Nitrite Oxidase and Nitrate Reductase in *Nitrobacter agilis*

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Nitrite oxidase and nitrate reductase in *Nitrobacter agilis* were shown to be separate enzymes. The best separation of the two systems was achieved by ammonium sulphate fractionation. The effects of various compounds, including antimycin A, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide and chlorate, also clearly distinguish between the two enzyme reactions. The relationship between the two opposing reactions in *Nitrobacter* is discussed.

Wallace & Nicholas (1968) described an enzyme system in the chemoautotrophic nitrifying bacterium *Nitrobacter agilis* that mediates the reduction of nitrate to nitrite. This enzyme system operates in the reverse direction to the following main energy-yielding reaction of this bacterium:

It has been implied that the nitrite oxidase (catalysing the reaction $\text{NO}_2^- \rightarrow \text{NO}_3^-$) and nitrate reductase (catalysing the reaction $\text{NO}_3^- \rightarrow \text{NO}_2^-$) in *Nitrobacter* may be a single enzyme system (Straat & Nason, 1965), whereas other workers consider them to be separate enzymes (Wallace & Nicholas, 1968). Kiesow (1964) proposed that during nitrate reduction ATP is produced.

In the present paper two separate enzymes for nitrite oxidation and nitrate reduction in *N. agilis* are described.

MATERIALS AND METHODS

Materials

NADH, FMN and antimycin A were purchased from Calbiochem (Los Angeles, Calif., U.S.A.); diphenylamine and α' -bipyridyl were from British Drug Houses Ltd., Poole, Dorset; sulphanilamide and *N*-(1-naphthyl)ethylene-diamine dihydrochloride were from Hopkin and Williams Ltd., Chadwell Heath, Essex; HOQNO* was from Sigma Chemical Co. (St Louis, Mo., U.S.A.); di-(*p*-aminophenyl) sulphone was from Fluka A.-G., Buchs, Switzerland. All other chemicals were analytical-grade reagents. Solutions were made up in double-distilled water.

* Abbreviation: HOQNO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide.

Culture of organism

The strain of *N. agilis* used was supplied by Dr M. I. H. Aleem, Department of Microbiology, University of Kentucky, Lexington, Ky., U.S.A. The organism was grown as described by Wallace & Nicholas (1968). The cells were collected by continuous-flow centrifugation at 30000g in a Sorvall RC-2 refrigerated centrifuge. The batch-culture system produced about 6 g. wet wt. of cells, equivalent to 1 g. of protein, every 3 days from two 40 l. containers.

Method for disrupting the cells

Freshly harvested cells were washed until free of nitrite and then disrupted by an ultrasonic probe (20 keyc./sec. for 20 min.) as described by Wallace & Nicholas (1968). Cell debris and unbroken cells were removed by centrifugation at 10000g for 15 min., and the final extract was brownish red. This fraction contained 6–18 mg. of protein/ml., depending on the weight of cells used for extraction in a given volume of buffer.

Enzyme assays

Nitrite oxidase. The assay was carried out in open test tubes (2 cm. $\times$ 15 cm.) containing phosphate buffer (KH_2PO_4 – Na_2HPO_4), pH 7.0 (115 μ moles), NaNO_2 (1.25 μ moles) and 0.1 ml. of the cell-free extract in a total volume of 2.5 ml. After preincubation for 5 min. at 30° in a reciprocating unit the reaction was started by addition of the enzyme. After a further 30 min. the residual nitrite was determined in a suitable portion (0.1 ml. or 0.05 ml.) of the reaction mixture. There was a 1:1 ratio between nitrite consumed and nitrate produced.

Nitrate reductase. The assay was carried out under anaerobic conditions in 20 ml. Thunberg tubes. The contents of each tube were: tube: phosphate buffer, pH 7.0 (115 μ moles), FMN (12.5 nmole) and 0.5 ml. of cell-free extract; side arm: NADH (2 μ moles) and KNO_3 (3 μ moles); the total volume was 3 ml. The tubes were rigorously

evacuated and the contents preincubated for 5 min. at 30° before the NADH and KNO_3 were mixed with the rest of the reaction mixture. Incubation was continued for 30 min. at 30° and then 0.1 ml. of 1M-zinc acetate and 1.9 ml. of 95% (v/v) ethanol were added to remove any residual NADH, which would otherwise interfere with the subsequent diazotization of nitrite (Medina & Nicholas, 1957). After standing for 10 min. the contents were centrifuged at 3000g for 5 min. and a suitable portion (0.1 ml. or 0.2 ml.) of the supernatant solution was used for nitrite determination.

