 [1959].

isomerase^{25, 26}). Similarly fructose and mannose may be determined after phosphorylation with hexokinase and ATP to give fructose and mannose-6-phosphate, respectively. Sucrose can be assayed after the addition of invertase to the complete system. If phosphoglucose isomerase is added to this system then the sensitivity of the sucrose assay is doubled since for each mole of fructose a mole of TPN will be reduced. Other oligosaccharides can be analysed similarly with the aid of hydrolytic enzymes. The method is also suitable for the determination of ATP (see p. 543) and TPN.

Sources of Error

These have already been described in the sections on "Purity of the enzyme preparations" and "Deproteinization".

Specificity

In the absence of contaminating enzymes the method is specific for glucose and glucose-6-phosphate. The latter is removed by deproteinization with $\text{Ba}(\text{OH})_2 + \text{ZnSO}_4$ or with *West's* reagent.

Other Methods for the Enzymatic Determination of Glucose

Glucose can also be assayed with glucose oxidase (glucose aerodehydrogenase, notatin)^{27, 28} (see below) or glucose dehydrogenase²⁹.

Determination with Glucose Oxidase and Peroxidase

Hans-Ulrich Bergmeyer and Erich Bernt

Glucose oxidase (GOD) specifically oxidizes β -D-glucopyranose¹⁻⁵). At 20° C α -D-glucose is oxidized 150 times more slowly⁵). In spite of this, the enzyme can be used to determine glucose in solutions containing an equilibrium mixture of the isomers, because even highly purified GOD preparations still usually contain mutarotase⁶). Also the time taken for the reaction is so chosen that all the α -glucose is oxidized.

Glucose was first determined with GOD manometrically⁴) and then later colorimetrically⁷⁻⁹). The optimum conditions, limits of error and range of application for the routine determination of

25) (from muscle) *M. W. Slein* in *S. P. Colowick and N. O. Kaplan: Methods in Enzymology*. Academic Press, New York 1955, Vol. I, p. 299.

26) (from yeast) *E. Noltmann and F. H. Bruns*, *Biochem. Z.* 330, 514 [1958].

27) *C. E. Coulthard, R. Michaelis, W. F. Short, G. Sykes, G. E. H. Skrimshire, A. F. B. Standfast, J. H. Birkinshaw and H. Raistrick*, *Biochem. J.* 39, 24 [1945].

28) *R. Bentley* in *S. P. Colowick and N. O. Kaplan: Methods in Enzymology*. Academic Press, New York 1955, Vol. I, p. 340.

29) *H. J. Strecker* in *S. P. Colowick and N. O. Kaplan: Methods in Enzymology*. Academic Press, New York 1955, Vol. I, p. 335.

1) *W. Franke and M. Deffner*, *Liebigs Ann. Chem.* 541, 117 [1939].

2) *D. Keilin and E. F. Hartree*, *Biochem. J.* 39, 293 [1945].

3) *D. Keilin and E. F. Hartree*, *Biochem. J.* 42, 221 [1948].

4) *D. Keilin and E. F. Hartree*, *Biochem. J.* 42, 230 [1948].

5) *D. Keilin and E. F. Hartree*, *Biochem. J.* 50, 331 [1952].

6) *D. Keilin and E. F. Hartree*, *Biochem. J.* 50, 341 [1952].

7) *E. R. Froesch and A. E. Renold*, *Diabetes* 5, 1 [1956].

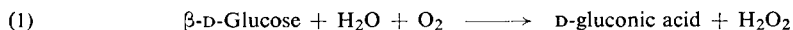
8) *A. S. Keston*, Abstracts of Papers, 129th Meeting Amer. chem. Soc. 1956, 31 C.

9) *J. D. Teller*, Abstracts of Papers, 130th Meeting Amer. chem. Soc. 1956, 69 C.

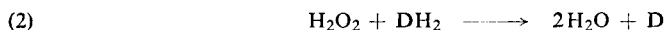
glucose in blood have been extensively studied (*e.g.* refer to¹⁰⁻¹⁶). With the colorimetric method the measurements can be made with simple colorimeters. As no disaccharides are present in blood the "true glucose" value is obtained even with impure glucose oxidase preparations (see p. 130 and 974). For the analysis of mixtures of sugars it is necessary to use highly purified enzyme preparations. Glucose in blood, urine (*e.g.*¹⁷), corn syrup¹⁸), hydrolysates of polysaccharides¹⁹) and fermentation liquors²⁰) has been determined with glucose oxidase.

