L-Glutamate

Determination with Glutamic Dehydrogenase

Erich Bernt and Hans-Ulrich Bergmeyer

Principle

Glutamic dehydrogenase (GlDH) catalyses the reaction:

(1) L-Glutamate + DPN^+ + H_2O $\rightleftharpoons$ α-oxoglutarate + DPNH + NH_4^+

The equilibrium lies far to the left. However, by trapping the α-oxoglutarate with hydrazine, and with a large excess of DPN and alkaline medium (pH 9), the L-glutamate can be quantitatively oxidized to α-oxoglutarate. The increase of optical density at 340 or 366 mμ due to the formation of DPNH is a measure of the reaction.

Reagents

1. Hydrazine hydrate, *ca.* 24% (w/v)
2. Glycine, A. R.
3. Sulphuric acid, A. R., 1 N
4. Diphosphopyridine nucleotide, DPN
   free acid; commercial preparation, see p. 1010.
5. Glutamic dehydrogenase, GlDH
   crystalline, from ox liver; free from ammonium sulphate. Commercial preparation, see p. 978.

Additional for the analysis of samples containing protein:

6. Perchloric acid, A. R., sp. gr. 1.67; *ca.* 70% (w/w)
7. Dipotassium hydrogen phosphate, K_2HPO_4
8. Tripotassium phosphate, K_3PO_4

Purity of the enzyme preparations

The GlDH should have a specific activity of at least 3 units*)/mg. The enzyme must be free from glutaminase.

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

Prepare all solutions with fresh, doubly distilled water. Sterilize the containers to prevent bacterial contamination.

I. Glycine-hydrazine buffer (0.5 M glycine; 0.4 M hydrazine; pH 9):
   Dissolve 3.75 g. glycine and 5.50 g. 24% hydrazine hydrate in doubly distilled water, adjust to pH 9 with *ca.* 14.8 ml. 1 N H_2SO_4 and make up to 100 ml.

II. Diphosphopyridine nucleotide (*ca.* 3×10^{-2} M β-DPN):
   Dissolve 100 mg. DPN in 5 ml. doubly distilled water.

III. Glutamic dehydrogenase, GlDH (*ca.* 10 mg. protein/ml.):
   If necessary, dilute the suspension with 0.15 M Na_2SO_4 solution.

*) A unit is the amount of enzyme which oxidizes 1 μmole L-glutamate in 1 min.

Additional for the analysis of samples containing protein:

IV. Perchloric acid (*ca.* 0.6 M):
Dilute 5.2 ml. 70% $HClO_4$ (sp. gr. 1.67) to 100 ml. with doubly distilled water.

V. Phosphate solution (2 M; pH *ca.* 12):
Dissolve 21.2 g. K_3PO_4 and 18.3 g. K_2HPO_4 in doubly distilled water and make up to 100 ml.

Stability of the solutions

Store the DPN solution at 0–4° C and make up freshly after *ca.* 2 weeks. Check the specific activity of the GlDH suspension each month; store the suspension at 0–4° C. The buffer solution is stable at room temperature.

Procedure

Preparation of the sample

Dilute glutamic acid preparations, protein hydrolysates and other amino acid mixtures so that they contain less than 150 μg. L-glutamic acid/ml. To obtain a homogeneous solution of meat extracts, soup cubes, *etc.*, heat in water until just boiling, cool and filter. The fat remains on the filter paper.

Remove ammonium ions (see "Sources of Error") before the determination by lyophilizing a solution of the sample which has been adjusted to pH 9. If in spite of dilution the sample is strongly coloured, isolate the glutamate by adsorption on a anion exchange resin (*e.g.* Amberlite IRA-400) and elution with 1 N NaOH. Analyse the eluate.

Blood: Thoroughly mix 5.00 ml. blood with 5.00 ml. perchloric acid (solution IV) and centrifuge for 10 min. at 3000 g. Adjust 3.00 ml. of the supernatant to pH *ca.* 9 with 0.8 ml. phosphate solution (V), allow to stand for 10 min. in an ice bath and then filter through a small, fluted filter paper. Allow to warm to room temperature; analyse 1.00 ml. of the filtrate.

Tissue: Homogenize with 4 parts by weight of perchloric acid (solution IV) and centrifuge for 10 min. at 3000 g. Adjust 3.00 ml. of the supernatant to pH *ca.* 9 with 1.80 ml. phosphate solution (V), allow to stand for 10 min. in an ice bath and then filter through a fluted filter paper. Allow to warm to room temperature; analyse 1.00 ml. of the filtrate.

Spectrophotometric measurements with protein-free samples

Wavelength: 340 or 366 mμ; light path: 1 cm.; final volume: 3.45 ml.; room temperature (20–24° C).

For each series of measurements prepare a reagent blank containing water instead of sample *). Measure against air or water.