On some occasions nitrate reductase was determined under aerobic conditions in open tubes (2 cm. $\times$ 15 cm. test tubes) by using the same procedure.

Separation of the two enzymes

Centrifugation. Low-speed-centrifugation (up to 20000g) studies were done in a Sorvall SS1 centrifuge at 2° with 50 ml. polypropylene centrifuge tubes.

Higher speeds up to 144000g were achieved in a Spinco model L ultracentrifuge at 2° fitted with an aluminium rotor (Al 50).

Ammonium sulphate fractionation. Fractionation of the crude cell-free extract with solid $(\text{NH}_4)_2\text{SO}_4$ was carried out at 2°. The $(\text{NH}_4)_2\text{SO}_4$ was added slowly with continuous stirring to the extract, which was then left standing for a further 30 min. The precipitate was separated by centrifuging at 10000g for 15 min. The pellet was resuspended in a small volume of 0.05M-phosphate buffer, pH 7.0, and excess of $(\text{NH}_4)_2\text{SO}_4$ removed by dialysis against a large volume of running water at 2° for 6 hr.

Spectral studies

Spectral studies on cell-free extracts were done with a Unicam SP.800 recording spectrophotometer. Difference spectra of oxidized and reduced samples were examined under aerobic and anaerobic conditions in 1 cm. quartz cuvettes containing 3 ml. of cell suspension (approx. 6 mg. of protein/ml. in 0.05M-phosphate buffer, pH 7.0).

Cell suspensions were oxidized by bubbling a fine stream of O_2 through them for 2 min. They were reduced by addition of a few crystals of either $\text{Na}_2\text{S}_2\text{O}_4$ or NaNO_2 (approx. 5 mg.).

Anaerobic spectra were recorded of samples that had been rigorously freed from air by evacuation with a high-vacuum pump and then flushed with O_2 -free N_2 . This was done in 1 cm. quartz cuvettes fitted with Thunberg attachments. Addition of reducing agents to the cell suspension was made from the side arm.

Chemical determinations

Nitrite. This was determined by the colorimetric method of Griess and Ilosvay, given by Snell & Snell (1944), in which a suitable portion of solution, containing up to 60 nmol of nitrite, was made to 1 ml. with water, and 1 ml. of 1% (w/v) sulphanilamide in 1.51M-HCl and 1 ml. of 0.02% (w/v) *N*-(1-naphthyl)ethylenediamine dihydrochloride were added. The intensity of the colour, which was allowed to develop for 20 min., was determined in a Hilger colorimeter (filter 55).

Nitrate. This was determined by the colorimetric method

of Szekely (1965, 1967) involving diphenylamine-di-(*p*-aminophenyl) sulphone. This reagent was prepared by dissolving 50 mg. of diphenylamine and 2 g. of di-(*p*-aminophenyl) sulphone in 225 ml. of water, to which 600 ml. of 18M- H_2SO_4 was added slowly with stirring, followed by 100 ml. of 18M- H_3PO_4 , and the volume was made up to 1 l. with 18M- H_2SO_4 . A sample containing up to 100 nmol of nitrate was diluted to 1 ml. with water in a 25 ml. conical flask, 0.1 ml. of NaCl (2000 p.p.m.) was added and the mixture was cooled in an ice bath. Then 10 ml. of the diphenylamine reagent was added slowly, with thorough mixing, over a period of 10 min. The flask was kept cold (2°) for a further 30 min. and then the colour was developed for a further 2 hr. at 30°. The intensity of the blue colour was determined in a Hilger colorimeter (filter 61) with 1 cm. cuvettes. Reagent blanks were used and the calibration curve obeyed Beer's Law over the range 5–100 nmol of nitrate.

Protein. This was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) after precipitation of the protein with 5% (w/v) trichloroacetic acid. Bovine serum albumin was used as a standard.