Principle

Glucose oxidase (GOD) catalyses the reaction:



δ -Gluconolactone which is first formed is spontaneously hydrolysed to D-gluconic acid²¹). The hydrogen peroxide is decomposed in the indicator reaction (2) with peroxidase (POD) and the oxygen liberated oxidizes a hydrogen donor DH₂ (*e.g.* *o*-dianisidine) to a coloured derivative D:



The amount of the dye D formed from DH₂ is a measure of the glucose oxidized. The absorption spectrum of the dye formed from *o*-dianisidine has a wide maximum around 460 m μ . The extinction coefficient depends on the experimental conditions, therefore the measured optical density is related to a glucose standard. The measurements are made at 436 m μ or an adjacent wavelength.

The method described here is based on the procedure of *Hugget* and *Nixon*²²) (other authors use, for example, *o*-toluidine²³) or 2,6-dichlorophenolindophenol²⁴) as the dye).

Reagents

1. Sodium dihydrogen phosphate, NaH₂PO₄·2H₂O

2. Disodium hydrogen phosphate, Na₂HPO₄·2H₂O

3. D-Glucose, A. R.

4. *o*-Dianisidine hydrochloride

commercial preparations of *o*-dianisidine (free base) are usually very coloured. It can be recrystallized from 25% acetone with the addition of charcoal. To prepare the hydrochloride dissolve 10 g. of the recrystallized base in 200 ml. distilled water + 8.5 ml. conc. HCl and add about 1 000 ml. acetone. Filter off the crystals, wash with acetone and ether, and dry *in vacuo* over KOH

5. Perchloric acid, A. R., sp. gr. 1.67; ca. 70% (w/w)

¹⁰) *H. Schön* and *B. Bucke*, *Ärztl. Lab.* 4, 49 [1958].

¹¹) *C. H. Mellinshoff* and *W. Kohleick*, *Med. Klin.* 54, 712 [1959].

¹²) *J. Eismann*, *Dtsch. med. J.* 10, 534 [1959].

¹³) *J. E. Middleton*, *Brit. med. J.* 4, 824 [1959].

¹⁴) *L. Kramer Jacobsen*, *Scand. J. clin. Lab. Invest.* 12, 76 [1960].

¹⁵) *H. Südhof* and *P. K. Riegel*, *Klin. Wschr.* 38, 138 [1960].

¹⁶) *E. Raabo* and *T. C. Terkildsen*, *Scand. J. clin. Lab. Invest.* 12, 402 [1960].

¹⁷) *V. Marks*, *Clin. chim. Acta* 4, 395 [1959].

¹⁸) *R. L. Whistler*, *L. Hough* and *J. W. Hysin*, *Analytic. Chem.* 25, 1215 [1953].

¹⁹) *H. H. Schlubach* and *K. Repenning*, *Angew. Chem.* 71, 193 [1959].

²⁰) *M. Damodaran* and *K. Singh*, *J. sci. Ind. Res. (India)* 13 B, 419 [1954].

²¹) *R. Bentley* and *A. Neuberger*, *Biochem. J.* 45, 584 [1949].

²²) *A. St. G. Hugget* and *D. A. Nixon*, *Biochem. J.* 66, 12 P [1957].

²³) *L. L. Salomon* and *J. E. Johnson*, *Analytic. Chem.* 31, 453 [1959].

²⁴) *L. A. Dobrick*, *J. biol. Chemistry* 231, 403 [1958].

6. Glucose oxidase, GOD

dry powder; commercial preparation, see p. 974. Crude preparations often contain insoluble material which should be centrifuged or filtered off.

7. Peroxidase, POD

dry powder; commercial preparation, see p. 990.

Purity of the enzyme preparations

For the procedure described here, especially because of the ratio of GOD : POD used, the POD preparation should have a k_4 value*) of at least 35000, which corresponds to a purpurogallin number**) of about 70. The GOD should have a Q_{O_2} of >7000 (see p. 974). The catalase content of the GOD preparation should be below 3 units***)/mg.

If the sample to be analysed contains starch, sucrose, lactose or maltose, then the GOD preparation should not contain more than 0.1% amylase, invertase, lactase or maltase (relative to the specific activity of the GOD).

Preparation of Solutions **)** (for *ca.* 20 determinations)

Prepare all solutions with fresh, doubly distilled water.