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*) DPN and hydrazine form a compound which absorbs at 303 mμ and which also has considerable absorption at 366 mμ. This blank reaction is very rapid in the first few seconds (optical density increase up to 0.030), then it creeps up with a rate of $\Delta E/30$ min. = 0.005 to 0.020. It is therefore necessary to have exactly the same reaction time after addition of DPN for the experimental and blank cuvettes. This also holds for any lengthy reactions which include DPN and hydrazine in the assay mixture, for example, the determination of L-lactate (p. 266) and L-glycerol-1-phosphate (p. 215).

Pipette into 1 cm. cuvettes:

3.00 ml. buffer (solution I)
0.20 ml. sample (doubly distilled water in blank)
0.20 ml. DPN solution (II).

Mix with a plastic spatula and read the initial optical density. Mix in

0.05 ml. GlDH suspension (III).

Allow the experimental and blank cuvettes to stand for *ca.* 30 min. (for each cuvette there must be the same time interval between adding the DPN solution and measuring the final optical density, see "Sources of Error") and then measure the final optical density. Calculate the differences between the final and initial optical densities for the experimental and blank cuvettes ($\Delta E_{sam.}$ and $\Delta E_{blk.}$). $\Delta E = \Delta E_{sam.} - \Delta E_{blk.}$ is used for the calculations.

Calculations

For measurements at 340 mμ: $\frac{\Delta E \times 3.45}{6.22 \times 0.2} = \Delta E \times 2.77 =$ μmoles L-glutamate/ml. sample

For measurements at 366 mμ: $\frac{\Delta E \times 3.45}{3.30 \times 0.2} = \Delta E \times 5.23 =$ μmoles L-glutamate/ml. sample

where
3.45 = assay volume [ml.]
0.2 = volume of the sample in the assay mixture [ml.]
6.22 = extinction coefficient of DPNH at 340 mμ [cm.²/μmole]
3.30 = extinction coefficient of DPNH at 366 mμ [cm.²/μmole]

To convert to μg. it is necessary to multiply the values by the molecular weight of glutamic acid (146).

Example

Liquid soup seasoning: 810 mg. was diluted to 100 ml. with distilled water (8.10 mg./ml.). This was diluted a further 1 : 50 for the assay and 0.2 ml. of this solution was analysed. Measurements against air at 366 mμ. Optical density difference of the sample: 0.200; optical density difference of the reagent blank: 0.085; $\Delta E = 0.115$.

$0.115 \times 5.23 = 0.6$ μmoles L-glutamate/ml. sample
$0.6 \times 146 = 87.5$ μg. L-glutamic acid/ml. sample.

As the weight of the sample was 8.10 mg./ml. and the initial dilution was 1 : 50, the soup seasoning contained

$$\frac{0.0875 \times 50 \times 100}{8.10} = 54\% \text{ L-glutamic acid.}$$

Spectrophotometric measurements with samples containing protein

Wavelength: 340 or 366 mμ; light path: 2 cm.; final volume 4.25 ml.; room temperature (20–24° C).

For each series of measurements prepare a reagent blank containing water instead of sample *) Measure against air or water.

Pipette into 2 cm. cuvettes:

1.00 ml. neutralized filtrate (sample)
3.00 ml. buffer (solution I)
0.20 ml. DPN solution (III).

*) See footnote on p. 385.

Mix with a plastic spatula and measure the initial optical density. Mix in

0.05 ml. GlDH suspension (IV).

Allow the experimental and blank cuvette to stand for 60 min. (for each cuvette there must be the same time interval between adding the DPN solution and measuring the final optical density, see "Sources of Error"). Measure the final optical density. Calculate the differences between final and initial optical densities for the experimental and blank cuvettes ($\Delta E_{sam.}$ and $\Delta E_{blk.}$). $\Delta E = \Delta E_{sam.} - \Delta E_{blk.}$ is used for the calculations.

Calculations

Under the given conditions the reaction proceeds stoichiometrically up to 30 μg. L-glutamic acid in the assay mixture. With a final volume in the cuvette of 4.25 ml. and with a light path of 2 cm., $\Delta E = 0.100$ at 340 mμ corresponds to 0.0342 μmoles (= 4.99 μg.) L-glutamate, or to 0.0644 μmoles (= 9.40 μg.) L-glutamate at 366 mμ.

To obtain the glutamate content per ml. blood or per gram fresh weight allowance must be made for the dilution occurring on deproteinization and neutralization. Blood contains *ca.* 80% of its weight as water; 1.0 ml. blood weighs 1.06 g. 5.00 ml. blood = 5.3 g. gives $5 + (5.3 \times 80/100) = 9.24$ ml. extract after deproteinization; 3 ml. of this is neutralized with 0.8 ml. phosphate solution and 1.0 ml. of this 3.8 ml. is taken for the assay. The dilution factor is $(9.24/5) \times (3.8/3) \times (1/1) = 2.34$.

