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Effect of vanadium on nitrate reductase activity in tomato leaves

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Abstract

The activity of nitrate reductase in cell-free extracts from tomato leaves is completely inhibited by 100 μM $NaVO_3$ or $VOCl_2$. In experiments *in vivo* vanadium ions inhibit the activity of the enzyme in 50 to 60 per cent. Addition of 1 mM vanadium to the medium on which tomato seedlings are grown causes after 24 h almost complete inhibition of nitrate reductase activity in cell-free extracts of the enzyme. Inhibition with vanadium may be abolished in experiments *in vitro* if the extract is treated with a rather concentrated EDTA solution or Sephadex G-25. The data obtained in this study indicate that vanadium ions in relatively low concentrations inhibit the activity of nitrate reductase in tomato seedlings both *in vivo* and *in vitro*.

The role of vanadium as a microelement fulfilling definite functions in developmental processes of plants is as yet little known. The experiments of Bortel (1936, 1939) and of Jensen and Spencer (1947) indicate that this microelement may substitute molybdenum in the activation of nitrogen binding by *Azotobacter chroococcum, A. vinilandii* and certain *Clostridium* strains. Arnon and Wessel (1953) found that vanadium is indispensable for the growth of *Scenedesmus obliquus*. This suggests in turn a specific role of this microelement. It results, however, from the investigations of Jensen and Betty (1943) and Jensen (1947) that the function of molybdenum cannot be taken over by vanadium in a number of microorganisms.

The role of vanadium in higher plants is still less known. The investigations of Arnon and Stout (1939) demonstrated that substitution of vanadium for molybdenum in medium containing nitrates as the sole source of nitrogen leads in tomatoes to accumulation of nitrates, and further to nitrogen deficit. A number of other plant species such as pea, soya bean or flax reacted to vanadium by growth and development disturbances (Hewitt and Nicholas, 1963). Vanadium also abolishes enhanced transpiration of tomatoes cultured on medium with

nitrate ions (Buczek 1967). Moreover, it is known from the studies of Spencer (1959) that this microelement inhibits nitrate reductase activity in wheat germs.

The present study was undertaken in order to examine the influence of vanadium on the activity of nitrate reductase extracted from tomato leaves (experiments *in vitro*) in discs cut from tomato leaves (experiments *in vivo*) and in tomato seedlings with roots cut off, placed for 24 h in medium with vanadium added.

MATERIAL AND METHODS

Plant cultures. Tomato seedlings of the variety 'Karzełek puławski', were sown on cheesecloth in one-quart dishes. After germination the seedlings were placed in one-litre glass jars with Knop's nutrient solution. The macronutrient solution was supplemented with the following micronutrient Fe-citrate — 50.0; H_3BO_3 — 0.5; $MnSO_4 . 5H_2O$ — 0.05; $CuSO_4 . 5H_2O$ — 0.05; $H_2MoO_4 . 4H_2O$ — 0.05 mg/l. The pH equal to 6.8 was if necessary adjusted to this value by means of 0.1 N NaOH. The plants were grown in a greenhouse. Arter 15 to 20 days the plants were selected and the leaves were used for enzyme assay. The experiments were made in May and June.

In vitro assays

Preparation of enzyme (cell-free extracts). One gram of fresh leaves was ground in 4 times its weight of 0.1 M Tris HCl buffer (pH 7.8) containing 10 mM cysteine. Homogenisation was done in a cold mortar containing cold acid-washed quartz sand. The macerate was pressed through four layers of cheesecloth into plastic centrifuge tubes and the filtrate was centrifuged at 20,000 g for 20 min. The cell-free supernatant was used as the source of enzyme and for soluble protein determination. All of the above operations were carried out at 0° to 3° with cold mortar and reagents.