I. Buffer-enzyme mixture (0.12 M phosphate; pH 7; 40 μ g. POD/ml.; 250 μ g. GOD/ml.):
Dissolve 2.07 g. $Na_2HPO_4 \cdot 2H_2O$, 1.09 g. $NaH_2PO_4 \cdot 2H_2O$, 6 mg. POD and 38 mg. GOD in doubly distilled water and make up to 150 ml.

II. Chromogen (5 mg. *o*-dianisidine hydrochloride/ml.):

Dissolve 10 mg. *o*-dianisidine hydrochloride in doubly distilled water and make up to 2 ml.

III. Glucose reagent:

Add 0.5 ml. solution II to 50 ml. solution I with vigorous stirring.

IV. Glucose standard solution (91 μ g. D-glucose/ml.):

Commercially available glucose contains *ca.* 9% moisture. Dissolve 100 mg. glucose in doubly distilled water, add 25 ml. perchloric acid (sp. gr. 1.67; *ca.* 70% w/w) and make up to 1000 ml. For each new standard solution check the glucose concentration with hexokinase and glucose-6-phosphate dehydrogenase (see p. 117).

V. Perchloric acid (*ca.* 0.34 M):

Dilute 2.9 ml. 70% perchloric acid to 100 ml. with doubly distilled water.

*) According to²⁵⁾ $k_4 = \frac{1}{a_0 \times e} \times \frac{x}{t}$. Where a_0 = initial concentration of guaiacol in the assay, e = enzyme concentration, x/t = hydrogen peroxide decomposed per sec.

***) According to²⁶⁾ the purpurogallin number is the amount of purpurogallin (mg.) which is formed from pyrogallol by 1 mg. peroxidase in 500 ml. assay mixture in 5 min. at pH 7.0.

****) According to²⁷⁾ a unit is the amount of enzyme which liberates half the active oxygen contained in an H_2O_2 solution of any concentration in 100 sec. at 25°C.

*****) Complete reagent kits are available commercially, see p. 1035.

25) P. George, J. biol. Chemistry 201, 413 [1953]; B. Chance and A. S. Maehly in S. P. Colowick and N. O. Kaplan: Methods in Enzymology. Academic Press, New York 1955, Vol. II, p. 764.

26) R. Willstätter and A. Stoll, Liebigs Ann. Chem. 416, 21 [1917].

27) H.-U. Bergmeyer, Biochem. Z. 327, 255 [1955].

Stability of the solutions

Prepare the glucose reagent freshly each day. In the preparation of the glucose reagent do not pipette solution I, but pour. If any precipitate forms the reagent can be filtered. Although the glucose standard solution is protected from bacterial contamination by the perchloric acid, it should be stored in a refrigerator. It is stable for at least a year.

Procedure

Experimental material

Blood: Collect from the lobe of the ear with a blood sugar pipette (graduated to the tip). Analyse venous blood as soon after collection as possible. Haemolysis does not interfere. Addition of citrate, oxalate, fluoride or ethylene-diamine-tetra-acetate to blood in the normal concentrations has no significant effect on the determination. For reasons which are not at present known, 10 mg. % more glucose is found if the supernatant after perchloric acid deproteinization is neutralized. Therefore neutralization must be avoided. Deproteinization with zinc sulphate-NaOH can be used directly without further modification and it gives the same values as with perchloric acid deproteinization without neutralization. Trichloroacetic acid can also be used for deproteinization.

If the blood sample cannot be determined immediately, it should be pipetted into perchloric acid solution (V), centrifuged and the clear supernatant decanted off. This can be stored in a refrigerator until the next day. After long storage in acid solution the polysaccharides contained in the sample slowly hydrolyse, resulting in glucose values which are too high. We found in the deproteinized supernatant an increase in the glucose values of 6% per 24 hours. If the mixture was not centrifuged after deproteinization then the increase was *ca.* 10% per 24 hours.

Serum and plasma: If the samples are analysed immediately, then deproteinization is unnecessary. Dilute the samples 1 : 10 with distilled water.

Urine: Urine which only contains small amounts of protein also need not be deproteinized. Reducing compounds such as ascorbic acid, uric acid, glutathione, creatinine, *etc.* must be removed, because they would interfere with the analysis. According to²³⁾ anion and cation exchange resins are best for this purpose. We proceed as follows:

Preparation and regeneration of the resins: Treat the anion exchange resin, Amberlite IRA-400 with 5% (w/v) NaOH and the cation exchange resin, Amberlite IR-120 with 5% (w/v) HCl, wash with distilled water until the washings are neutral in reaction. Prepare a glass tube (10 cm. long, 1 cm. diameter, with a glass tap at the bottom) with a glass wool plug, *ca.* 3 ml. Amberlite IR-120, another glass wool plug, *ca.* 3 ml. Amberlite IRA-400 and sufficient distilled water so that surface of the resin is covered.

