INTERFERENCE OF NADH WITH THE REACTION ON NITRITE IN NITRATE REDUCTASE ESTIMATION

INEKE STULEN

Botanisch Laboratorium afd. Plantenfysiologie, Rijksuniversiteit, Groningen

SUMMARY

In the assay of nitrate reductase, using NADH as electron donor, excess NADH interferes in the reaction on the formed nitrite. A method is described by which the excess NADH is removed by adsorption on activated charcoal.

1. INTRODUCTION

Nitrate reductase activity in plant extracts is generally assayed by determining the nitrite formed. NADH is used as electron donor. In experiments with an enzyme preparation from radish cotyledons it appeared that excess NADH interfered in this assay.

Interference of NADH with the reaction on nitrite has been reported by MEDINA & NICHOLAS (1957). According to these authors its effect is of little importance in the determination of nitrate reductase activity, in estimation of nitrite reductase activity it is more serious. In the present experiments, which were carried out with a nitrate reductase preparation from radish cotyledons, it appeared that excess NADH seriously interfered in the nitrate reductase assay.

2. METHODS

One gram of radish cotyledons was frozen in liquid nitrogen and pulverized with mortar and pestle. Four ml of the extraction medium, consisting of a mixture of 0.01 M potassium phosphate, 0.01 M EDTA and 10^{-4} M cysteine, were added and thoroughly mixed with the powder by means of the pestle. The pH of the reaction mixture was 8.2. The homogenate was centrifuged at 1800 g for 25 minutes, after which the supernatant was used as enzyme preparation. All actions were carried out at 0–4 °C.

The reaction mixture in which the enzyme assay was performed consisted of: 1.0 ml 0.1 M potassium phosphate (pH 8.2); 0.2 ml 0.1 M KNO_3; 0.1–0.2 ml enzyme preparation; 45–600 mμ mol NADH and distilled water to make a final volume of 2.0 ml. After 20 min incubation at 27 °C 1 ml 1 % (w/v) sulphanilamide in n HCl and 1 ml 0.01 % (w/v) N-(1-naphtyl) ethylenediamine-dihydrochloride was added. With sulphanilamide nitrite forms a diazo compound which is coupled with N-(1-naptyl) ethylenediamine-dihydrochloride to form a red dye (SNELL & SNELL 1949). After 30 minutes the extinction was determined

at 540 mμ in a Unicam SP 600 spectrophotometer. The activity of the enzyme preparation was expressed as mμ mol nitrite formed/20 min. The extraction and assay of the enzyme were based on the methods described by BEEVERS *et al.* (1965).

3. RESULTS

Up to a certain concentration of NADH the quantity of nitrite found increased, at higher concentrations this quantity decreased again (*fig. 1a*). This decrease was due to inhibition of the colour formation in the nitrite reaction since it occurred also when no enzyme preparation was present and nitrite had been added to various quantities of NADH (*table 1*).

Fig. 1b shows the inhibition of the nitrite reaction found upon addition of various quantities of NADH as percentage of the maximum nitrite concentration found in the experiment of *fig. 1a*. Apparently, when testing the activity of various enzyme preparations, the percentage inhibition of the nitrite reaction is not the same for one quantity of NADH added, but depends on the activity of the enzyme preparation. At high activities much of the added NADH is used in the reduction of nitrite, and, hence, less remains to interfere with the reaction on nitrite. It follows that the removal of excess NADH after incubation is an absolute necessity for reliable results.

In nitrate reductase tests excess NADH is either not removed (FILNER 1966; HAGEMAN & FLESHER 1960; KANNANGARA & WOOLHOUSE 1967; WALLACE & PATE 1956) or it is removed by treatment with Ba or Zn acetate-ethanol (PANEQUE & LOSADA 1966; SANDERSON & COCKING 1964), by treatment with alcohol dehydrogenase-acetaldehyde (HEWITT & NOTTON 1966; SADANA & MCELROY 1957) or by a combination of the last two treatments (SPENCER 1959).

To test the efficacy of these methods in the present investigation both were tested on radish cotyledon material.

For treatment with Zn acetate-ethanol 0.1 ml of an 1 M solution of Zn acetate and 10,0 ml ethanol 96% were added to 2.0 ml incubated reaction mixture in the cold. The mixture was shaken and centrifuged at 0°C. Nitrite was determined in the supernatant.