Enzyme assay. Nitrate reductase activity was assayed in the following incubation medium: 1 ml of 0.1 M potassium phosphate buffer pH 7.5, 0.2 ml 50 mM KNO_3, 0.2 ml 2.7 mM NADH, 0.2 ml of extracts, substrates when used and bidistilled water to make up the final volume to 2 ml. Vanadium was added as $NaVO_3$ or $VOCl_2 . 2H_2O$. The addition of enzyme extract initiated the reaction. After incubation for 30 min at 27°, the reaction was interrupted by adding 0.2 ml M zinc acetate followed by 5 ml of 95% ethanol. Then the centrifuge tubes were placed in a refrigerator for 10 min., subsequently centrifuged at 10,000 g for 5 minutes. For estimation of nitrite 1 to 3 ml of supernatant was removed. One millilitre of 1% (w/v) sulphanilamide in N HCl and 1 ml of

0.01% (w/v) N-(1-naphthyl)ethylenediamine dihydrochloride solution was added to the above aliquot, and the mixture was throughly mixed. After 30 minutes the absorbancy was read at 540 nm with a Specol Zeiss photometr. The nitrites content was formed on a standard curve. The results were computed for total activity (mμ moles of NO_2 formed per gram fresh leaves) and for specific activity (mμ moles of $N0_2$ formed per mg protein per hour).

Protein analyses. Soluble protein was precipitated by the addition of trichloroacetic acid (10% final concentration) to samples of extract. For the removal of chlorophyll, the precipitates were washed three times with a mixture of ethanol and ether (3 : 1). The precipitates were redissolved in 1 N NaOH, and protein was determined by the method of Lowry *et al.* (1951). The standard was bovine serum albumin, fraction V.

In vivo assays. The procedure described by Klepper *et al.* (1971) with some modification was used for *in vivo* assays. The experiments were made on 9-mm discs cut from tomato leaves and weighed before infiltration. The discs (approximately 0.25 g in all cases) were then placed in 50-ml flasks containing 15 ml of infiltration medium. The medium consisted of 0.1 M KNO_3, 0.1 M phosphate buffer (pH 7.5) and vanadium salt when used. The flasks containing the infiltration medium and discs were evacuated. All of the above operations were carried out at 0° to 3°. Evacuation was stopped when the discs were visibly wetted and sank below the surface of the medium. The discs were then removed into 50-ml Erlenmayer flasks containing 5 ml of fresh infiltration medium. The Erlenmayer flasks were then incubated in a water bath at 33° with gentle shaking, and in complete darkness. At time intervals, 0.2 ml of the medium was removed for nitrite determination according to the method described above. The results are expressed as mμmoles of NO_2 per gram fresh weight.

Experiments with tomato seedlings. The 20 days old tomato seedlings were cut off in the root-neck region and the rootless seedlings were placed in flasks containing Knop's medium (control) and in flasks containing Knop's medium with 1 mM of $NaVO_3$ or $VOCl_2$. After 24 h incubation in green-house (16-h photo-period and 8-h dark period), the leaves were taken for extraction of enzyme at the end of the photo-period. The cell-free exctract was either centrifuged at 20,000 g for 20 min or treated with Sephadex G-25. The procedure described by Sanderson and Cocking (1964) was used in treatment with Sephadex. Supernatant (0.2) or 0.2 ml of eluate were used for determination of nitrate reductase activity according to the method described above. Vanadium salt was not added to the incubation medium.

All reagents for the assays were obtained from Calbiochem and Ciech.

RESULTS

Nitrate reductase isolated from various plant species shows maximum activity within a rather narrow pH range. In the present experiments (Fig. 1), pH 7.5 was optimal for enzyme activity in cell-free extracts in the presence of NADH as coenzyme and 0.1 M phosphate buffer.

