Nitrate

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As the existing colour reactions for nitrate are less sensitive and less specific than the *Griess-Ilosvay* reaction for nitrite, nitrate is usually determined after reduction to nitrite. However, the usual reducing agents are not sufficiently specific and it is normally difficult to obtain a quantitative reduction of nitrate to nitrite. *Egami* and *Sato*[1] found that certain strains of *Escherichia coli* contain nitrate reductase, but no nitrite reductase. Nitrate reductase reduces nitrate to nitrite in the presence of a hydrogen donor (*e.g.* formic acid) and an intermediary hydrogen carrier (*e.g.* methylene blue). It has been used for the microdetermination of nitrate[2].

Principle

Nitrate reductase catalyses the reduction of nitrate with leuco-methylene blue (MbH_2):

(1) $$NO_3^- + MbH_2 \longrightarrow NO_2^- + Mb + H_2O$$

The leuco-methylene blue is regenerated by the reaction of formate with methylene blue, which is catalysed by the formic dehydrogenase contained in the nitrate reductase preparations:

(2) $$Mb + HCO_2^- + H_2O \longrightarrow MbH_2 + CO_2 + OH^-$$

The nitrite formed in reaction (1) is determined colorimetrically by the *Griess-Ilosvay* reaction.

Reagents

1. Sodium formate
2. Sodium dihydrogen phosphate, NaH_2PO_4
3. Disodium hydrogen phosphate, $Na_2HPO_4 \cdot 12\ H_2O$
4. Methylene blue*)
5. Uranyl acetate, $UO_2(Ac)_2 \cdot 2\ H_2O$
6. Sulphanilic acid
7. Sodium acetate, anhydrous
8. Acetic acid, glacial
9. α-Naphthylamine
10. Hydrochloric acid, conc.
11. Nitrate reductase
    from *E. coli* strain *Yamagutchi***). For a description of the preparation, see Appendix, p. 639.

*) Flavine adenine nucleotide (FAD) (available commercially as the free acid, see p. 1012) can be used instead of methylene blue.

**) Other strains are suitable, providing they contain no nitrite reductase. Common *E. coli* strains, when grown under the conditions described in the Appendix (p. 639), contain relatively large amounts of nitrate reductase and only small amounts of nitrite reductase. For routine work a suspension of the bacteria can be used[3]. *R. M. Hill et al.*[4] and *G. H. Garner et al.*[5] have described the use of viable cells of other types of bacteria for determination of nitrate.

1) *F. Egami* and *R. Sato*, J. chem. Soc. Japan *68*, 39 [1947]; *69*, 160 [1948]; Proc. japan. Acad. *24*, 29 [1948].
2) *F. Egami, K. Iida, T. Doke* and *S. Taniguchi*, Bull. chem. Soc. Japan *27*, 619 [1954].
3) *R. Williams*, J. Lab. clin. Med. *41*, 157 [1953].
4) *R. M. Hill, H. Pivnick, W. E. Engelhard* and *M. Bogard*, Agric. Food Chem. *7*, 291 [1959].
5) *G. B. Garner, J. S. Baumstark, M. E. Muhrer* and *W. H. Pfander*, Analytic. Chem. *28*, 1589 [1956].

Purity of the enzyme preparations

The preparation obtained according to p. 639 contains no nitrite reductase. If the enzyme preparation is obtained from other strains of *E. coli*, it is necessary to check for the absence of nitrite reductase. The enzyme preparation must contain formic dehydrogenase.

Preparation of Solutions

I. Phosphate buffer (0.2 M; pH 7.2):
Dissolve 0.778 g. NaH_2PO_4 and 5.162 $Na_2HPO_4 \cdot 12\ H_2O$ in 100 ml. distilled water.

II. Uranyl acetate (saturated):
Add 10 g. $UO_2(Ac)_2 \cdot 2\ H_2O$ to 100 ml. distilled water and heat to 40°C. Allow to cool to room temperature and decant the supernatant.

III. *Griess-Ilosvay* reagent:
a) Dissolve 10.5 g. sulphanilic acid and 6.8 g. Na acetate in 300 ml. acetic acid + 600 ml. distilled water. Boil for 3 min. and then dilute to 1000 ml.
b) Add 5.0 g. α-naphthylamine to 1000 ml. boiling water and then add 5 ml. conc. HCl.
c) Just before use mix equal parts of solutions a) and b).

IV. Methylene blue-formate (5×10^{-3} M Mb; 0.2 M formate):
Dissolve 187 mg. methylene blue*) and 1.36 g. Na formate in 100 ml. phosphate buffer (solution I).

V. Nitrate reductase (*ca.* 2 units**)/ml.):
Suspend 20 mg. of the preparation obtained according to p. 639 in 10 ml. phosphate buffer (solution I).

Stability of the solutions

The enzyme suspension is stable in the dark at 0 to 5°C for 1 – 2 weeks. All the other solutions are stable at room temperature.