RESULTS

Nitrite oxidase and nitrate reductase

Relative activities. Cell-free extracts prepared by ultrasonic treatment contain a nitrite-oxidizing system with a normal Michaelis-Menten-type rate curve over the range 0–4.0 mM-sodium nitrite (Fig. 1). This system had substrate K_m 0.38 mM and observed $V_{\max}$ 0.68 μmole of NO_2^- oxidized/30 min./mg. of protein.

Fig. 1. Lineweaver-Burk plot for nitrite oxidase (●) and nitrate reductase (▲). Enzyme assays are described in the Materials and Methods section. Substrates used: for nitrite oxidase, NaNO_2 ; for nitrate reductase, KNO_3 . Units of velocity (v): for nitrite oxidase, μmoles of NO_2^- oxidized/30 min./mg. of protein; for nitrate reductase, nmol of NO_2^- formed/30 min./mg. of protein.

Table 1. *Effect of inhibitors on nitrite oxidase and nitrate reductase*

Crude cell-free extracts were prepared by ultrasonic treatment and assays were carried out as described in the Materials and Methods section. All inhibitors were dissolved in 95% (v/v) ethanol, and controls were run with the same amount of ethanol without inhibitor. Preincubation of enzyme and inhibitor was done for 5 min. before the reaction was commenced in some cases. Results are expressed as percentage inhibition of the controls without inhibitor and ethanol.

Inhibitor	Final concn. of inhibitor	Inhibition (%)			
		Nitrite oxidase		Nitrate reductase	
		No preincubation	5 min. preincubation	No preincubation	5 min. preincubation
Ethanol	2% (v/v)	0	2	6	3
Amytal	3 mM	12	22	0	23
	6 mM	16	30	0	30
Antimycin A	0.1 mM	63	63	4	3
	0.2 mM	64	89	7	16
HOQNO	0.1 mM	23	29	6	11
	0.3 mM	35	68	8	15
$\alpha\alpha'$ -Bipyridyl	3.2 mM	0	0	0	0
	7.5 mM	6	8	0	15
	15.0 mM	9	24	11	45

Fig. 2. Lineweaver-Burk plots showing competitive inhibition of nitrate reductase by KClO_3 . Enzyme assays are described in the Materials and Methods section. $\blacktriangle$, No KClO_3 ; $\blacksquare$, final concn. $5 \mu\text{M}$ - KClO_3 ; $\bullet$, final concn. 0.01 mM - KClO_3 . Units of activity, v , are $\mu\text{moles of NO}_2^-$ formed/30 min./mg. of protein.

The same extracts contained a nitrate reductase that showed a Michaelis-Menten-type rate curve over the range $0\text{--}0.6 \text{ mM}$ -potassium nitrate, but at higher concentrations substrate inhibition occurred (Fig. 1). The nitrate reductase had substrate K_m 1.05 mM and observed V_{max} $0.18 \mu\text{mole of NO}_2^-$ formed/30 min./mg. of protein.

Effect of pH and ionic strength on the two systems. The nitrite-oxidizing system had optimum pH 6.8, and the activity was the same in phosphate buffer (0.05 M and 0.10 M) and in tris-hydrochloric acid buffer (0.05 M and 0.10 M).

The nitrate reductase, which had optimum pH 7.1, had greater activity in the more-concentrated buffer

solutions (0.10 M -phosphate and 0.10 M -tris-hydrochloric acid buffers), and was more active in phosphate buffer (0.05 M and 0.10 M) than in tris-hydrochloric acid buffer (0.05 M and 0.10 M).

Effect of inhibitors. The effect of various inhibitors on both enzymes is shown in Table 1.

Antimycin A and HOQNO strongly inhibited oxidation of nitrite, but had little effect on reduction of nitrate. Both systems were retarded to a similar extent by Amytal, whereas $\alpha\alpha'$ -bipyridyl at the highest concentration tested (15 mM) inhibited nitrate reduction significantly but had only a small effect on nitrite oxidation.

Chlorate was a competitive inhibitor of nitrate reductase (Fig. 2), giving complete inhibition at a final concentration of 0.13 mM -potassium chlorate. At concentrations greater than 0.13 mM , potassium chlorate stimulated nitrite oxidation; the stoichiometry between nitrite utilization and nitrate appearance was 1:1. Thus the oxidation of nitrite increased progressively with increasing concentrations of chlorate and analysis showed that equivalent amounts of nitrate were formed.