The influence of substrate concentration is shown in fig. 2. KNO_3 concentrations of the order of 5, 10 and 20 mM activate the enzyme

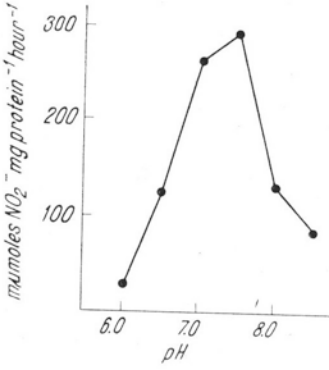

Fig. 1

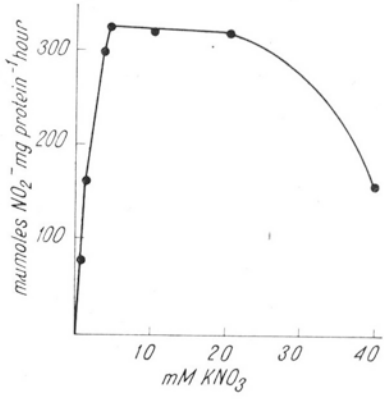

Fig. 2

Fig. 1. Effect of pH on nitrate reductase activity in tomato leaf extracts. The concentration of KNO_3 and phosphate buffer was 5mM and 0.1 M respectively.

Fig. 2. Effect of nitrate concentration on nitrate reductase activity in tomato leaf extracts. pH - 7.5.

equally during 30-min incubation. In further *in vitro* experiments a substrate concentration of 5 mM was used, corresponding to 10 μM of KNO_3 in 2 ml of the incubation mixture.

Both sodium metavanadate and vanadyl chloride proved to be effective inhibitors of nitrate reductase extracted from tomato leaves. Fig. 3 shows the relative values of nitrate reductase inhibition by the action of $NaVO_3$ and $VOCl_2$. Vanadyl chloride was the stronger inhibitor. A 20 μM concentration of $VOCl_2$ per 1 l. of incubation medium inhibited the enzyme activity in 50 per cent, whereas vanadium in the form of $NaVO_3$ produced identical inhibition only in concentrations as high as 60 μM. Both vanadium compounds in 100 μM concentration caused almost complete inhibition of nitrate reductase activity.

In the range of optimal substrate concentrations (5—20 mM) no differences in enzyme inhibition by vanadium ions were observed. As seen in fig. 4, the extent of inhibition is the same at substrate concentrations of 5, 10 or 20 mM. Neither were there any noticeable differences when supraoptimal substrate concentrations were applied.

Complete inhibition of the enzyme activity due to vanadium chloride in cell-free extract can be abolished by the addition to the incubation mixture of 1 mM EDTA. The same EDTA concentration, however, abolishes inhibition only in 50 per cent if it is caused by sodium metavanadate. When EDTA concentration is increased in the incubation mixture to 2.5 mM, the inhibitory influence of vanadyl chloride completely disappears, but the inhibitory effect of $NaVO_3$ does not change.

Vanadium in 1 mM or 10 mM concentrations, when infiltrated into discs cut out of tomato leaves causes almost immediate inhibition of the

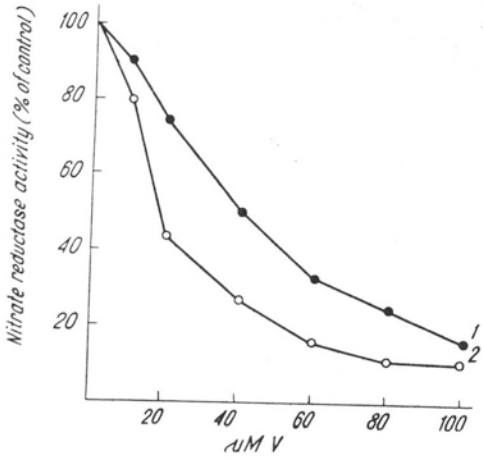

Fig. 3

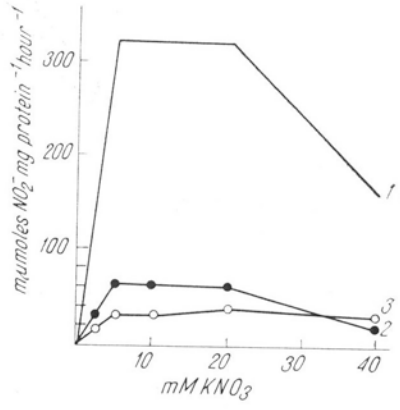

Fig. 4

Fig. 3. Influence of $NaVO_3$ and $VOCl_2 . 2H_2O$ on inhibition of nitrate reductase activity in tomato leaf extracts. Incubation medium was the same as in Fig. 1. *1* - $NaVO_3$; *2* - $VOCl_2$.

