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INCREASE IN NITRATE REDUCTASE ACTIVITY IN THE PRESENCE OF SUCROSE IN BEAN LEAF SEGMENTS

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Key Word Index—*Phaseolus vulgaris*; bean leaves; nitrate reductase; sucrose effects.

Abstract—The supply of sucrose to leaf segments from light-grown bean seedlings caused a substantial increase in substrate inducibility of *in vivo* and *in vitro* nitrate reductase activity but only a small increase in total protein. Cycloheximide and chloramphenicol inhibited the increase in enzyme activity by nitrate and sucrose. The *in vivo* decline in enzyme activity in nitrate-induced leaf segments in light and dark was protected by sucrose and nitrate. The supply of NADH also protected the decline in enzyme activity, but only in the light. *In vitro* stability of the extracted enzyme was, however, unaffected by sucrose. The size of the metabolic nitrate pool was also enhanced by sucrose. The experiments demonstrate that sucrose has a stimulatory effect on activity or *in vivo* stability of nitrate reductase in bean leaf segments, which is perhaps mediated through increased NADH level and/or mobilization of nitrate to the metabolic pool.

INTRODUCTION

The activity of nitrate reductase (NRA), one of the key enzymes of nitrogen assimilation, is regulated by a variety of chemical compounds [1]. Carbohydrates in general may regulate enzyme activities by acting as a source of energy, carbon and coenzymes (for redox enzymes) for induced synthesis or activation of the existing enzyme molecules. Besides these general effects, sugars have specific effects on nitrate reductase (NR) levels in different plants. In maize roots, glucose has been reported to be effective in inducing NRA [2]. Decline in *in vivo* NRA due to excision in bean seedlings is prevented by the exogenous supply of sucrose, glucose and fructose in the presence of nitrate [3]. The exogenous supply of sucrose to excised pea roots also enhances induction of NRA [4]. In an earlier investigation with bean leaf segments, sucrose enhanced *in vivo* NRA in the presence of nitrate, although only a small increase was obtained with glucose [5]. Different postulates have been advanced to explain the mechanism of regulation of NRA by sucrose and other sugars [2–4], although none appears to be conclusive. The aim of experiments carried out in the present investigation was to provide more information for elucidating a possible mechanism of increase in NRA by sucrose.

RESULTS

Time course of increase in NRA in the presence of nitrate and sucrose

When leaf segments were floated either on distilled water or on sucrose, the *in vivo* NRA increased slowly after a lag of about 0.5 hr, up to 5 hr at least at similar rates (Fig. 1). Further incubation up to 8 hr had no effect on enzyme activity. This trend of increase with time was observed in KNO_3 and KNO_3 + sucrose also, although the enzyme activity in these treatments was significantly higher at 3 hr or for longer incubation periods. The maximum enzyme activity was observed with KNO_3 + sucrose treatments.

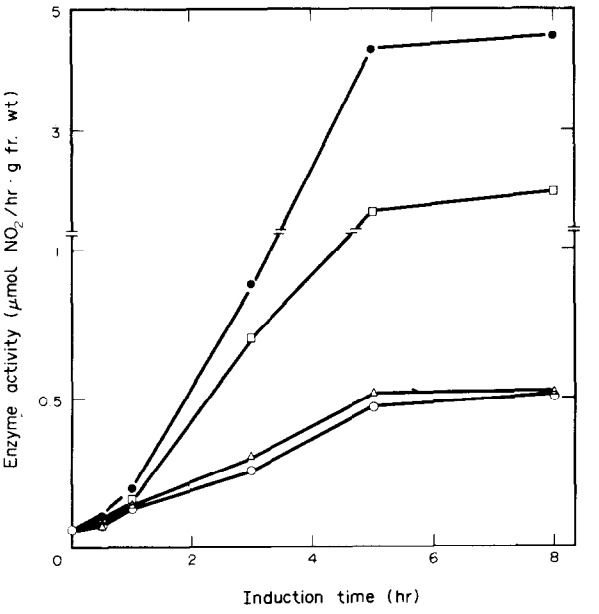

Fig. 1. Time course of increase in nitrate reductase activity in the presence of nitrate and sucrose. Leaf segments were floated on the desired solution and *in vivo* enzyme activity was determined at different time intervals. Open circles—control, triangles—sucrose, squares—KNO_3, closed circles—KNO_3 + sucrose.

In vitro *NRA in leaf segments floated on nitrate in the presence or absence of sucrose*

A stimulatory effect of sucrose on NRA was also

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observed when the enzyme was assayed *in vitro*. For example, the extractable level of NRA in the leaf segments incubated for 8 hr were: control 58, sucrose 73, KNO_3 493 and KNO_3 + sucrose 1974 nmol NO_2/hr per g fr.wt.