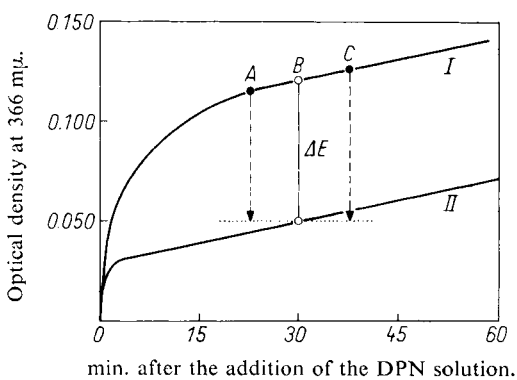

Fig. 1. Course of the optical density in the determination of glutamate with GlDH. Effect of inaccurate timing on the ΔE values.

Curve I: Experimental cuvette
Curve II: Blank cuvette
A: Measured too early in comparison to the 30 min. blank cuvette (ΔE too small)
B: Measured at the right time
C: Measured too late in comparison to the 30 min. blank cuvette (ΔE too large)

With a 20% homogenate (= 1/5 tissue), neutralization of 3.00 ml. with 1.8 ml. phosphate solution and the use of 1.00 ml. of this solution for the assay, gives a dilution factor of $(5/1) \times (4.8/3) \times (1/1) = 8.0$.

Therefore for measurements at 340 mμ:

$\Delta E \times 117$ = μg. L-glutamic acid/ml. blood
$\Delta E \times 399$ = μg. L-glutamic acid/g. tissue

or for measurements at 366 mμ:

$\Delta E \times 220$ = μg. L-glutamic acid/ml. blood
$\Delta E \times 752$ = μg. L-glutamic acid/ml. tissue.

Example

1 g. mouse liver was analysed and the following measurements were made at 366 mμ:

	Reagent blank	*Sample*
Initial optical density:	0.146	0.319
Final optical density:	0.176	0.580
	$\Delta E_{blk.} = 0.030$	$\Delta E_{sam.} = 0.261$

$$\Delta E = \Delta E_{sam.} - \Delta E_{blk.} = 0.231$$

$0.231 \times 752 = 174$ μg. L-glutamic acid/g. liver

Specificity

Glutamine, D-glutamate, L-aspartate, pyrrolidonecarboxylic acid and other derivatives of glutamic acid do not react.

Sources of Error

Ammonium ions interfere with the determination, therefore they must be removed (see "Procedure"). Inaccurate timing of the addition of the DPN solution to the blank and experimental cuvettes leads to false results (Fig. 1).

Determination with Glutamic Dehydrogenase and Lactic Dehydrogenase

Hans-Ulrich Bergmeyer and Rudolf Czok

Principle

Glutamic dehydrogenase (GlDH) catalyses the reaction:

(1) L-Glutamate + DPN^+ + H_2O $\rightleftharpoons$ α-oxoglutarate + DPNH + NH_4^+

At pH 7 the equilibrium lies far to the left[1]. It can be displaced entirely in favour of α-oxoglutarate by use of high DPN concentrations, absence of NH_4^+ions at the start of the reaction and continuous re-oxidation of the DPNH formed[2]. Therefore reaction (1) is coupled with that catalysed by lactic dehydrogenase (LDH):

(2) Pyruvate + DPNH + H^+ $\rightleftharpoons$ lactate + DPN^+

Reaction (2) must be sufficiently rapid in order to keep the DPNH concentration low and the DPN concentration high until all the glutamate is converted to α-oxoglutarate. After heat denaturation of the GlDH and LDH, the α-oxoglutarate is determined according to equation (1) by the further addition of GlDH and NH_4^+ ions (refer also to p. 324). The decrease in the optical density at 366 mμ due to the oxidation of DPNH is a measure of the reaction. If the sample contains α-oxoglutarate it must be estimated separately and subtracted.

Reagents

1. Potassium dihydrogen phosphate, KH_2PO_4, A. R.
2. Disodium hydrogen phosphate, $Na_2HPO_4 \cdot 2H_2O$, A. R.
3. Triethanolamine hydrochloride, A. R.
4. Ammonium acetate, A. R.
5. Sodium pyruvate
   commercial preparation, see p. 1027.
6. Perchloric acid, A. R., sp. gr. 1.67; *ca.* 70% (w/w)
7. Potassium hydroxide, A. R.

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[1] *J. A. Olson* and *C. B. Anfinsen*, J. biol. Chemistry *202*, 841 [1953].
[2] *H.-U. Bergmeyer*, unpublished.

8. Ethylene-diamine-tetra-acetic acid, EDTA
disodium salt, EDTA-$Na_2H_2 \cdot 2H_2O$ (Trilon B, Titriplex III, Versene)
9. Diphosphopyridine nucleotide, DPN
free acid; commercial preparation, see p. 1010.
10. Reduced diphosphopyridine nucleotide, DPNH
sodium salt, DPNH-Na_2; commercial preparation, see p. 1011.
11. Lactic dehydrogenase, LDH
crystalline, from rabbit muscle[3], suspension in 2.2 M ammonium sulphate solution. Commercial preparation, see p. 986.
12. Glutamic dehydrogenase, free from ammonium sulphate, GlDH
crystalline, from ox liver, dissolved in 50% (v/v) glycerol solution. Commercial preparation, see p. 978.