Fig. 4. Effect of nitrate concentration on vanadium inhibition of enzyme in a cell-free extracts. Incubation medium was the same as for Fig. 1.
1 — Control; *2* — $NaVO_3$; *3* — $VOCl_2$

Table 1

Effect of EDTA added to incubation medium on vanadium inhibition of nitrate reductase activity in tomato leaf extracts

Incubation medium	Control		1 mM EDTA		2.5 mM EDTA	
	Activity*	%	Activity*	%	Activity*	%
KNO_3 5 mM	89.96	100	129.4	100	115.0	100
KNO_3 5 mM + $NaVO_3$ 100 μM	17.52	20	64.2	50	63.5	55
KNO_3 5 mM + $VOCl_2$ 100μM	14.16	16	126.7	98	116.1	100

*) Activity as mμmoles NO_2^- per mg protein per hour.

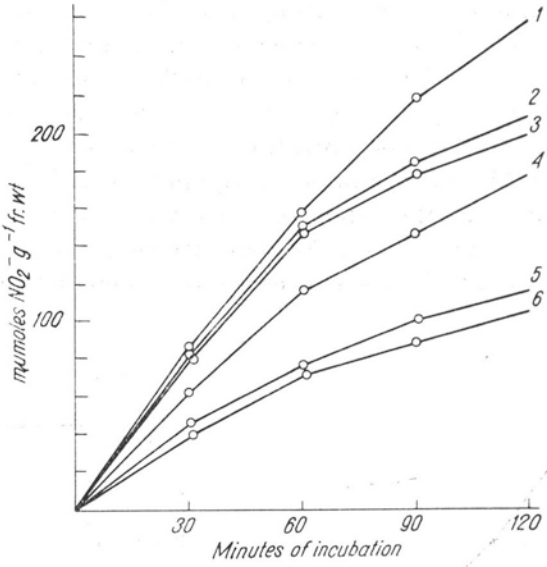

Fig. 5. Effect of $NaVO_3$ on *in vivo* inhibition of nitrate reductase activity. The incubation medium was composed of 0.1 m KNO_3 and 0.1 M phosphate buffer (pH 7.5).

1 — Control; *2* — 0.001 mM; $NaVO_3$; *3* — 0.1 mM $NaVO_3$; *4* — 0.1 mM $NaVO_3$; *5* — 1.0 mM $NaVO_3$; *6* — 10.0 mM $NaVO_3$

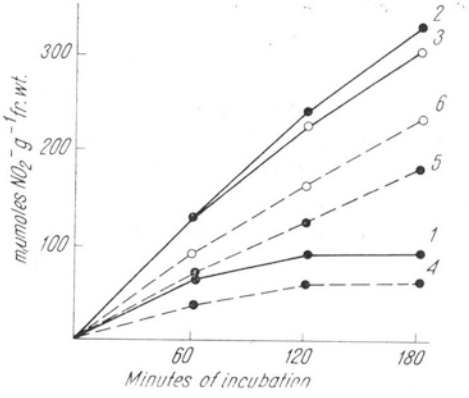

Fig. 6. Effect of nitrate concentration on *in vivo* vadanium inhibition of enzyme.

1 — 10 mM KNO_3; *2* — 100 mM KNO_3; *3* — 200 mM KNO_3; *4* — 10 mM KNO_3 and 1 mM $NaVO_3$; *5* — 100 mM KNO_3 and 1 mM $NaVO_3$; *6* — 200 KNO_3 and 1 mM $NaVO_3$

enzyme. At lower vanadium concentration the enzyme inhibition is noticeable only after 90 min of incubation (Fig. 5). Experiments *in vivo* demonstrated that vanadium did not completely inhibit the enzyme activity as it did *in vitro*.