Separation of the two enzymes

Centrifugation. Attempts were made to separate the two systems by high-speed centrifugation (Table 2). The nitrite-oxidizing system was associated with large particles and was almost entirely sedimented by centrifugation at $136000g$ for 1 hr. At the same time 50–70% of the nitrate reductase was found in the dark-red pellet, and the

Table 2. *Separation of nitrite oxidase and nitrate reductase by centrifugation*

Crude cell-free extracts were prepared and assays performed as described in the Materials and Methods section. All pellet fractions were resuspended in 0.05 M-phosphate buffer, pH 7.0, with a Teflon-glass homogenizer. The units of total activity are μ moles of NO_2^- oxidized or formed/30 min./fraction, and the units of specific activity are μ moles of NO_2^- oxidized or formed/30 min./mg. of protein.

Speed of centrifugation (g)	Initial vol. of extract (ml.)	Fraction	Nitrite oxidase			Nitrate reductase		
			Total activity	Specific activity	Distribution of activity recovered (%)	Total activity	Specific activity	Distribution of activity recovered (%)
10 000	10	Supernatant	126.0	0.80		13.95	0.087	
20 000	10	Supernatant	61.26	0.70	49	8.77	0.10	63
		Pellet	64.59	0.88	51	5.07	0.07	37
33 000	10	Supernatant	46.35	0.59	38	7.92	0.10	59
		Pellet	75.85	0.96	62	5.43	0.069	41
76 000	10	Supernatant	5.76	0.10	6	3.84	0.069	37
		Pellet	86.70	1.03	94	6.60	0.079	63
136 000	10	Supernatant	0.10	0.002	1	3.14	0.065	30
		Pellet	84.18	1.04	99	7.35	0.091	70

Table 3. *Separation of nitrite oxidase and nitrate reductase by ammonium sulphate fractionation*

The cell-free extract was prepared and enzyme assays were carried out as described in the Materials and Methods section. Excess of $(\text{NH}_4)_2\text{SO}_4$ was removed by dialysis against a large volume of running water for 6 hr. Units of total activity are μ moles of NO_2^- oxidized or formed/30 min./fraction and the units of specific activity are μ moles of NO_2^- oxidized or formed/30 min./mg. of protein.

Saturation with $(\text{NH}_4)_2\text{SO}_4$ (%)	Volume of extract used (ml.)	Fraction	Nitrite oxidase			Nitrate reductase		
			Total activity	Specific activity	Distribution of activity recovered (%)	Total activity	Specific activity	Distribution of activity recovered (%)
0 (original extract)	44	10 000g supernatant	330.0	1.1		68.8	0.23	
0-33	12	Supernatant	9.98	0.16	23	7.01	0.11	93
		Precipitate	32.66	16.10	77	0.49	0.02	7
0-44	20	Supernatant	0	—	0	39.6	0.50	91
		Precipitate	57.32	9.60	100	0.90	0.03	9
0-55	6	Supernatant	0	—	0	1.08	0.03	11
		Precipitate	17.20	7.20	100	4.22	0.17	89
0-70	6	Supernatant				0.80	0.01	1
		Precipitate				4.50	0.06	99

remainder of the enzyme was in the clear pink supernatant fraction.

Ammonium sulphate treatment. Ammonium sulphate fractionation of crude cell-free extracts produced a better separation of the nitrite oxidase and nitrate reductase systems than was achieved by ultracentrifugation (Table 3). There was, however, a considerable loss in enzyme activities after this treatment, about 30% and 60% for nitrite oxidase and nitrate reductase respectively.

At 33% saturation with ammonium sulphate, 77% of the nitrite oxidase was precipitated but 93% of the nitrate reductase remained in the

supernatant fraction. At 44% saturation, all the nitrite oxidase was precipitated but 91% of the nitrate reductase remained in the supernatant fraction. Most of the nitrate reductase was precipitated between 44% and 70% saturation.

Simultaneous operation of nitrite oxidase and nitrate reductase

With crude extracts nitrate reductase was not detected under aerobic conditions, but partly purified preparations, which were almost free of nitrite oxidase, reduced nitrate to nitrite in air.