Table 1. Inhibition of reaction on nitrite (25 mμ mol) at various amounts of NADH in absence of enzyme preparation

NADH	inhibition
in mμ mol	in %
45	11
75	19
105	24
300	37
705	63

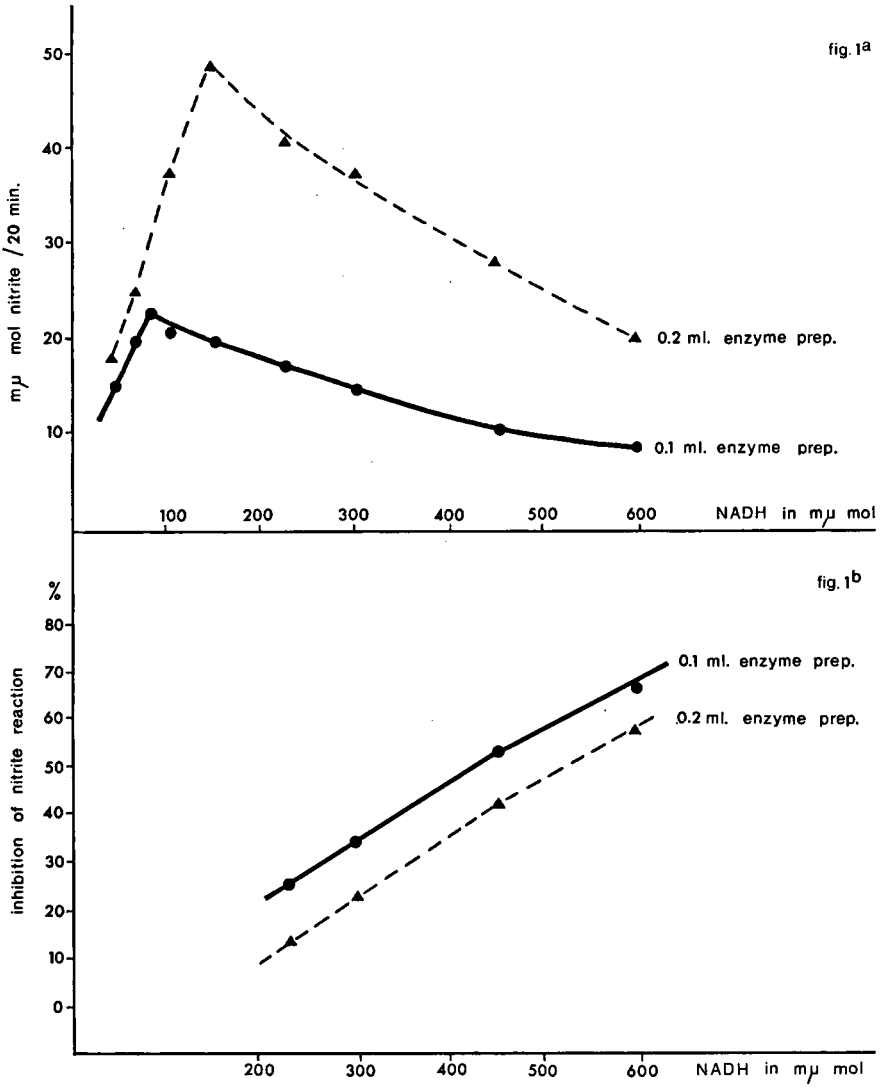

Fig. 1a. Activity of enzyme preparation with various quantities of NADH
Fig. 1b. Inhibition of nitrite reaction at various quantities of NADH

Before treatment with alcohol dehydrogenase-acetaldehyde the reaction mixture was adjusted to pH 7.0 by addition of a diluted HCl solution. Then 120 μmol units of alcohol dehydrogenase and 0,1 ml 1M acetaldehyde were added. The solution was shaken and incubated for 30 min at room temperature. After this period nitrite was determined.

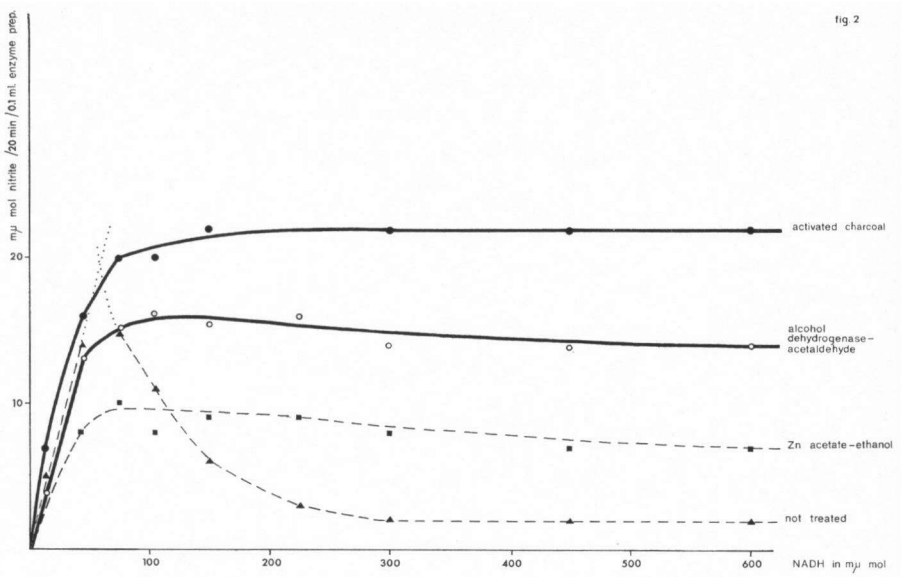

Fig. 2. Reversal of inhibition of the reaction on nitrite at various treatments

Zn acetate-ethanol and alcohol dehydrogenase-ethanol treatments only partially reversed the inhibition of the reaction by NADH on nitrite, and to a different degree (*fig. 2*). Moreover the nitrite concentration found was lower than the concentration obtained when the NADH was just used in the reduction of nitrate. This concentration was determined by extrapolating the curve in *fig. 2* for the untreated mixture. The difference is not due to adsorption of nitrite on precipitated Zn phosphate or protein, since standard curves were used, made from known quantities of nitrite added to a not incubated reaction mixture without NADH, to which the same treatments were applied as to the incubated reaction mixture. As the results with these methods, applied to radish cotyledon material, were unreliable, another treatment was devised.