Purity of the enzyme preparations

The GlDH should have a specific activity of at least 175 units (defined according to[4])/mg. protein and the LDH at least 12000 units/mg. protein. Both enzymes must be free from glutaminase. The GlDH must not contain more than 0.02 units LDH/ml.

Preparation of Solutions

I. Phosphate buffer (0.2 M; pH 7.6):
Mix 88.5 ml. of a solution of 35.65 g. $Na_2HPO_4 \cdot 2H_2O$/1000 ml. doubly distilled water with 11.5 ml. of a solution of 27.22 g. KH_2PO_4/1000 ml. doubly distilled water.

II. Triethanolamine buffer (0.2 M; pH 7.6):
Dissolve 9.3 g. triethanolamine hydrochloride in *ca.* 200 ml. doubly distilled water, add 3.7 g. EDTA-$Na_2H_2 \cdot 2H_2O$. adjust to pH 7.6 with about 9 ml. 2 N NaOH and dilute to 250 ml. with doubly distilled water.

III. Ammonium acetate (2 M):
Dissolve 1.54 g. ammonium acetate in doubly distilled water and make up to 10 ml.

IV. Diphosphopyridine nucleotide (*ca.* 2.5×10^{-2} M β-DPN):
Dissolve 20 mg. DPN in 1 ml. doubly distilled water.

V. Reduced diphosphopyridine nucleotide (*ca.* 8×10^{-3} M β-DPNH):
Dissolve 7 mg. DPNH-Na_2 in 1 ml. triethanolamine buffer (solution II).

VI. Sodium pyruvate (1 M):
Dissolve 1.1 g. sodium pyruvate in doubly distilled water and make up to 10 ml.

VII. Lactic dehydrogenase, LDH (*ca.* 2 mg. protein/ml.):
Lactic dehydrogenase is commercially available only as a suspension in ammonium sulphate solution. It should be dialysed for three successive 4 hour periods against 70 volumes 0.01 M phosphate buffer pH 6.5 (containing 10^{-3} M EDTA) each time. This results in a 2 to 3-fold dilution of the enzyme preparation.

VIII. Glutamic dehydrogenase, GlDH (*ca.* 20 mg. protein/ml.):
Use the commercially available in 50% (v/v) glycerol solution.

IX. Perchloric acid (*ca.* 6% w/v):
Dilute 52 ml. 70% $HClO_4$ to 1000 ml. with doubly distilled water.

X. Potassium hydroxide (*ca.* 2 N):
Dissolve 11.2 g. KOH in doubly distilled water and make up to 100 ml.

[3] *H. J. Hohorst*, Biochem. Z. *328*, 509 [1956].
[4] *G. Beisenherz* et al., Z. Naturforsch. *8b*, 555 [1953]; refer also to p. 33.

Stability of solutions

Store the DPNH solution at 0—5°C and prepare freshly each week. There is no significant change in the specific activity of the dialysed LDH when stored at 0—5°C for 4—6 days. Older solutions should have their activity checked, and, if necessary, a larger volume should be used for the assay. All other solutions keep practically indefinitely at 0—5°C. At this temperature some of the phosphate in the phosphate buffer crystallizes out; before use care should be taken to obtain complete solution.

Procedure

Experimental material and deproteinization

Deproteinize blood or tissue according to the method given in the chapter on "Pyruvate" (p. 254). To neutralize the perchloric acid extract, titrate with 2 N KOH (solution X) to pH 7.3—7.8 (indicator paper). Allow the solution to stand for 15 min. at 0°C and then centrifuge off the precipitated $KClO_4$.

To remove ammonium ions before the determination adjust the pH of urine to 9.5 and lyophilize[5]. The same applies to all samples that contain ammonia.

Dilute glutamic acid preparations, protein hydrolysates and other amino acid mixtures, so that the portion taken for analysis contains at the most 50 μg. (*ca.* 0.3 μmoles) L-glutamate. Heat and filter meat extracts, soup flavourings, *etc.* to obtain complete solution. The fat remains on the filter paper.

Preliminary enzymatic reaction

Pipette into a centrifuge tube with a graduation mark at 4 ml.:

3.00 ml. (deproteinized) neutralized sample
0.75 ml. phosphate buffer (solution I)
0.04 ml. sodium pyruvate solution (VI)
0.12 ml. DPN solution (IV)
0.04 ml. LDH solution (VII)
0.05 ml. GlDH solution (VIII).

Incubate the mixture in a water bath for 30 min. at 37°C. To inactivate the enzymes heat for at least 15 min. in a boiling water bath and then cool in cold water. Wash down the water condensed on the upper part of the tube with the reaction solution by careful inclination of the tube. If necessary, make up to 4 ml. with doubly distilled water. Centrifuge for 5 min. at 3000 g and discard precipitate. Determine the α-oxoglutarate formed from L-glutamate in a portion of the supernatant.

