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SHORT COMMUNICATION

THE EFFECT OF CYCLOHEXIMIDE ON THE INDUCTION OF NITRATE AND NITRITE REDUCTASE IN *LEMNA MINOR* L.

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Abstract—Cycloheximide has been shown to inhibit the synthesis of nitrate and nitrite reductase in *Lemna minor*. At levels above 5 μg/ml it causes complete inhibition of the induction of both these enzymes. The enzymes are sequentially induced. Addition of cycloheximide at any stage during the induction prevents further synthesis of the two enzymes, suggesting that it interferes with protein synthesis at the "translation" level.

INTRODUCTION

THE NITRATE and nitrite reductases of *L. minor* have been shown to be adaptive enzymes,[1] being induced by nitrate and nitrite and repressed by ammonia and various amino acids. The nitrate reductase induced in the presence of nitrate but in the absence of sucrose is NADH-specific. When nitrate is supplied as the inducer there is a 15–20-fold increase in specific activity.

Cycloheximide, (3 2(3,5-Dimethyl-2-oxocyclohexyl)2-hydroxyethyl glutarimide) has been shown to inhibit protein synthesis in a number of organisms[2–5] and there are indications that it interferes with a number of other processes including nucleic acid synthesis[2] and flowering.[6] The induction of isocitrate lyase[2] and the induction of a permease for choline sulphate[7] are inhibited by cycloheximide. Much of the work using this inhibitor suggests that it interferes with protein synthesis after the production of messenger RNA,[5] affecting in some way the translation of the message and hence the synthesis of the polypeptide chain.

RESULTS

Plants of *L. minor* from a clone (S.1.), pre-grown on an ammonia-containing medium were sub-cultured on a nitrate medium containing varying levels of cycloheximide. Samples were harvested 80 hr after the transfer and assayed for nitrate and nitrite reductase activity (Table 1). Levels of cycloheximide above 5 μg/ml can be seen to inhibit completely the induction of both enzymes.

[1] B. F. FOLKES, A. P. SIMS and A. H. BUSSEY, In *Recent Aspects of Nitrogen Metabolism in Higher Plants*, University of Bristol, Long Ashton Symposium, Academic Press (1967).
[2] L. MORRIS, *J. Exp. Botany* **18**, 54 (1967).
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[4] H. L. ENNIS and M. LUBIN, *Science* **146**, 1474 (1964).
[5] M. J. CHRISPEELS and J. E. VARNER, *Plant Physiol* **42**, 1008 (1967).
[6] C. ROSS and M. KRINNER, *Plant Physiol.* **42**, S-20 (1967).
[7] P. NISSEN, *Plant Physiol.* **42**, S-42 (1967).

TABLE 1. THE EFFECT OF VARIOUS CONCENTRATIONS OF CYCLOHEXIMIDE ON THE INDUCTION OF NITRATE AND NITRITE REDUCTASE

Cycloheximide μg/ml	Specific activity Δ A_{540}/hr/100 μg protein		Activity as % fully induced level	
	Nitrate reductase	Nitrite reductase	Nitrate reductase	Nitrite reductase
0	0·32	0·64	100·0	100·0
0·5	0·25	0·50	78·0	78·0
1·0	0·06	0·15	18·8	23·0
5·0	0·01	0·03	3·0	4·5
10·0	Trace	Trace	0·0	0·0
50·0	Trace	Trace	0·0	0·0

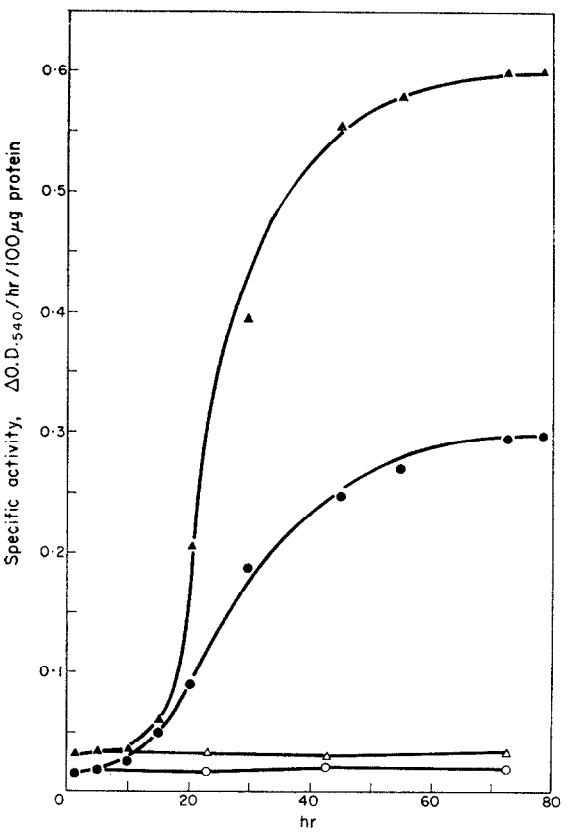

FIG. 1. THE INDUCTION OF NITRATE AND NITRITE REDUCTASE.

Closed symbols; grown on 5×10^{-3} M KNO_3.
Nitrate reductase ●—●—● Nitrite reductase ▲—▲—▲

Open symbols; grown on 5×10^{-3} M KNO_3 + 10 μg/ml cycloheximide.
Nitrate reductase ○—○—○ Nitrite reductase △—△—△

The time course of induction was followed in an experiment (Fig. 1) where the plants were grown in a continuous culture apparatus. The plants were allowed to grow to suitable numbers on a medium containing ammonia. The ammonia level was then diluted out by switching to a nitrogen-free medium for 6 hours prior to the switch to the nitrate medium. A replica experiment was carried out in which 10 μg/ml cycloheximide was added to the nitrate medium.