Table 4. *Nitrite oxidation and nitrate reduction under aerobic and anaerobic conditions*

The cell-free extract was prepared by ultrasonic treatment. Anaerobic assays were made in Thunberg tubes, and aerobic assays in open test tubes, as described in the Materials and Methods section. Units of activity are $\mu\text{moles of NO}_2^-$ oxidized or formed/30 min./mg. of protein.

Enzyme preparation	Enzyme system	Condition of assay ...	Activity	
			Aerobic	Anaerobic
Crude cell-free extract	Nitrite oxidation		0.70	0
			0.71	0
	Nitrate reduction		0	0.14
			0	0.15
136000g $\times$ 1 hr. supernatant fraction	Nitrite oxidation		0.056	0
			0.037	0
	Nitrate reduction		0.041	0.20
			0.041	0.21

Table 5. *Simultaneous operation of nitrite oxidase and nitrate reductase*

Cell-free extracts were prepared by ultrasonic treatment as described in the Materials and Methods section. The amounts of KNO_3 , FMN and NADH used are as given for the nitrate reductase assay in the Materials and Methods section. The units of activity are $\mu\text{moles of NO}_2^-$ oxidized or formed/30 min./mg. of protein. Results from duplicate experiments are given.

Added cofactors and substrates	Conditions of assay	Nitrite oxidized	Nitrite formed
—	Aerobic	0.598	
		0.602	
—	Anaerobic		0.143
			0.150
KNO_3 (1 mM)	Aerobic	0.600	
		0.605	
FMN (4 μM)	Aerobic	0.595	
		0.600	
NADH (0.6 mM)	Aerobic	0.630	
		0.630	
KNO_3 (1 mM)	Aerobic	0.500	
FMN (4 μM)		0.490	
NADH (0.6 mM)			

Thus the supernatant fraction left after centrifuging at 136000g for 1 hr. reduced nitrate to nitrite under aerobic conditions at a fairly low rate (0.04 $\mu\text{mole of NO}_2^-$ formed/30 min./mg. of protein) compared with that under anaerobic conditions (0.20 $\mu\text{mole of NO}_2^-$ formed/30 min./mg. of protein) (Table 4). This preparation, however, contained some nitrite oxidase activity.

Constituents of the nitrate reductase assay (nitrate, FMN and NADH) when added singly had little or no effect on the rate of nitrite oxidation, but when they were added together the overall rate of nitrite oxidation was decreased (Table 5). The decrease in rate (0.1 $\mu\text{mole of NO}_2^-$ oxidized/30 min./mg. of protein) corresponded approximately to the rate of nitrite formation by the extract under

Fig. 3. Difference spectra of oxidized versus reduced cell-free extracts. The base line (—) is the difference spectrum of two oxidized samples; the full line (—) is the difference spectrum of an oxidized versus a reduced sample of crude cell-free extract containing 4 mg. of protein/ml.; the broken line (---) is the difference spectrum of an oxidized versus a reduced sample of the supernatant fraction left after centrifuging the crude extract at 136000g for 1 hr., final concentration 3.4 mg. of protein/ml. of supernatant fraction. Experiments were done with a Unicam SP.800 recording spectrophotometer fitted to an expansion recorder.

anaerobic conditions (0.15 $\mu\text{mole of NO}_2^-$ formed/30 min./mg. of protein).

Spectral studies on cell-free extracts

Difference spectra of oxidized versus reduced cell-free extracts, illustrated in Fig. 3, show five

main absorption bands at the following wavelengths: 605–597, 550, 520, 438 and 418 nm. These were obtained when the cell-free extract was reduced in an open cuvette with sodium dithionite or sodium nitrite. Identical difference spectra were also obtained when the crude extracts were reduced with NADH in the absence of air.

The difference spectrum (dithionite-reduced) of the 136 000g supernatant fraction had absorption bands at 550, 520 and 418 nm.

DISCUSSION

The above results support the hypothesis that there are two separate enzyme systems involved in nitrite oxidation and nitrate reduction in *N. agilis*.