Procedure: Introduce 0.5 ml. urine onto the column and allow to drop through at the rate of 5 drops/min. into a 50 ml. volumetric flask. Add sufficient distilled water to the column so that the resin is always covered with liquid. After about 20 ml. has run through the column, increase the flow rate to 50 to 100 drops/min. (washing the resin with distilled water). Collect a total of 50 ml. of eluate and mix thoroughly in the volumetric flask. Use 0.2 ml. of this deionized urine (which has been diluted 1 : 100) for the analysis.

The amounts of resin given here are sufficient to remove at least 5 mg. ascorbic acid and glutathione and >0.3 mg. uric acid (corresponding to a concentration in the urine of 1% or 0.06%, respectively). No loss of glucose occurs in the process.

Foodstuffs: According to the protein content (if it causes coloration of the sample) deproteinize as for blood.

Deproteinization

Pipette successively into a 10 ml. centrifuge tube:

1.00 ml. perchloric acid (solution V)

0.10 ml. sample (*e.g.* blood).

Wash out the pipette by sucking the liquid up and down several times and mix thoroughly with a thin glass rod. Centrifuge for 5–10 min. at least at 3000 g, pour off the clear supernatant into a dry test tube and analyse 0.20 ml. of this.

Enzymatic reaction and colorimetric measurements

Wavelength: 436 m μ (430–480 m μ); light path: 1 cm.; final volume: 5.2 ml.; room temperature. Each series of measurements requires a reagent blank and a glucose standard.

Bring the glucose reagent (solution III) to room temperature before use.

Pipette successively into test tubes:

Reagent blank: 5.00 ml. glucose reagent (solution III)
0.20 ml. distilled water

Glucose standard: 5.00 ml. glucose reagent (solution III)
0.20 ml. glucose standard solution (IV)

Sample: 5.00 ml. glucose reagent (solution III)
0.20 ml. deproteinized sample.

Mix thoroughly and allow to stand for 30–40 min. at 20–22°C. For the measurements pour the solutions into cuvettes. Measure the optical densities E_{sample} and E_{standard} against the reagent blank.

Set up a series of measurements in such a way (*e.g.* addition of glucose reagent at minute intervals) that an average incubation time of 35 min. is obtained.

Calculations

Standard curves are linear up to *ca.* 80 $\mu\text{g.}$ (*ca.* 0.45 μmoles)/ml. This corresponds to a blood sugar concentration of *ca.* 450 mg.%. With optical densities above 0.600 (measured against the reagent blank) the accuracy of the photometer readings are too low. In this case, dilute the sample or deproteinized supernatant with doubly distilled water and analyse again.

For the calculations the measured optical densities are related to that of the glucose standard. This contains 18.2 $\mu\text{g.}$ glucose/tube, corresponding to 1 mg. glucose/ml. blood or serum under the given conditions of deproteinization.

Therefore
$$\frac{E_{\text{sample}}}{E_{\text{standard}}} \times 100 = \text{mg. \% glucose in blood or serum.}$$

With samples which have not been deproteinized allow for any preliminary dilution and that the volume of sample is 0.2 ml.

General formula:

$$\frac{E_{\text{sample}} \times 5 \times 0.0182 \times \text{dilution factor}}{E_{\text{standard}}} = \frac{E_{\text{sample}} \times 0.091 \times \text{dilution factor}}{E_{\text{standard}}} = \text{mg. glucose/ml. sample}$$

where 5 = conversion from 0.2 ml. to 1 ml.
0.0182 = mg. glucose in the standard tube

Example

1. *Blood.* 0.1 ml. of blood was collected in the morning, deproteinized and the clear supernatant was poured into a test tube. Measurements were made in the afternoon: $E_{\text{sample}} = 0.104$; $E_{\text{standard}} = 0.170$.

$$\frac{0.104}{0.170} \times 100 = 61 \text{ mg. \% glucose. Normal values (see below).}$$

2. *Hard caramels.* 1.55 g. were dissolved in doubly distilled water and made up to 100 ml. (15.5 mg./ml.). Diluted 1:10 for the assay; 0.2 ml. of this solution was analysed. $E_{\text{sample}} = 0.245$; $E_{\text{standard}} = 0.185$.

$$\frac{0.245 \times 0.091 \times 10}{0.185} = 1.20 \text{ mg. glucose/ml.}$$

As the weight taken was 15.5 mg./ml. the hard caramels contained 7.75% glucose.