Spectrophotometric measurements

Wavelength: 366 mμ; light path: 2 cm.; final volume: 3.5 ml. Measure against distilled water or air.

Pipette successively into the cuvette:

3.00 ml. supernatant (from preliminary enzymatic reaction)
0.30 ml. triethanolamine buffer (solution II)
0.10 ml. ammonium acetate solution (III)
0.05 ml. DPNH solution (V).

Mix, read the initial optical density E_1 and then mix into the cuvette

0.05 ml. GlDH solution (VIII).

[5] *V. Klingmüller*, personal communication.

Measure the optical density E_2 after 5—10 min., when the reaction should be complete. After a further 5 min. read the optical density once again. If it is smaller than E_2 then the GlDH contains more LDH than is stated under "Purity of the enzyme preparations". A linear decrease in optical density occurring at the end of the α-oxoglutarate reaction is due to the reduction according to equation (2) of the pyruvate which is present in high concentration. This error can be eliminated by graphical extrapolation (refer to p. 39) from the start of the reaction (addition of GlDH).

The optical density difference $\Delta E = E_1 - E_2$ is used for the calculations.

Since L-glutamate is measured as α-oxoglutarate, a separate determination of the α-oxoglutarate already present in the sample is necessary*), refer to "α-Oxoglutarate" p. 324.

The measurement is carried out under the conditions just described, but instead of 3 ml. of the supernatant from the enzymatic incubation, 2.25 ml. of the deproteinized, neutralized extract + 0.75 ml. doubly distilled water are used. ΔE is corrected for the optical density difference $\Delta E_{oxoglutarate}$ (see "Calculations").

Calculations

$$\Delta E_{glutamate} = \Delta E - \Delta E_{oxoglutarate}$$

$$\frac{\Delta E_{glutamate} \times V \times 1.33}{\varepsilon \times d \times v} = \mu\text{moles L-glutamate/ml. deproteinized, neutralized sample}$$

where

V = assay volume (3.5 ml.)

ε = extinction coefficient of DPNH (cm.²/μmole) $\varepsilon_{366} = 3.30$[3]; $\varepsilon_{340} = 6.22$[6]; $\varepsilon_{334} = 6.09$[7]

d = light path (2 cm.)

v = volume of sample taken for the enzymatic reaction or of the supernatant after deproteinization (3 ml.)

1.33 = conversion of the volume of supernatant taken for the α-oxoglutarate determination (3 ml.) to the volume of the enzymatic incubation (4 ml.)

The method is suitable for glutamate concentrations from 0.2 to 1 mM in the neutralized extract. This is the range obtained when whole blood is deproteinized with three volumes of $HClO_4$. With lower concentrations the sensitivity can be doubled by measuring at 334 or 340 mμ. In this case half the volume of the DPNH solution should be taken.

Sources of Error

Ammonium ions in concentrations higher than 3 mM in the deproteinized, neutralized extract inhibit the conversion to α-oxoglutarate, because they displace the equilibrium (1). The presence of 2 mM urea in the extract interferes with the determination; uric acid, ascorbic acid, creatinine and creatine have no effect[8].

Specificity

Glutamine is not determined. D-Glutamate, L-aspartate, pyrrolidonecarboxylic acid and other derivatives of glutamate are not oxidized by glutamic dehydrogenase[1] and do not interfere with the determination[8].

*) Owing to the lability of keto acids in neutral extracts the value obtained for α-oxoglutarate is not absolutely identical with the α-oxoglutarate content of the untreated sample.

6) *A. Kornberg* and *W. E. Pricer:* Biochemical Preparations. Wiley, New York 1953, Vol. 3, p. 20.

7) *G. Beisenherz, Th. Bücher* and *K. H. Garbade* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1955, Vol. I, p. 391.

8) *H. v. Bernuth*, Ph. D.-Thesis, Universität Hamburg, 1959.

Determination with Glutamic Dehydrogenase and the 3-Acetylpyridine Analogue of DPN

Helmut Holzer, Hans-Dieter Söling and Irene Witt

The spectrophotometric determination of L-glutamate with glutamic dehydrogenase (GlDH) from bovine liver and the 3-acetylpyridine analogue of DPN (AP-DPN), without the use of a trapping agent, is made possible by the favourable redox potential of $AP\text{-}DPN^+/AP\text{-}DPNH$ in contrast to $DPN^+/DPNH$ (see p. 394).

Principle

Glutamic dehydrogenase catalyses the reaction:

(1) Glutamate + $AP\text{-}DPN^+$ + H_2O $\rightleftharpoons$ α-oxoglutarate + AP-DPNH + NH_4^+

At pH 7 the equilibrium is to the left, while alkaline pH favours the oxidation of glutamate. In order to obtain quantitative oxidation of glutamate the assay must be carried out with high concentrations of GlDH and AP-DPN, as well as in the absence of NH_4^+ ions, since the affinity of GlDH for glutamate in the assay with AP-DPN is low ($K_M = 4.45 \times 10^{-3}$ M).