The differential effects of certain electron-transfer-chain inhibitors (antimycin A and HOQNO) on the two systems, and the reasonably distinct separation of the enzymes by centrifugation and ammonium sulphate fractionation (Tables 3 and 4), clearly support this idea. Although 91% of the total nitrate reductase activity of the cell-free preparation could be accounted for in fraction 4 (Table 4) in the complete absence of the nitrite-oxidizing system, no nitrite oxidase preparation was produced that was completely free from nitrate reductase activity. Because of this, the possibility still remains that a small proportion (less than 9% of the total recovered) of the nitrate reductase activity might be due to a reversal of the nitrite oxidase system under the anaerobic conditions of the reductase assay, as postulated by Kiesow (1964). However, in view of the small amount of activity involved here, it does not detract from the concept of two separate and distinct enzymes.

The effect of various inhibitors on the nitrite-oxidizing system was found to be in accord with reports in the literature (Van Gool & Laudelout, 1966). Antimycin A and HOQNO are known to inhibit at a site between cytochromes *b* and *c* in the mammalian electron-transfer system, whereas Amytal is thought to be specific for a site between nicotinamide nucleotide and flavoprotein (Ernster & Lee, 1964). Assuming that these inhibitors act in the same way in *Nitrobacter*, it seems that cytochrome components are not essential for nitrate reductase activity; however, an NADH-flavoprotein is required. Inhibition of nitrate reductase by the metal-chelating agent $\alpha\alpha'$ -bipyridyl indicates a requirement by the enzyme for a metal. Molybdenum is known to be a component of all nitrate reductase systems studied thus far (Nicholas, 1963), and this element has been found to be an essential micro-nutrient for *Nitrobacter* (Finstain & Delwiche, 1965).

Chlorate inhibits the growth of, and affects

nitrite oxidation by, *Nitrobacter* (Lees, 1955). These two effects appear to be separate, since the bacteriostatic effect operates at much lower concentrations than does the action of chlorate on nitrite oxidation. In this study chlorate was found to be a competitive inhibitor of nitrate reductase (Fig. 2), an effect that has been described for other enzymes utilizing nitrate. Chlorate also stimulated the rate of nitrite oxidation in cell-free extracts (Table 2), presumably by acting as an alternative electron acceptor to oxygen for the cytochrome system. This latter effect has also been observed by Lees & Simpson (1957).

In *Nitrobacter winogradskyi* the nitrite-oxidizing system is known to be associated with membrane structures (Aleem & Nason, 1959, 1960; Tsien & Laudelout, 1968), and this is probably the case in *N. agilis*. In the latter organism Straat & Nason (1965) reported that the cytochrome components were solubilized by digitonin. In the present work nitrite oxidase was associated with membrane fragments that were found in the 136 000g pellet fraction. The nitrate reductase system was distributed equally between the supernatant and pellet fractions after centrifugation at 136 000g for 1 hr. and did not appear to be linked to membrane structures.

Butt & Lees (1958) and Van Gool & Laudelout (1966, 1967) identified cytochromes of the *c* and *a* types as participants in nitrite oxidation. In the difference spectrum (Fig. 3) the bands at 605–597 and 438 nm. are associated with the α - and γ -peaks respectively of cytochrome *a*, whereas the bands at 550, 520 and 418 nm. are those of the α -, β - and γ -peaks respectively of cytochrome *c*. The 136 000g supernatant fraction contained the soluble cytochrome *c* only (Fig. 3). The absence of the terminal oxidase accounts for the very low activity of nitrite oxidase in this fraction.

Straat & Nason (1964, 1965) suggested that nitrate reductase in *N. agilis* mediates electron transfer via the cytochrome pathway of the bacterium, since it was strongly inhibited by antimycin A and HOQNO. The system that they described was associated with cytochrome oxidase, which was solubilized after treatment with digitonin. Their results are difficult to interpret since nitrite formation was followed in an open tube after addition of reduced cytochrome *c*, which would be readily oxidized by air.

Kiesow (1964, 1967) claimed that nitrate reduction in *N. winogradskyi* is part of an anaerobic 'back-reaction' involved in the energy metabolism of the cell. However, nitrate reduction in *N. agilis* has been shown not to be associated with a phosphorylating system (Wallace & Nicholas, 1968).

The results of this work agree with the conclusions of Wallace & Nicholas (1968) that oxidative

and reductive pathways for nitrite and nitrate are separate in *Nitrobacter*.

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