Effect of sucrose and nitrate supply on RNA and protein contents and in vivo *NRA during short term incubation*

The supply of sucrose and nitrate during a 5 hr incubation caused a slight increase in protein and very little increase in total RNA (Table 1). However, the increase in NRA during the same period was far greater, suggesting that the increase in NRA was specific and not due to elevation in general protein or translatable RNA pool.

Effect of protein synthesis inhibitors on increase in in vivo *NRA in the presence or absence of sucrose and nitrate*

The induction of NRA by nitrate or nitrate + sucrose was inhibited by cycloheximide and chloramphenicol, although the inhibition with the former was more pronounced than with the latter (Table 2). With cycloheximide the inhibition of NRA was almost complete, particularly in the presence of sucrose. With chloramphenicol, the inhibition was more severe in the absence of sucrose than in its presence.

Effect of sucrose and nitrate on in vivo *and* in vitro *stability of NRA in light and in the dark*

When leaf segments treated with nitrate for 24 hr were transferred to distilled water in light, the *in vivo* NRA declined slowly over the 4 hr experimental period (Fig. 2a). In the presence of nitrate, the NRA did not change up to 2 hr but increased slightly at 3 and 4 hr of incubation. Sucrose also maintained the original NRA up to 2 hr but it increased the NRA considerably and more than nitrate at 3 and 4 hr. When sucrose and nitrate were supplied together, a slightly higher level of NRA than sucrose alone was observed at 1, 2 and 3 hr but the enzyme levels were almost similar in both at 4 hr.

The rate of decline in *in vivo* NRA in the dark was slightly more than that in light (Fig. 2b). Further, nitrate had no effect on this decline. However, sucrose or sucrose + KNO_3 promoted a more or less linear increase in NRA. The NRA in sucrose + nitrate was higher than that in sucrose alone over the whole time course.

In another experiment (data not shown), the enzyme extract from the leaf segments treated with nitrate for

Table 1. Effect of sucrose and nitrate on RNA, total protein and *in vivo* nitrate reductase activity in bean leaf segments

Treatment	Total RNA (mg/g fr. wt)	Total protein (mg/g fr. wt)	NRA (μmol NO_2/hr per g fr. wt)
Water (control)	7.34 ± 0.30 (100)	20.31 ± 0.79 (100)	0.47 ± 0.04 (100)
Sucrose	7.46 ± 0.20 (102)	22.55 ± 0.80 (111)	0.51 ± 0.03 (108)
KNO_3	7.58 ± 0.20 (103)	23.61 ± 0.50 (117)	1.63 ± 0.38 (346)
Sucrose + KNO_3	7.82 ± 0.10 (106)	24.07 ± 0.83 (119)	4.36 ± 0.22 (928)

Leaf segments were floated on the desired solution for 5 hr in light. The values relative to the control are given in parentheses.

Table 2. Effect of protein synthesis inhibitors on the increase in *in vivo* nitrate reductase activity in the presence of nitrate and sucrose

	NRA (μmol NO_2/hr per g fr. wt)		
	Inhibitor used		
Incubation media	None	Chloramphenicol (1 g/l.)	Cycloheximide (5 mg/l.)
Sucrose	0.51 ± 0.03 (100)	0.37 ± 0.03 (73)	0.04 ± 0.01 (9)
KNO_3	1.63 ± 0.38 (100)	0.68 ± 0.01 (42)	0.27 ± 0.01 (17)
Sucrose + KNO_3	4.36 ± 0.22 (100)	2.77 ± 0.02 (64)	0.09 ± 0.0 (2)

Details as in Table 1.