Normal Values in Blood, Serum and Plasma

As the method described here is specific for glucose, the measured values are lower than those obtained by measuring the reducing power. Normal values for blood, serum and plasma are given in Table 1. Table 2 shows the differences between the values obtained with this method and those obtained by measurements of the reducing power (method of *Hagedorn* and *Jensen*²⁸⁾).

Table 1. Normal values for glucose in blood, serum and plasma.
Determined enzymatically with GOD-POD

Range [mg. %]	Mean (± 2 SD) [mg. %]	Sample	Author
57-92	72 (± 8.6)	Blood	Südhof ²⁹⁾
60-100	80 (± 4)	Blood	Raabo et al. ¹⁶⁾
54-90	—	Capillary blood	Kreutzer ³⁰⁾
43-95	69 (± 13)	Capillary blood	Middleton ¹³⁾
48-88	68	Venous blood	Renold ³¹⁾
59-95	77	Venous blood	Hugget ³²⁾
49-97	73	Capillary blood	Schön ³³⁾
67-97	82	Serum	Teller ⁹⁾
75-107	90.9 (± 7.9)	Plasma	Saifer et al. ³⁴⁾

Table 2. Differences between the values for glucose determined by measurement of the reducing power²⁸⁾ and those obtained enzymatically with GOD-POD.

Difference (residual reduction) [mg. %]	Mean (± 2 SD) [mg. %]	Sample	Author
18-34	24	Blood	Eismann ¹²⁾
4-54	25 (± 12)	Blood	Südhof ²⁹⁾
—	27.9 (± 12.8)	Blood	Südhof et al. ¹⁵⁾
—	11.9 (± 8.6)	Liquor	Südhof et al. ¹⁵⁾
10-15	—	Blood	Kramer, Jacobsen ¹⁴⁾
—	20	Blood	Kreutzer ³⁰⁾
5.6-33.3	23.5	Blood	Mellinghof et al. ¹¹⁾
—	14.3	Blood	Schön et al. ¹⁰⁾

28) H. C. Hagedorn and B. N. Jensen, *Biochem. Z.* 135, 46 [1923]; 137, 92 [1923].

29) H. Südhof: *Therapie des Monats.* Boehringer, Mannheim (Germany) 1960, p. 203.

30) H. H. Kreutzer, *Lecture Symp. d'Enzymologie*, Gent (Belgium) April 2nd, 1960.

31) A. E. Renold, see ref.¹³⁾.

32) A. St. G. Hugget, see ref.¹³⁾.

33) H. Schön, see ref.¹³⁾.

34) A. Saifer and S. Gerstenfeld, *J. Lab. clin. Med.* 51, 448 [1958].

Modifications

a) More accurate measurements

In spite of measuring against a reagent blank the glucose standard curves often do not pass through the origin, but cut the ordinate. The point of intersection with the ordinate can be as much as $E = 0.060$ and depends on the enzyme preparation. With samples in which the glucose content is very different from the standard (equivalent to 100 mg. %) this displacement of the standard curve is important. In such cases, for accurate measurements it is not sufficient to relate the optical density of the unknown to that of the standard. Instead several standards containing 0.05, 0.10, 0.15 and 0.20 ml. glucose standard solution (IV) should be prepared. Plot the optical densities (measured against the reagent blank) (ordinate) against the amounts of glucose (abscissa) and read off from this standard curve the amounts of glucose corresponding to the optical densities of the samples.

b) Rapid measurements

The GOD-POD reaction goes to completion more rapidly at higher temperatures. On the other hand the colour fades more quickly; the optical density decreases after reaching an optimum value. At 37°C the measurements are made after about 20 min. and the colour is fixed by addition of H_2SO_4 ³⁴. At the end of the reaction (ca. 20 min.) add to the tube

5.00 ml. 50% (w/w) H_2SO_4

and mix (slight warming is not harmful). The red dye is stable. Measure at 546 m μ against a reagent blank.