Procedure

Experimental material

The method has been used for the determination of nitrite in human urine, horse serum, sea water, rain water, extracts of spinach leaves, algae culture medium, *etc.*

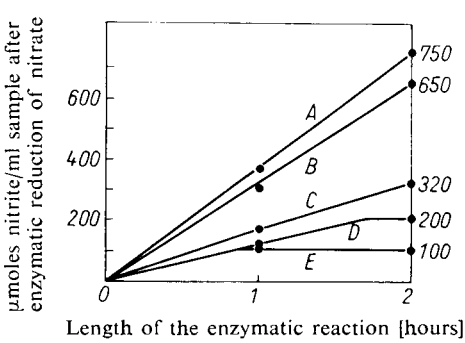

Fig. 1.
Reaction rate with high substrate concentrations
Curve A: 10 µmoles nitrate/ml. sample
Curve B: 1 µmoles nitrate/ml. sample
Curve C: 0.5 µmoles nitrate/ml. sample
Curve D: 0.2 µmoles nitrate/ml. sample
Curve E: 0.1 µmoles nitrate/ml. sample

*) 4 mg. FAD can be used instead of 187 mg. methylene blue.
**) A unit is the amount of enzyme which under the conditions described here forms 1 µmole NO_2^- in 20 min. from 2 ml. 0.01 M KNO_3 solution.

The sample to be analysed should contain between 0.01 and 0.1 μmoles nitrate/ml. (determine the approximate concentration in a preliminary experiment, see Fig. 1). If the sample contains more than 0.1 μmole nitrate/ml. dilute with distilled water.

Method

Preliminary remarks: If the sample contains nitrite as well as nitrate, then the nitrite must be determined in a portion of the sample before reduction of the nitrate, and the total nitrite determined in a second portion after reduction of the nitrate. The nitrate content of the sample is given by the difference in the two values.

Enzymatic reaction:

Pipette into Thunberg tubes (see p. 31):

Experimental	*Control*
1.0 ml. enzyme solution (V)	1.0 ml. enzyme solution (V)
0.5 ml. methylene blue-formate solution (IV)	0.5 ml. methylene blue-formate solution (IV)
2.0 ml. sample	2.0 ml. distilled water.

Evacuate the tubes and incubate for 1.5 to 2 hours at 37° C.

Nitrite determination:

Wavelength: 525 mμ; light path: 1 cm.; Measure against the control.

Pipette into centrifuge tubes:

Experimental	*Control*
2.0 ml. uranyl acetate solution (II)	2.0 ml. uranyl acetate solution (II)
3.0 ml. from the enzymatic reaction mixture	3.0 ml. from the enzymatic reaction mixture

To decolorize the mixture add a little alumina to each tube and centrifuge until clear. Measure the optical density of the clear supernatant (E_1).

To

4.0 ml. of each clear supernatant

add

1.0 ml. Griess-Ilosvay reagent (solution IIIc).

After 20 min. read the optical density E_2.

Calculations

Obtain from a standard curve the μmoles nitrate/ml. sample corresponding to the measured optical density difference $\Delta E = E_2 - E_1$.

Sources of Error

Among the inhibitors of nitrate reductase are [6-8] cyanide, azide, *o*-phenanthroline, α,α'-dipyridyl, thiourea and high concentration of salts (*e.g.* sea water; in this case add 1 ml. distilled water to the enzymatic reaction mixture; final volume of the mixture 4.5 instead of 3.5 ml.).

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[6] *K. Ilida* and *S. Taniguchi*, J. Biochem. *46*, 1041 [1959].
[7] *E. Itagaki* and *S. Taniguchi*, J. Biochem. *46*, 1419 [1959].
[8] *S. Taniguchi* and *E. Itagaki*, Biochim. biophysica Acta *31*, 294 [1960].

Specificity

The method is completely specific for nitrate, since apart from nitrate, nitrate reductase only reduces chlorate[4], and the *Griess-Ilosvay* reaction is specific for nitrite. Crude nitrate reductase preparations may contain a nitro-reductase which catalyses the reduction of aromatic nitro compounds. This contaminant does not cause any interference in the analysis of biological samples.

Appendix

Preparation of the nitrate reductase

Grow *E. coli* strain *Yamagutchi* for 8 hours anaerobically in a peptone broth containing 0.1% KNO_3, 0.2% K_2HPO_4, 2% glucose, amino acids (casein hydrolysate) and yeast extract. Collect the cells by centrifuging and wash with distilled water to remove all the nitrite. Grind the frozen cells for 30 min. in a cold mortar with double their weight of Al_2O_3 powder. Grind the resulting brei for 10 min. with five times the cell weight of cold 0.1 M phosphate buffer (pH 7.1). Centrifuge in the cold for 20 min. at 2000 g. Discard the precipitate. Centrifuge the supernatant in the cold for 40 min. at 20000 g. The sediment contains nitrate reductase and formic dehydrogenase and is used as the enzyme preparation.

Store the preparation as a suspension in distilled water at 0—5° C. It keeps for about 2 weeks.