Enzymatic Microdetermination of Nitrate

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As the color reactions for nitrate so far known are less sensitive and less specific than the Griess-Ilosvay reaction for nitrite, nitrate has often been estimated as nitrite after reduction. However, ordinary reducing agents are not sufficiently specific and it is generally difficult to reduce nitrate quantitatively to nitrite.

We tried, therefore, to reduce nitrate to nitrite quantitatively by a specific enzyme—nitrate reductase,* and to determine nitrate indirectly by the Griess-Ilosvay reaction for nitrite.

Concerning nitrate reductase, R. Sato and one of us (Egami)[1] have shown that a cell-free extract obtained by the ultrasonic destruction of certain strains of *Escherichia coli* contains nitrate reductase, but not nitrite reductase, and nitrate can be reduced to nitrite by the enzyme preparation in the presence of an appropriate hydrogen donor such as formic acid and an intermediary hydrogen carrier such as methylene blue.

Analytical Procedure

Enzyme Preparation.—A strain of *E. coli* (given by Dr. S. Yamagutchi) is cultured at first on the KNO_3(0.1%), peptone (1%), bouillon (1%), agar plate for 15—17 hours at pH 7.4, 37°C. The preparatory culture is inoculated on the peptone bouillon agar plate without nitrate and incubated for 15—17 hours at 37°C. The bacterial cells harvested from 2—3 litre plate culture are washed several times by centrifugation until the supernatant is free from nitrite. The bacterial cells are suspended in 50—60 ml. of distilled water and destroyed by ultrasonic irradiation (490 K.C. for 30—40 min.). Then the bacterial debris are centrifuged off (7000×g. for 30—40 min.) at 0°C. To the cooled supernatant, is added an equal volume of saturated ammonium sulfate solution (adjusted to pH 7.0) under stirring. The formed precipitate containing nitrate reductase and formic dehydrogenase is centrifuged (7000×g. for 30 min.) at 0°C and dissolved in 20—30 ml. of cold water. The enzyme solution is dialysed against running water for 24 hours. Then the enzyme solution is freeze-dried, and 700—800 mg. of the dried preparation is obtained. The dried preparation in evacuated ampoule maintains its activity for at least 3 months in an ice-box.

Solutions required. — a. Enzyme solution: The freeze-dried powder is dissolved in distilled water (2mg./1 ml.).

b. Methylene blue or FAD**(1/200 M), sodium formate (1/5 M), phosphate buffer (1/5 M) (pH

* In this connection, it should be added that R. Williams et al. have used living cells of *E. coil* as reducing catalyst. Their method is for clinical use and less accurate (*J. Lab. Clin. Med. St Louis*, **41**, 157, (1953)).

1) F. Egami and R. Sato, *J. Chem. Soc. Japan*, **68** 39, (1947); **69** 160 (1948); *Proc. Japan Acad.*, **24** 29 (1948).

* Flavin adenine dinucleotide.

* If FAD is used in place of methylene blue, the process of decoloration may be omitted.

7.2) mixed solution.

c. Saturated aqueous solution of uranyl acetate.

d. Griess-Ilosvay reagent:

Solution 1. Sulfanilic acid (10.5 g.), sodium acetate (6.8 g.) and glacial acetic acid (300 ml.) are dissolved in 600 ml. of distilled water. The soluion is boiled for 3 min. and diluted to 1 l.

Solution 2. α-Naphthylamine (5.0 g.) is added to 1 l. of boiling water, then 5 ml. of conc. hydrochloric acid is added to the solution.

Before use, equal volumes of the two solutions are mixed together.

Method of analysis—1. Place 1 ml. of solution a, 0.5 ml. of solution b and 2 ml. of test solution into a Thunberg tube.

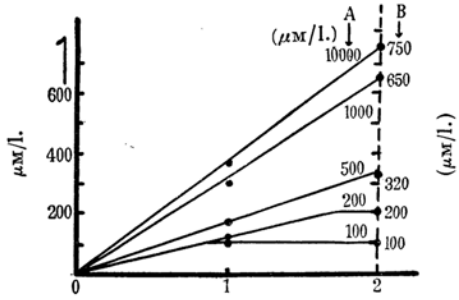
2. Evacuate the tube by aspiration.
3. The tube is allowed to stand at 37°C for 1.5—2.0 hours. Here the reduction takes place.
4. To 2 ml. of solution c in a centrifugal tube, are added 3 ml. of the reaction mixture and a small amount of kaolin powder for decoloration.
5. After centrifugation, to 4 ml. of the clear supernatant is added 1 ml. of Griess-Ilosvay reagent.
6. Then, it is colorimetrically estimated as usual. The authors measured the extinction at 530 mμ with Coleman's spectrophotometer. As blank test solution, the similarly treated solution with 2 ml. of distilled water in place of the test solution should be used.