In experiments *in vitro* (Fig. 4) no relation between the degree of inhibition and substrate concentration could be found. *In vivo* this relation is rather distinct (Fig. 6). An increase of substrate concentration

Table 2

Effect of EDTA added into incubation medium contained 0.2 ml of cell-free extracts, on vanadium inhibition of nitrate reductase activity. The extracts was obtained from 20 days tomato seedings. The seedlings were grown in Knop medium (24 hr) with or without vanadium salts

Culture solution	Incubation medium			
	Buffer		Buffer+1 mM EDTA	
	Activity	%	Activity	%
Knop medium	127.5	100	285.0	100
Knop medium +1 mM $NaVO_3$	26.0	20	113.0	40
Knop medium +1 mM $VOCl_2$	19.3	15	228.0	80

Activity as for Table 1.

in the incubation medium from 0.1 to 0.2 M KNO_3, the $NaVO_3$ concentration remaining unchanged (1 mM), markedly weakens the influence of vanadium.

When 20-day tomato seedling with root cut off are kept in Knop medium with 1 mM sodium vanadate or vanadyl chloride the enzyme activity in the cell-free extract is completely inhibited (Table 2). In these experiments vanadium salt was not added to the incubation medium containing the enzyme. It would seem, therefore, that the inhibition was caused by vanadium accumulated in the plant tissues during 24-h incubation of the lower part of the stems in Knop medium with vanadium added. Treatment with Sephadex of enzyme ectract obtained from such seedlings abolishes completely the inhibitory action of vanadium (Table 3). Similarly, the addition of EDTA to

Table 3

Comparison of nitrate reductase activity in cell-free extracts prepared with two methods. Plants cultures were the same as for Table 2

Culture solution	Preparation of cell-free extracts			
	Centrifuged at 1700 x g then treated with Sephadex G-25		Centrifuged at 20000 x g	
	Activity	%	Activity	%
Knop medium	147.2	100	132.0	100
Knop medium + $NaVO_3$ (10^{-3}M)	163.8	115	25.0	19

Activity as for Table 1.

the enzyme extract, obtained from seedlings inhibited for 24 h with vanadium, abolishes in 80 per cent the inhibitory action of $VOCl_2$, and in a much lower degree the inhibition due to $NaVO_3$ (Table 2). These results are consistent with those obtained in experiments *in vitro*.

DISCUSSION

The above described experiments show that vanadium ions inhibit nitrate reductase activity in cell-free extract of tomato leaves. Inhibition is almost complete at a vanadium concentration fifty times lower as compared to the substrate concentration.

In experiments *in vivo* inhibition of enzyme activity is also observed under the influence of vanadium ions. In contrast, however, to the *in vitro* studies, inhibition is not complete. If the substrate concentration in the filtration medium is increased in *in vivo* experiments, the influence of vanadium inhibiting the enzyme is weaker. It would seem that it is not so much the competition between substrate and inhibitor in the activation or inhibition of the enzyme that comes into play, but rather a competition in free diffusion of these ions in the cytoplasm of the cells. Since from the investigations of Ritenour *et al.* (1967) it is known that nitrate reductase is localized in the cytoplasm or on the outer surface of the chloroplast membranes, it is suggested that the presence in the cytoplasm of free vanadium ions would condition effective enzyme inhibition.

These suppositions find confirmation in the present experiments with plants which accumulated vanadium ions from the medium during 24-h culture. The cell-free extracts of leaves of these seedlings, namely, show a complete disappearance of nitrate reductase activity. If these extracts, however, are treated with Sephadex or EDTA, then enzyme activity is completely restored. This suggests, that the presence of free ions in the cytoplasm is the condition for enzyme inhibition. It also indicates a very labile bond between vanadium and the enzyme molecule. It is but little probable that the inhibition would involve competition between vanadium ions and molybdenum. The latter, as known from the studies of Nicholas and Nason (1954 a, b and 1955), is rather stronghly bound with the flavine nucleotide, and can only be separated from the flavoprotein by long-lasting dialysis in the presence of cyanides. It would seem therefore, that the presence of vanadium in the cytoplasm, like the addition of vanadium to the enzyme extract, produces inhibition of the enzyme activity. The mechanism of this inhibition is not known so far.