In cases of extreme urgency a reaction time of 5 min. can be used. As the reaction is still proceeding, the sample, glucose standard and reagent blank must be stopped at exactly the same time by the addition of 50% (w/w) H_2SO_4 . The errors in this method due to inaccurate timing are very large.

c) Series of measurements with automatic analysers

The method has been adapted by *Malmstedt* and *Hicks*³⁵) for automatic measurements. The measurements are made after 1 min. and the error is only 2%.

d) Semi-quantitative determination with test paper

Filter paper strips soaked with the enzymes and dye (*o*-toluidine) can be used for the semi-quantitative determination of glucose, especially of glucose in urine (*e.g.* refer to³⁶⁻³⁹). A modification for the determination in blood has been described by *Kohn*⁴⁰). For the measurements a few centimetres of the paper strip are dipped in the urine, allowed to react in the air and after a few minutes the colour of the paper is compared with a colour chart.

Commercial preparations: "Tes-Tape" (Eli Lilly Company, Indianapolis, Ind., USA); "Glucotest" (C. F. Boehringer & Soehne GmbH., Mannheim, Germany), colour: green. Paper in rolls in a plastic container. — "Clinistix" (Ames Comp., Nuffield House, Picadilly, London W. 1., England), colour: blue. Separate strips in a carton.

*Doyle et al.*⁴¹) have determined glucose in the mucus of the cervix uteri (fertility test) by introduction of "Tes-Tape" bound to a plastic rod.

Specificity

The determination is specific for D-glucose. Mannose, altrose and galactose only give about 1% of the value with glucose. Fructose, lactose, raffinose, maltose, arabinose, xylose, sorbose, inositol,

³⁵) *H. V. Malmstedt* and *G. P. Hicks*, *Analytic. Chem.* 32, 394 [1960].

³⁶) *J. A. Hunt*, *C. H. Gray* and *D. E. Thorogood*, *Brit. med. J.* 1956, 586.

³⁷) *J. P. Comer*, *Analytic. Chem.* 28, 1748 [1956].

³⁸) *H. S. Seltzer*, *J. Amer. med. Assoc.* 162, 1234 [1956].

³⁹) *I. R. Leonards*, *J. Amer. med. Assoc.* 163, 260 [1957].

⁴⁰) *I. Kohn*, *Lancet* 272, 213 [1957].

⁴¹) *J. B. Doyle*, *F. J. Ewers jr.* and *D. Sapit*, *J. Amer. med. Assoc.* 172, 1744 [1960].

melibiose, sucrose, α -methylglucoside and phosphorylated sugars do not react with GOD⁴²). Relative to β -glucose = 100 the reaction rates with other sugars are⁵⁾: α -glucose 0.64; mannose 0.98; altrose 0.16; galactose 0.14; talose 0–0.015; allose 0; gulose 0; idose 0.018; 6-methylglucose 1.85; 4,6-dimethylglucose 1.22; according to⁴³⁾: 2-deoxyglucose 25; 6-deoxy-6-fluoroglucose > 3; xylose > 0.4.

Sources of Error

Interference with the analysis will occur if the sample (*e.g.* starch syrup) contains disaccharides and if the GOD preparation is contaminated with enzymes (*e.g.* maltase, invertase, lactase, amylase) which will liberate glucose from these disaccharides. In this case, the reaction does not come to a stop; the optical density continues to increase. Interference due to small optical density increases after the end of the reaction can be corrected for: the optical density is read at 5 to 10 min. intervals and extrapolated to zero time (see p. 39).

A source of error in the determination of glucose in urine is the presence of large amounts of reducing substances in the sample (*e.g.* vitamin C^{44,45}), uric acid⁴⁵⁾). For example, 25 mg. % ascorbic acid reduces the final value by 15%. These substances compete with the peroxidase for the hydrogen peroxide formed. Catalase competes in the same way (possible contaminant of the GOD preparation). In such cases, the standard curves are non-linear even with relatively low glucose values.

Addition of 1–3 g. fluoride, oxalate or formaldehyde to 100 ml. of blood interferes with the determination. The glucose increases with time after the addition. Simultaneous addition of two of the substances in a concentration of 0.1% leads to a considerable increase in the glucose values. The reason is not yet known.

⁴²⁾ E. Bernt, unpublished.

⁴³⁾ A. Sols and G. de la Fuente, *Biochim. biophysica Acta* 24, 206 [1957].

⁴⁴⁾ H. H. Kreutzer and H. J. H. Kreutzer, *Huisarts en Wetenschap* 3, 1 [1960].

⁴⁵⁾ H.-U. Bergmeyer and E. Bernt, unpublished.