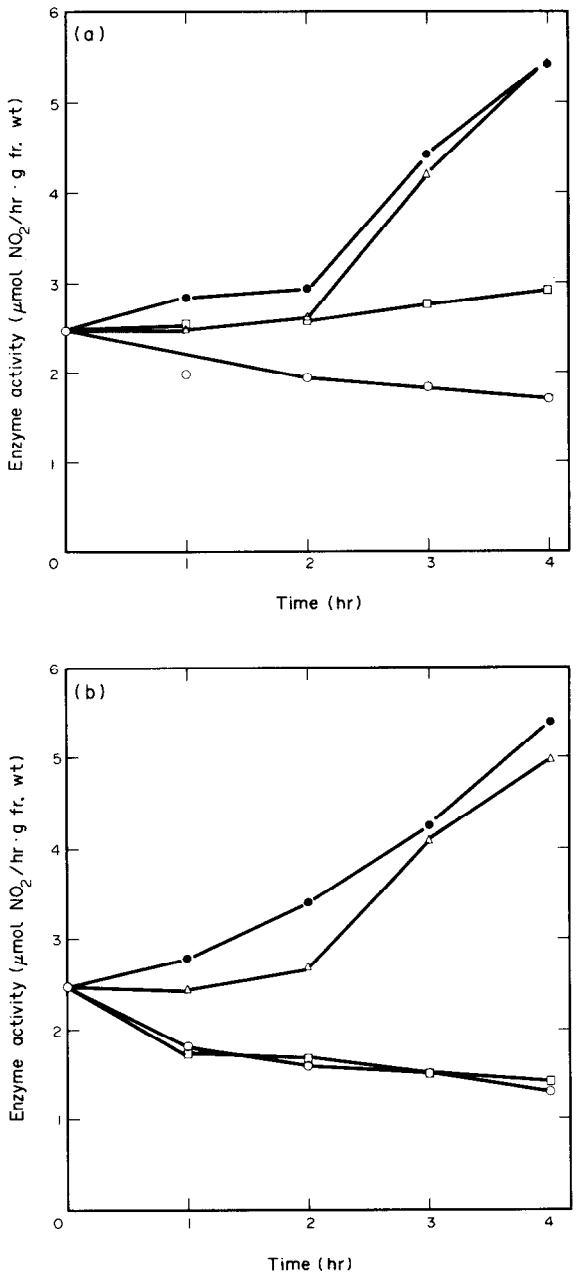

Fig. 2. (a) Effect of sucrose on the *in vivo* stability of nitrate reductase activity in light. Leaf segments were floated on nitrate for 24 hr and then they were transferred to the respective medium in light. The enzyme activity was assayed *in vivo* at different time intervals. Symbols same as in Fig. 1. (b) Effect of sucrose on the *in vivo* stability of nitrate reductase activity in the dark. Leaf segments were floated on nitrate for 24 hr and then they were transferred to the respective medium in the dark. Other details as in (a).

24 hr was stored at 10° in the presence of nitrate or nitrate + sucrose, and the NRA was determined periodically over 2 hr. The decline in NRA in either case was similar. Similarly when enzyme extracted from leaves floated on KNO_3 + sucrose for 24 hr was stored under similar conditions, the NRA declined at almost similar rates. In each case, the residual activity after 2 hr was *ca* 15–19 % of the original activity.

Effect of NADH in the presence and absence of nitrate on in vivo *stability of pre-induced enzyme in light and dark*

During a 5 hr treatment of nitrate-treated leaf segments, the *in vivo* NRA declined to *ca* 69 % of the original value in light (Table 3). This decline was prevented by NADH, nitrate and NADH + nitrate. In dark, the enzyme activity declined to 40 % and NADH and nitrate supplied either singly or together caused a slight increase in this level.

Effect of nitrate and sucrose on the endogenous level of nitrate

The amount of anaerobically produced nitrite was taken as a measure of the endogenous nitrate available for metabolic reduction. This amount was low in the leaf segments floated on either water or sucrose (Table 4). On the other hand, incubation with nitrate or nitrate + sucrose resulted in higher nitrite production, the level being higher in the latter treatment.

Table 3. Effect of NADH and nitrate on the stability of pre-induced enzyme in light and in the dark

Incubation media	Relative NRA	
0 hr value	100	
Values after 5 hr in	Light	Dark
Water (control)	69	40
KNO_3	121	60
NADH	113	52
KNO_3 + NADH	131	62

The leaf segments were floated on nitrate for 24 hr in light. The *in vivo* enzyme activity after this period is expressed as 100 units in the table. The leaf segments were then transferred to the desired solution for 5 hr either in light or in the dark at 25° and the enzyme activity was determined.

Table 4. Effect of nitrate and sucrose on metabolic nitrate in the leaf segments

Treatment	μmol NO_2/g fr. wt
Water (control)	0.55 ± 0.04 (100)
Sucrose	0.63 ± 0.04 (114)
KNO_3	5.81 ± 0.44 (1056)
Sucrose + KNO_3	9.01 ± 0.79 (1638)

Leaf segments were floated on the desired solution for 24 hr in light. Values relative to control are given in parentheses.

DISCUSSION

The supply of sucrose increases NRA both *in vivo* and *in vitro* in green bean leaves. This increase appears to be specific, as the increase in total protein or RNA is very small compared to the increase in NRA under similar conditions. In fact, the observed increase in protein may be the consequence of elevated NR levels rather than its prerequisite.