Reagents

1. Potassium dihydrogen phosphate, KH_2PO_4, A. R.
2. Disodium hydrogen phosphate, $Na_2HPO_4 \cdot 2H_2O$, A. R.
3. 3-Acetylpyridine analogue of diphosphopyridine nucleotide, AP-DPN *)
4. Glutamic dehydrogenase, GlDH (NH_4^+-free!)
   from bovine liver, crystalline; commercial preparation, see p. 978.

Preparation of Solutions

I. Phosphate buffer (0.066 M; pH 8.2):
   Dissolve 9.078 g. KH_2PO_4 in 1000 ml. distilled water and 11.876 g. $Na_2HPO_4 \cdot 2H_2O$ in 1000 ml. distilled water. Mix 3.3 parts by volume of the KH_2PO_4 solution with 96.7 parts by volume of the Na_2HPO_4 solution.

II. 3-Acetylpyridine analogue of diphosphopyridine nucleotide (*ca.* 10^{-2} M AP-DPN):
   Dissolve 5 mg. AP-DPN in 1 ml. doubly distilled water.

III. Glutamic dehydrogenase, GlDH (20 mg. protein/ml.):
   Use commercially available suspension of 20 mg. protein in 1 ml. 0.15 M Na_2SO_4 solution (pH 7.0).

Procedure

For the preparation and extraction of experimental material (blood, tissue, *etc.*) see the determination of pyruvate (p. 254).

Spectrophotometric measurements

Wavelength: 366 mμ (λ_{max} for AP-DPNH = 363 mμ); glass cuvettes, light path: 1.0 cm.; final volume 3.0 ml. The light path and final volume can be altered so as to make the test

*) obtainable from the Pabst Laboratories, Milwaukee, Wisconsin, USA.

more sensitive. Temperature: 37°C. Incubate the cuvettes in a thermostatically controlled water bath or constant temperature cuvette chamber. Read experimental and control cuvettes against a water blank.

Bring buffer and sample to 37°C.

Pipette successively into the cuvettes:

Experimental cuvette	*Control cuvette*
2.37 ml. buffer (solution I)	2.37 ml. buffer (solution I)
0.40 ml. AP-DPN solution (II)	0.40 ml. AP-DPN solution (II)
sample + water to 2.97 ml.	water to 2.97 ml.

Record the optical densities of both cuvettes. If the change in optical density in both cuvettes is not greater than 0.001 to 0.002 per 30 sec., mix into both cuvettes

0.03 ml. GlDH suspension (III) (*ca.* 0.6 mg. protein).

The reaction is considered complete (usually after 30—40 minutes) when the change in optical density with time in both cuvettes falls to a low rate. A control containing all the components of the assay system, except the enzyme, shows no significant optical density change with time. Subtraction of the optical density difference between the experimental and control cuvette before the start of the reaction with GlDH from the optical density difference between the experimental and control cuvette on completion of the reaction yields ΔE required for the calculations.

Since the only optical density change occurring in the control cuvette is that caused by the absorption of the enzyme and the dilution of the assay mixture on addition of the enzyme, this cuvette can usually be omitted in order to conserve expensive AP-DPN. In this case, the change in optical density caused by the absorption of the enzyme and by the dilution of the assay mixture must be obtained by a further addition of the enzyme to the experimental cuvette after completion of the reaction.

Calculations

$$\frac{\Delta E \times V}{\varepsilon \times d} = \mu\text{moles glutamate/cuvette}$$

ΔE is the increase in optical density occurring on addition of GlDH, corrected as detailed above. The extinction coefficient ε of AP-DPNH is 9.1 cm.^{2}/μmole[1] at 366 mμ, d is the light path of the cuvette in cm., V is the final volume of the test mixture in ml.

Specificity

The estimation is virtually specific for L-glutamate. The optical density change with time using equimolar amounts of DL-norleucine, DL-norvaline or DL-serine is less than 0.5% of that with L-glutamate.

Appendix

Initial velocity of glutamate oxidation

The initial velocity of the reactions with 3.3×10^{-5} M AP-DPN or DPN were compared. In 0.16 M phosphate buffer (pH 8.0) and with 13.4 mg. protein/l. the ratio of $V_{DPN} : V_{AP\text{-}DPN}$ was 1.94 : 1.

[1] *J. M. Siegel, G. A. Montgomery* and *R. M. Bock*, Arch. Biochem. Biophysics *82*, 288 [1959].

Michaelis constants

The reaction rates with 5.5×10^{-4} M to 1.65×10^{-2} M glutamate or with 2.5×10^{-5} M to 5.0×10^{-4} M AP-DPN were measured in 0.165 M phosphate buffer (pH 8.0) at 23.5°C. The constants were calculated according to *Lineweaver* and *Burk*[2]. At 1.36×10^{-2} M glutamate the K_M for AP-DPN is 3.85×10^{-4} M, at 3.33×10^{-4} M AP-DPN the K_M for L-glutamate is 4.45×10^{-3} M[3].