Table 4

Effect of vanadium ions on transpiration of tomato seedlings

Culture solution	Transpiration in per cent of control	
	$-NaVO_3$	$+ NaVO_3$
Without N	100	100
With NO_3	166	99
With NH_4	108	93

In an earlier paper (Buczek 1967) the author demonstrated that nitrate ions enhance transpiration (Table 4) as compared to that of plants growing on ammonium salts as only nitrogen source. Tomato seedlings in experiments with roots cut off were placed in potometres with medium deficient in nitrogen (–N), with nitrate medium ($+NO_3$) and in ammonium medium ($+NH_4$). Addition to the medium of vanadium salts in a 1 mM/l. of medium concentration reduced transpiration stimulated by NO_3 ions to the level of transpiration of the control plants. The present experiments in which it has been demonstrated that vanadium ions are a strong inhibitor of nitrate reductase both *in vitro* and *in vivo* seem to confirm the hypothesis of a correlation between metabolic processes and the loss of water in the transpiration process, and particularly between the reduction of nitrate ions and the transpiration intensity.

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Wpływ wanadu na aktywność reduktazy azotanowej w liściach pomidorów

Streszczenie

Przeprowadzono badania nad wpływem jonów wanadu na aktywność reduktazy azotanowej w liściach pomidorów. Doświadczenia wykonano *in vitro*, posługując się wyciągami enzymu z liści, oraz *in vivo*, stosując test krążków (Klepper i wsp. 1971) wyciętych z liści pomidorów. Badano również aktywność reduktazy azotanowej wyodrębnionej z liści siewek, które uprzednio umieszczano na okres 24 godzin w pożywce z dodatkiem 1 mM $NaVO_3$. Stwierdzono, że aktywność reduktazy azotanowej w bezkomórkowych ekstraktach z liści ulega całkowitemu zahamowaniu po dodaniu do medium inkubacyjnego $NaVO_3$ lub $VOCl_2$ w stężeniu 100μM na litr medium. Dodanie EDTA do medium inkubacyjnego zawierającego enzym, substrat i jony wanadu, znosi całkowicie inhibicję wywołaną chlorkiem wanadylu i w 50% inhibicję wywołaną metawanadanem sodu. W doświadczeniach *in vivo*, jony wanadu hamują aktywność enzymu jedynie w 50—60%. Wanad w stężeniu 1 mM na litr pożywki, w której umieszczono siewki pomidorów (dolna, odcięta od korzenia część łodygi zanurzona do 1 cm) powoduje po 24 godzinach całkowitą inhibicję aktywności reduktazy azotanowej mierzoną w wyciągach enzymu. Hamowanie to można całkowicie usunąć, jeżeli wyciągi enzymu oczyści się na kolumnie z Sephadexem G-25, względnie potraktuje EDTA. Powyższe dane wskazują, że jony wanadu hamują aktywność reduktazy azotanowej w siewkach pomidorów tak *in vivo* jak *in vitro*.

Badania powyższe potwierdzają również wcześniejszą hipotezę autora o istnieniu zależności pomiędzy redukcją azotanów a intensywnością transpiracji. Mianowicie stwierdzono (Buczek 1967), że jony azotanowe wpływają na zwiększenie transpiracji w porównaniu do transpiracji roślin rosnących na solach amonowych. Dodanie do pożywek soli wanadu w stężeniu 1 mM powoduje zmniejszenie transpiracji stymulowanej jonami NO_3 do poziomu transpiracji roślin kontrolnych. Obecne badania, w których wykazano, że jony wanadu są skutecznym inhibitorem reduktazy azotanowej w liściach pomidorów, zdają się potwierdzać hipotezę o istnieniu zależności transpiracji od procesów metabolicznych.