Since nucleotides may be absorbed on activated charcoal (KELLER & CORI 1955) the incubated reaction mixture was treated with activated charcoal ("Carbo adsorbens", Brocades, Amsterdam).

To 4.0 ml reaction mixture 0,05 g activated charcoal was added. The solution was mixed for 15 sec on a Vortex vibrator and filtered through 2 layers of Blue Band S/S filterpaper. Nitrite was determined in 2.0 ml of the filtrate.

A standard curve was obtained by determining known quantities of nitrite in a not incubated reaction mixture without NADH after treatment with activated charcoal. *Fig. 2* shows that the charcoal treatment reverses the inhibition of the nitrite reaction by NADH. The concentration of nitrite found was higher than with the previous treatments. It is at about the same level as the concentration derived by extrapolation from the curve of untreated mixtures. Moreover by the

Table 2. Treatment with activated charcoal of not incubated mixtures of plant material, 0 or 600 mμ mol NADH and various amounts of nitrite

enzyme prep in ml	NADH in mμ mol	extinction at 540 mμ nitrite in mμ mol		
		20	40	60
0.4	600	0.242	0.430	0.659
		0.241	0.454	0.664
0.4	–	0.243	0.430	0.649
		0.240	0.457	0.661
0.2	600	0.240	0.457	0.660
		0.242	0.450	0.675
0.2	–	0.251	0.450	0.642
		0.242	0.449	0.670

addition of activated charcoal the reaction mixture is not further diluted, which is an additional advantage in determining low enzyme activities.

Treatment with activated charcoal gives reliable and reproducible results (*table 2*). From the results of *table 2* it is to be seen that 600 mμmol NADH is completely removed, and that the extinction values found are the same in presence of either 0.4 or 0.2 ml enzyme preparation. Therefore the amount of added plant material has no influence on the adsorption of NADH on the activated charcoal.

ACKNOWLEDGEMENT

The author wishes to express her thanks to prof. dr. M. H. van Raalte and to dr. J. J. Hofstra for their stimulating interest during this investigation.

REFERENCES

BEEVERS, L., L. E. SCHRADER, D. FLESHER & R. H. HAGEMAN (1965): The role of light and nitrate in the induction of nitrate reductase in radish cotyledons and maize seedlings. *Plant Physiol.* **40**: 691–698.

FILNER, P. (1966): Regulation of nitrate reductase in cultured tobacco cells. *Biochim. Biophys. Acta* **118**: 299–310.

HAGEMAN, R. H. & D. FLESHER (1960): Nitrate reductase activity in corn seedlings as affected by light and nitrate content of nutrient media. *Plant Physiol.* **35**: 700–708.

HEWITT, E. J. & B. A. NOTTON (1966): Effect of substituted uracil derivates on induction of nitrate reductase in plants. *Biochem. J.* **101**: 39c.

KANNANGARA, C. G. & H. W. WOOLHOUSE (1967): The role of CO_2, light and nitrate on the synthesis and degradation of nitrate reductase in leaves of Perilla frutescens. *New Phytol.* **66**: 553–561.

KELLER, P. J. & G. T. CORI (1955): The PR enzyme of muscle. In: *Methods in Enzymol.* **1**: 206–210. Ac. Press. Inc. N.Y.

MEDINA, A & D. J. D. NICHOLAS (1957): Interference by reduced pyridine nucleotides in the diazotization of nitrite. *Biochim. Biophys. Acta* **23**: 440–442.

PANEQUE, A. & M. LOSADA (1966): Comparative reduction of nitrate by spinach nitrate reductase with $NADH_2$ and $NADPH_2$. *Biochim. Biophys. Acta* **128**: 202–204.

SADANA, J. C. & W. D. MCELROY (1957): Nitrate reductase from Achromobacter Fischeri. *Arch. Biochem. Biophys.* **67**: 16–34.

SANDERSON, G. W. & E. C. COCKING (1964): Enzymic assimilation of nitrate in tomato plants. *Plant Physiol.* **39**: 416–422.

SNELL, F. D. & C. T. SNELL (1949); In: *Colorimetric methods of analysis:* 802–807 D. van Nostrand Company. Inc. N.Y.

SPENCER, D. (1959): A DPNH-specific nitrate reductase from germinating wheat. *Aust. J. Biol. Sci.* **12**: 181–191.

WALLACE, W. & J. S. PATE (1956): Nitrate reductase in the field pea. *Ann. Bot. N.S.* **29**: 655–671.