A slight increase in specific activity of nitrate reductase is detectable after 5–10 hr, but the increase in specific activity of nitrite reductase is detectable only after 10–15 hr. The induction of both enzymes is complete after 60–70 hr, there being however no induction in the presence of cycloheximide.

There is evidence that some inhibitors of protein synthesis are effective only at certain stages of induction.[8] Accordingly a series of cultures pre-grown on ammonia were set up, to which cycloheximide was added (10 μg/ml) at different times after the addition of nitrate. Samples for assay were taken prior to the addition of the inhibitor and 12 hr after its addition (Table 2). It is clear that there is no increase in specific activity after the addition of cycloheximide at any stage of the induction.

TABLE 2. THE EFFECT OF ADDING CYCLOHEXIMIDE AT VARIOUS TIMES AFTER THE START OF INDUCTION

Time cycloheximide added after start of induction (hr)	Specific activity (Δ A_{540}/hr/100 μg protein) Before the addition of cycloheximide		Specific activity (Δ A_{540}/hr/100 μg protein) 12 hr after the addition of cycloheximide	
	Nitrate reductase	Nitrite reductase	Nitrate reductase	Nitrite reductase
2	0·015	0·040	0·014	0·041
10	0·024	0·041	0·022	0·039
15	0·050	0·065	0·047	0·065
25	0·150	0·350	0·148	0·355
40	0·250	0·520	0·250	0·472

DISCUSSION

The level of cycloheximide causing complete inhibition of the induction in this system compares fairly closely with the levels reported by other investigators, the exception being the work of Syrett.[2] Using the isocitrate lyase system in *Chlorella* he found that levels above 2·5 μg/ml inhibited the synthesis of no more than 80 per cent of the total inducible enzyme.

The time course of the induction indicates clearly that the two enzymes, nitrate and nitrite reductase are not co-ordinately induced but appear to be sequentially induced. This confirms earlier experiments,[1] where varying ratios of nitrite to nitrate reductase were obtained under different nutritional conditions, suggesting the enzymes were not co-ordinately regulated. This contrasts markedly with the situation in yeast where there is co-ordinate regulation of nitrate, nitrite and hydroxylamine reductases.[1,9]

Inhibitors of protein synthesis such as Actinomycin D if added after the inducer prevent the synthesis of only part of the inducible enzyme. Actinomycin D is now known to interfere with m-RNA synthesis and the rise in specific activity, after the addition of the inhibitor, and

[8] A. KEPES and S. BEGUIN, *Biochim. Biophys. Acta* **123**, 546 (1966).
[9] A. H. BUSSEY, Ph.D. Thesis, University of Bristol.

in the presence of the inducer is attributed to the synthesis of the protein polypeptide chain from message synthesized before the addition of the inhibitor. Clearly with cycloheximide this is not the case since there is no increase in specific activity at any stage of induction after the addition of cycloheximide. The data in Table 2 while by no means conclusive tend to confirm the hypothesis that cycloheximide acts as an inhibitor of protein synthesis at the translation level, preventing the synthesis of the polypeptide chain from the m-RNA.

EXPERIMENTAL

Plants of *L. minor* were grown under sterile conditions in a modification of Hutner's Basic Medium,[10] containing either 5×10^{-3} M NH_4Cl or 5×10^{-3} M KNO_3, as the nitrogen source. They were grown at 25° under continuous light (50 lux).

Material for assay, approximately 100 plants, was thoroughly washed with H_2O and extracted in 3 ml 0·1 M phosphate buffer, pH 7·5, containing 5×10^{-4} M EDTA. The extractions were carried out in a glass homogenizer at 10°. The extract was filtered on glass fibre paper to remove the cell debris. A 2·5 ml aliquot of the filtered homogenate was desalted on a Sephadex G-25 column, $17{\cdot}5 \times 1{\cdot}0$ cm pre-equilibrated with 0·1 M phosphate buffer, pH 7·5 containing 5×10^{-4} M EDTA. Normally two such preparations were bulked for assay.

After desalting the enzymes were assayed under anaerobic conditions in Thunberg tubes at 25°. The reaction mixture for nitrate reductase was as follows: *Tube* 0·25 ml 0·1 M phosphate buffer, pH 7·5; 0·25 ml $1{\cdot}2 \times 10^{-2}$ M KNO_3; 0·5 ml enzyme extract (containing approximately 500 μg protein/ml). *Side arm* 0·4 ml Benzyl Viologen (32 mg/ml); 0·1 ml 3×10^{-3} M $NADH_2$. The reaction mixture for nitrite reductase was as follows: *Tube* 0·1 ml 0·1 M phosphate buffer pH 7·5; 0·25 ml 7×10^{-4} M $NaNO_2$; 0·5 ml enzyme extract. *Side Arm* 0·4 ml Benzyl Viologen (32 mg/ml); 0·25 ml 3×10^{-2} M $Na_2S_2O_4$.

Samples were withdrawn at 0, 10, 20 and 30 min intervals, the reaction being terminated by pipetting 1·0 ml of the reaction mixture into a solution of zinc acetate (0·25 ml) and ethyl alcohol (0·5 ml). After centrifuging, an aliquot of the supernatant was withdrawn and the nitrite produced or consumed was estimated using a modification of the sulphanilamide method.[9]

Protein was estimated using a modification of the Folin method.[9]

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[10] R. H. HUTNER. In *Growth and Differentiation in Plants*, Iowa State College Press (1953).