Sucrose may increase NRA either by increasing the synthesis of enzyme molecules or by activating the existing molecules. In our time course experiments (Fig. 1), the substrate induction of NR increased linearly after a lag phase of about 0.5 hr and reached almost maximum at 5 hr. The stimulatory effect of sucrose was observed after 1 hr, although it was more pronounced at 5 hr and onwards than at earlier times. This indicates that sucrose has a more pronounced effect on the stability of the enzyme than on its synthesis. Further, the increase in enzyme activity by sucrose is dependent upon protein synthesis, as cycloheximide inhibits the process (Table 2). The positive effect of sucrose on activity/stability of NR may be either through some of its metabolic products or through the mobilization of nitrate. The *in vivo* procedure of NR assay depends entirely on endogenous NADH [6]. Therefore, the supply of sucrose may cause an elevated level of *in vivo* NRA by increasing the endogenous pool of NADH. However, an increase in enzyme activity with sucrose was observed with the *in vitro* assay also and therefore the effect of sucrose appears to be on the enzyme level itself rather than on the co-enzyme level. Besides taking part in active reduction of nitrate, NADH is known to protect the inactivation of NR by endogenous inactivation factors in cucumber leaves [7]. Further, NR from rice seedlings is activated by pre-incubation with NADH [8, 9]. On the other hand, NR in spinach is inactivated by NADH and this inactivation is prevented by nitrate [10]. In the present investigation, the exogenous supply of NADH either in the presence or absence of nitrate prevented the *in vivo* decline of NRA in light, although it did not increase the activity to a level observed with sucrose. This observation indicates that prevention of enzyme inactivation by an elevated level of endogenous NADH during sucrose supply may be only partly responsible for the stimulation of NRA.

The stimulatory effect of sucrose was observed only when either nitrate was also present or when the leaf segments had been preinduced with it. It may be postulated, therefore, that the positive effect of sucrose on NRA is mediated through nitrate. Nitrate protects *in situ* inactivation of enzyme activity in several systems [11, 12]. Pistorius *et al.* [13] have suggested that nitrate prevents *in vivo* inactivation of nitrate reductase in *Chlorella vulgaris* by maintaining it in the oxidized form. When both sucrose and nitrate are supplied together, sucrose may increase the endogenous level of nitrate by accelerating its uptake, as has been observed in detached rice seedlings [14] and dwarf bean roots [3]. Besides, sucrose may also mobilize nitrate from the storage pool to the metabolic pool, as has been suggested for glucose effects in maize [2]. This latter suggestion may be pertinent to the stimulatory effect of sucrose on NRA in the leaves pre-induced with nitrate (Fig. 2a, b). An indirect measurement of the metabolic pool in the present investigation shows an increase in the pool size (Table 4). Besides modulating through its metabolic products or nitrate, sucrose may stimulate NRA through direct effects also. Sahulka and Lisa [4] have suggested that sucrose acts as co-inducer or de-repressor of NR synthesis.

Another important observation in the present investigation was an increase in enzyme activity during incubation of leaf segments in nitrate-free medium after 2 hr (Fig. 1). A similar trend of increase in nitrate-free samples has been observed in soybean [15]. It has been suggested that nitrate is synthesized from organic nitrogen in soybean tissues or in *Chlorella* and that in turn induces NRA [15].

EXPERIMENTAL

Plant material. Seeds of *Phaseolus vulgaris* L. cv Rajmah were surface-sterilized with 0.1% $HgCl_2$ and washed thoroughly with H_2O. Seedlings were grown in plastic pots containing washed sand in continuous light supplied by a mixture of incandescent bulbs and fluorescent tubes (*ca* 65 W/m^2) at 25 ± 2°. Seedlings were watered daily with modified (minus nitrogen) half-strength Hoagland's solution. Primary leaves from 8-day-old seedlings were used for various treatments. Leaf segments of *ca* 0.5 cm^2 were floated on the desired soln (pH 6) for the required time at 25° either in light or in the dark. Chloramphenicol (30 mg/l.) was added routinely to the incubation medium to prevent bacterial contamination. When used, the concentration of various salts were: KNO_3, 10 mM; sucrose, 5 mM; NADH, 0.1 mM.

Nitrate reductase activity. This was assayed either by the *in vivo* [6] or by the *in vitro* [16] procedure. Protein content was estimated by Lowry's method [17]. RNA was extracted and estimated by the methods of refs. [18] and [19], respectively. The size of the metabolic nitrate pool was estimated by a modified method from ref. [20]. About 200 mg of leaf sample was incubated in a medium containing 9 ml of 0.1 M NaPi buffer (pH 7.2) and 1 ml of *i*-PrOH, contained in a 25 ml dark vial. Partial anaerobiosis was created by bubbling N_2 gas in the incubation medium for 30 sec. The vials were stoppered tightly and kept in the dark at 30°. After 1 hr, the vials were heated to 100° for 2 min and then a suitable aliquot of the medium was withdrawn. Nitrite in the aliquot was estimated by diazotization and colour development with sulfanilamide and *N*-1-naphthylethylene diamine hydrochloride. Data presented in the paper are average values of three replicate experiments, with s.e. given in the tables.

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