Remarks.—(1) When nitrite coexists with nitrate, nitrite should be estimated at first as usual, and the value should be subtracted from the total value obtained after reduction.

(2) When the test solution is too concentrated (>100μM/l. nitrate), it must be diluted preliminarily, otherwise the complete reduction can not be attained in 2 hours, as is shown in Fig. 1. So before the accurate analysis, the approximate nitrate concentration must be estimated by measuring the nitrite produced in 2 hours. The test solution is then diluted to an appropriate concentration (10—100μM/l.), and the accurate analysis should be carried out.

Some Examples of Analyses

Some Examples of Analyses with Nitrate and Nitrite Solutions of the Known Concentration.—Some of the experimental results are summarized in Table I, II and III. As shown in Table I and II respectively, nitrate (10—100μM/l.) can be quantitatively estimated by this method and nitrite (50,100μM/l) be quantitatively recovered. Table III shows that the mixture of nitrate and nitrite is quite satisfactorily estimated as the total sum, so nitrate can be determined by subtracting the quantity of nitrite estimated directly as usual.



A: Nitrate concentration in test solution used in enzymatic reduction.

B: Nitrite concentration produced after 2 hours enzymatic reduction.

Fig. 1. Experimental scale for approximate estimation of nitrate concentration.

TABLE I

DETERMINATION OF NITRATE IN STANDARD SOLUTION

Nitrate concn. present (μM/l.)	Nitrite concn. determd. (μM/l.)	Nitrate recovery (%)
10	9.5, 10.5	95, 105
25	25.0, 26.0, 26.5	100, 104, 106
50	48.5, 48.0	97, 96
100	102.0, 99.0, 97.0	102, 99, 97

TABLE II

RECOVERY TESTS OF NITRITE IN NITRATE DETERMINATION

Nitrite concn. present (μM/l.)	Nitrite concn. determd. (μM/l.)	Nitrite recovery (%
50	52.0, 52.0	104, 104
100	98.0	98

TABLE III

DETERMINATION OF NITRATE IN MIXED SOLUTIONS OF NITRATE AND NITRITE

(1) Nitrate concn. present (μM/l.)	(2) Nitrite concn. present (μM/l.)	(3) Nitrite concn. preliminarily determd. (μM/l.)	(4) Nitrate plus nitrite concn. determd. (μM/l.)	(5) Nitrate concn. determd. :(4)—(3) (μM/l.)	(6) Nitrate recovery. (%)
17.0	19.5	19.5	35.0	15.5	91
20.0	6.0	6.0	26.0	20.0	100
20.0	20.0	20.0	38.0	18.0	90
20.0	40.0	40.0	61.0	21.0	105
100.0	40.0	40.0	144.0	104.0	104

Some Examples of Practical Applications.—The method was applied to the estimation of nitrate in human urine, horse serum, spinach leaf extract, sea water and rain water. As a small quantity of nitrate added to these natural test solutions were always quantitatively recovered as shown in Tables IV—VIII, it may be concluded that the method is satisfactory for the estimation of nitrate in these natural samples.

TABLE IV

DETERMINATION OF NITRATE IN HUMAN URINE

Human urine freshly obtained is diluted according to Remarks (2), then used as a sample for accurate analysis. The sample contains no nitrite.

Sample 1. (400 fold diluted)

	Nitrate concn. determd. (μM/l.)	(Mean) (μM/l.)	
Sample alone	15.0		Nitrate concn. in original human urine
	15.0	15.0	15.0μM/l. $\times$ 400 = 6.0 $\times$ 10^{-3}M/l. = 84 mg. nitrate-N/l.
Sample + 25μM/l. nitrate.	38.5		Recovery of 25μM/l. nitrate added (μM/l.)
	38.0	38.5	23.5

Recovery (%): 94

Sample 2. (100 fold diluted)

	Nitrate concn. determd. (μM/l.)	(Mean) (μM/l.)	
Sample alone	63.0		Nitrate concn. in original human urine
	61.5		63.0μM/l. $\times$ 100 = 6.3 $\times$ 10^{-3}M/l. = 88 mg.
	65.0	63.0	nitrate-N/l.
Sample + 25μM/l. nitrate	87.0		Recovery of 25μM/l. nitrate added (μM/l.)
	86.0	86.5	23.5

Recovery (%): 94

TABLE V

DETERMINATION OF NITRATE IN HORSE SERUM

To the freshly prepared horse serum, is added sodium fluoride (final concn. 1%) as an antiseptic.