Equilibrium constant

The equilibrium constant K is defined as

$$K = \frac{[\text{AP-DPNH}]\times[\alpha\text{-oxoglutarate}]\times[NH_3]\times[H^+]}{[\text{AP-DPN}^+]\times[\text{glutamate}]}$$

Glutamate was determined as described in the above assay. AP-DPN was determined by quantitative conversion to AP-DPNH with malic dehydrogenase at pH 9.5 and excess malate (refer to p. 332). The H^+ ion concentration was measured with a glass electrode after the equilibrium was reached. The equilibrium concentrations of AP-DPN, α-oxoglutarate and NH_3 were obtained from the spectrophotometric measurement of the AP-DPNH concentration at equilibrium. The value obtained[3] for K was 2.68×10^{-12} [moles/l.][2] at 31°C.

Determination with Glutamate-Pyruvate Transaminase

Gerhard Pfleiderer

Principle

The α-amino group of L-glutamic acid is transferred to pyruvate by glutamate-pyruvate transaminase (GPT). In addition to L-alanine, α-oxoglutaric acid is also formed and this can be reductively aminated with glutamic dehydrogenase (GlDH) and reduced diphosphopyridine nucleotide (DPNH) to give glutamate again. The glutamic acid acts catalytically and is not consumed in the over-all process.

(1) L-Glutamate + pyruvate $\overset{\text{GPT}}{\rightleftharpoons}$ α-oxoglutarate + L-alanine

(2) α-Oxoglutarate + NH_4^+ + DPNH $\overset{\text{GlDH}}{\rightleftharpoons}$ L-glutamate + DPN^+

The indicator reaction (2) proceeds quantitatively from left to right owing to the favourable equilibrium constant[1] ($K = 5.05\times10^4$ l./mole). At relatively low glutamate concentrations (about 0.07 to 1.4 μmoles/ml.) there is direct proportionality between the glutamate concentration and the rate of the coupled reaction. This can be followed spectrophotometrically at 340 or 366 mμ by the decrease in DPNH per unit time[2]. The advantage of this method over that of chromatographic analysis has been demonstrated[3].

Reagents

1. Potassium dihydrogen phosphate, KH_2PO_4, A. R.
2. Disodium hydrogen phosphate, $Na_2HPO_4\cdot2H_2O$, A. R.
3. Sodium pyruvate
   commercial preparation, see p. 1027.
4. L-Glutamic acid

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[2] *H. Lineweaver* and *D. Burk*, J. Amer. chem. Soc. *56*, 658 [1934].
[3] *H. D. Söling* and *H. Holzer*, Biochem. Z., *336*, 201 [1962].

[1] *J. A. Olson* and *C. B. Anfinsen*, J. biol. Chemistry *197*, 67 [1952]; *202*, 845 [1953].
[2] *G. Pfleiderer*, *I. Grein* and *Th. Wieland*, Ann. Acad. Sci. fennicae, Ser. A II, *60*, 381 [1955].
[3] *W. Thorn*, *H. Scholl*, *G. Pfleiderer* and *B. Mueldener*, J. Neurochem. *2*, 150 [1958].

5. Reduced diphosphopyridine nucleotide, DPNH
   disodium salt, DPNH-Na_2; commercial preparation, see p. 1011.
6. Ammonium acetate, A. R.
7. Glutamic dehydrogenase, GlDH
   from liver, crystalline suspension in 2.0 M ammonium sulphate solution; commercial preparation, see p. 978.
8. Glutamate-pyruvate transaminase, GPT
   from pig heart, suspension in 1.6 M ammonium sulphate solution; commercial preparation, see p. 977.

Purity of the enzyme preparations

Both enzyme preparations must not contain lactic dehydrogenase, otherwise the pyruvate will be reduced by DPNH, thus simulating a raised transaminase activity. Commercially available GlDH *) is sufficiently pure. GPT isolated according to[4] contains virtually no lactic dehydrogenase after the first purification step (adsorption on calcium phosphate gel). This enzyme should have a specific activity of 70—90 units**)/mg.

Preparation of Solutions

I. Phosphate buffer (M/15; pH 7.2):
   a) Dissolve 11.876 g. $Na_2HPO_4 \cdot 2H_2O$ in doubly distilled water and make up to 1000 ml.
   b) Dissolve 9.078 g. KH_2PO_4 in doubly distilled water and make up to 1000 ml.
   Mix a) and b) in the ratio of 72 : 28 volumes.

II. Sodium pyruvate (0.1 M):
   Dissolve 1.1 g. sodium pyruvate in doubly distilled water and make up to 100 ml.

III. Ammonium acetate (3 M):
   Dissolve 2.31 g. ammonium acetate in doubly distilled water and make up to 10 ml.

IV. Reduced diphosphopyridine nucleotide (1.2×10^{-2} M β-DPNH):
   Dissolve 50 mg. DPNH-Na_2 in 5 ml. doubly distilled water.

V. Glutamic dehydrogenase, GlDH (10 mg. protein/ml.):
   If necessary, dilute the crystalline suspension with 2.0 M ammonium sulphate solution.

VI. Glutamate-pyruvate transaminase, GPT (5—10 mg. protein/ml.):
   Use without dilution preparations obtained according to[4]. Dilute the commercial preparations with 1.6 M ammonium sulphate solution.

VII. Glutamate standard solution (500 μg./ml.):
   Dissolve 50 mg. L-glutamic acid in doubly distilled water, neutralize with NaOH and dilute with doubly distilled water to 100 ml.

Stability of the solutions

The phosphate buffer, ammonium acetate and pyruvate solutions are stable, well stoppered, in a refrigerator. The solution to be analysed and the glutamate standard solution should be used as soon as possible. Otherwise, like the DPNH solution, they are stored frozen. Crystalline suspensions of GlDH are stable for months at 0°C; GPT for 6—8 weeks.

*) From C. F. Boehringer & Soehne GmbH, Mannheim (Germany).
**) Units according to *Th. Bücher* et al. (see p. 33).
4) *L. Grein* and *G. Pfleiderer*, Biochem. Z. *330*, 433 [1958].

Procedure

Preliminary treatment of the experimental material

Deproteinize tissue extracts by boiling*) as described on p. 379.

Treat protein hydrolysates according to p. 379.

For the determination of glutamine in the presence of glutamate, heat a portion of the sample in 2 N H_2SO_4 for 2 hours in a boiling water bath, cool, neutralize with 2 N NaOH and dilute with distilled water to a known volume. Use this solution for the determination. The result is the sum of the glutamine and glutamic acid.

Standard curve

A standard curve should be prepared for each series of measurements. Take portions of the L-glutamate standard solution (VII) (0.08 to 0.56 ml., corresponding to 40 to 280 μg. L-glutamate) and measure the ΔE/minute under the test conditions described below. The rates, corrected if necessary, are plotted graphically, ΔE/minute (ordinate) versus μg. L-glutamate (abscissa). There must be strict linear proportionality between the corrected rates and the glutamate concentrations. The standard curve must cut the coordinates at the origin (see under "Sources of Error" p. 380). The standard curve is unnecessary if the results are calculated using a standard value (see below).

Spectrophotometric measurements

Wavelength: 340 mμ or 366 mμ; light path: 1 cm.; final volume: 4 ml.; room temperature must be constant for a series of measurements! Preferably work with a constant temperature cuvette chamber; at least allow all solutions to warm to room temperature before use. Prepare two determinations with different amounts of sample.

Pipette successively into the cuvettes:

0.20 ml. pyruvate solution (II)
0.10 to 0.20 ml. pre-treated sample or 0.08 to 0.56 ml. glutamate standard solution
0.07 ml. DPNH solution (IV)
0.02 ml. ammonium acetate solution (III)
0.01 ml. GlDH suspension (V)
buffer (solution I) to 3.96 ml.

Mix, observe for several minutes any small change in optical density which may occur (ΔE_1/minute). By mixing in

0.04 ml. GPT suspension (VI)

start the transaminase reaction. Take readings of the decrease in optical density at 60 second intervals for about 5 min. (ΔE_2/minute).

Calculations

Both the transaminase reaction and any reaction before addition of transaminase are linear. Therefore the values for the rates can be averaged. The corrected rate of the transaminase reaction is ΔE_2/minute $- \Delta E_1$/minute $= \Delta E$/minute. These values are used to prepare the standard curve if known

*) At pH 7.2 glutamine is not measurably converted to pyrrolidonecarboxylic acid when heated for 5 minutes at 100° C: *A. Meister*, J. biol. Chemistry *235*, PC 39 [1960].

amounts of glutamate have been added, or to obtain the glutamate concentration of unknown samples from the standard curve. Routine determinations can be calculated according to the following formula without reference to the standard curve:

$$\frac{\Delta E/\text{minute of sample}}{\Delta E/\text{minute of standard}} \times \mu g.\ \text{glutamate in standard} = \mu g.\ \text{glutamate/test mixture.}$$

The glutamine content of the sample is calculated from the difference of the measurement before and after acid hydrolysis. The molecular weights of glutamine (146.14) and glutamic acid (147.13) are very similar.

Sources of Error

See chapter on "Alanine" under "Sources of Error" (p. 380). Contamination of the enzyme preparations with lactic dehydrogenase interferes with the reaction (see under "Purity of the enzyme preparations"). Substances present in tissues or heavy metal contaminants may also interfere with the determination. The indicator enzyme, glutamic dehydrogenase, is very sensitive to high concentrations of anions and heavy metals. Other amino acids have virtually no effect on the rate of the reaction.

Specificity

The method is extremely specific. Only glutamate serves as amino donor and only α-oxoglutarate as acceptor in the GPT-reaction. D-Glutamate does not react. It can be determined if the value for L-glutamate obtained enzymatically is subtracted from the value for total glutamate determined with ninhydrin (after chromatographic or electrophoretic separation of the glutamate).