This sample contains no nitrite.

	Nitrate concn. determd. (μM/l.)	(Mean) (μM/l.)	
Sample alone	35.0, 34.0		Nitrate concn. in horse serum
	32.0, 33.0		34μM/l. = 0.5 mg. nitrate-N/l.
	34.0, 35.0	34.0	
Sample + 25μM/l. nitrate	57.0		Recovery of 25μM/l. nitrate added. (μM/l.)
	57.0	57.0	23.0
Sample + 50μM/l. nitrate	82.0		Recovery of 50μM/l. nitrate added.
	80.0		(μM/l.)
	80.0	81.0	47.0

Recovery (%): 92 (for 25μM/l. added); 94 (for 50μM/l. added)

TABLE VI

DETERMINATION OF NITRATE IN SPINACH LEAF EXTRACT

Minced young spinach leaves are immersed in boiling water, then homogenized and centrifuged (10000×g. for 30 min.). The green supernatant obtained—the dry weight of 1 ml. of this original extract amounts 3.2 mg.—is diluted 200 fold according to Remarks (2), then used as an sample for accurate analysis. This sample contains no nitrite.

	Nitrate concn. determd. (μM/l.)	(Mean) (μM/l.)	
Sample alone	36.0		Nitrate concn. in original spinach leaf extract
	36.0	36.0	$36\mu M/l. \times 200 = 7.2 \times 10^{-3} M/l. = 0.1$ mg. nitrate-N/ml.
			=0.03 mg. nitrate-N/mg. dry weight
Sample+ 25μM/l. nitrate	60.0		Recovery of 25μM/l. nitrate added (μM/l.) / Recovery (%)
	59.0		
	59.0	59.0	23.0 / 92
Sample+ 50μM/l. nitrate	86.0		Recovery of 50μM/l. nitrate added (μM/l.) / Recovery (%)
	84.0	85.0	49.0 / 98

TABLE VII

DETERMINATION OF NIRATE IN SEA WATER

Prior to the determination of nitrate, the nitrite concn. of the sea water is determined according to Remarks (1). In this case, it is necessary to lower the high salt concn. of the sea water, by which nitrate reductase activity is inhibited. So the ordinary analytical method is slightly modified by adding excess 1 ml. of distilled water at Step. 1 and converting the total volume of reaction mixture to 4.5 ml.

	Nitrite concn. preliminarily determd. (μM/l.)	Nitrate plus nitrite concn. determd. (μM/l.)	Nitrate concn. determd. (μM/l.)	(Mean.) (μM/l.)	
Sample alone	2.0	15.0	13.0		Nitrate concn. in sea water
	2.0	17.0	15.0	14.0	14μM/l. =0.20 mg. nitrate-N/l.
Sample+ 25μM/l. nitrate	2.0	40.5	38.5		Recovery of 25μM/l. nitrate added (μM/l.) / Recovery (%)
	2.0	39.5	37.5	38.0	24.0 / 96

TABLE VIII

DETERMINATION OF NITRATE IN RAIN WATER

Prior to the determination of nitrate, the nitrite concn. of the rain water is determined according to Remarks (1). In this case, the nitrate concn. of the test sample is very dilute. So, the ordinary analytical method is slightly modified by using 3 ml. of the test sample and dissolving the enzyme powder in 3.5 ml. reaction mixture.

	Nitrite concn. preliminarily determd. (μM/l.)	Nitrite plus nitrite concn. determd. (μM/l.)	Nitrate concn. determd. (μM/l.)	(Mean) (μM/l.)	
Sample alone	0.5	6.5	6.0		Nitrate concn. in rain water
	0.5	6.0	5.5	6.0	6.0μM/l. =0.08 mg. nitrate-N/l.
Sample+ 12.5μM/l. nitrate	0.5	18.5	18.0		Recovery of 12.5μM/l. nitrate added. (μM/l.) / Recovery (%)
	0.5	18.5	18.0	18.0	12.0 / 96

Summary

1) On the principle of the colorimetry of nitrite produced by enzymatic reduction, a method of microdetermination of nitrate was established.

2) This method can be applied to 10—100μM/l. nitrate solution. 2 ml. of the solution (1.2—12 μg. NO^-_3 or 0.28—2.8 μg. nitrate-N) is sufficient for an analysis.

3) This method is successfully applied to the estimation of nitrate in human urine, horse serum, spinach leaf extract, sea water and rain water.

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