CHAPTER III

RESULTS

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Phytochrome mediated photoregulation of glutamine synthetase and NAD kinase :

Development of both GS and NAD kinase is known to be light dependent. In the present study also it was found that the two enzymes were 4 and 17 fold higher respectively in light than in dark (Table-1). The exposure of dark grown seedlings to white light for different periods before assay on 9th day showed a time dependent increase in enzyme levels and the optimum was reached at about 102 and 78 h for GS and NAD kinase respectively (Fig. 1 & 2).

The involvement of phytochrome in light mediated regulation of the two enzymes was studied in 5-day old etiolated pea seedlings exposed to red or red followed immediately by far-red light for 5 min each. After the light exposure the seedlings were transferred to dark and assayed at different time intervals (Fig. 3 & 4). GS and NAD kinase activity increased from 8-12 h after exposure to red light. A maximum increase of 65% over the dark control level was reached at 30 h in case of GS and 175% at 48 h in case of NAD kinase. Further incubation in darkness decreased both the enzymes to dark level at 48 h and 96 h respectively. The red light mediated increase for both the enzymes was reversed to dark level by a subsequent exposure to far-red light. A 5 min exposure to red light was found to elicit a maximal response (Table-2). Increasing the period of red light exposure beyond 5 min decreased the phytochrome response and the decreasing effect was more

Table - 1 : GS and NAD kinase activity in terminal buds of pea seedlings grown in dark or continuous white light.

Growth condition	Enzyme Activity (nkat/g fr. wt.)	
	GS	NAD kinase
Dark	4.4	0.17
Light	19.6	3.10

Terminal buds from 6 day old seedlings were used for enzyme assay.

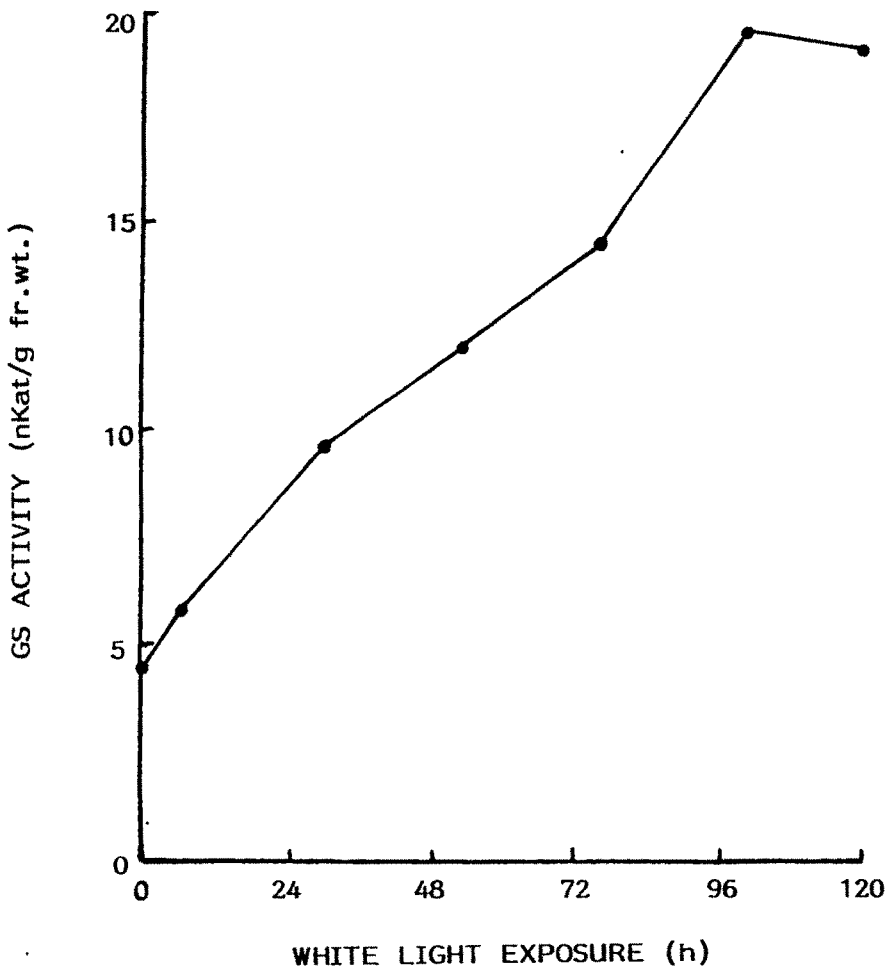

Fig.1 : Effect of white light exposure on GS activity in etiolated pea terminal buds.

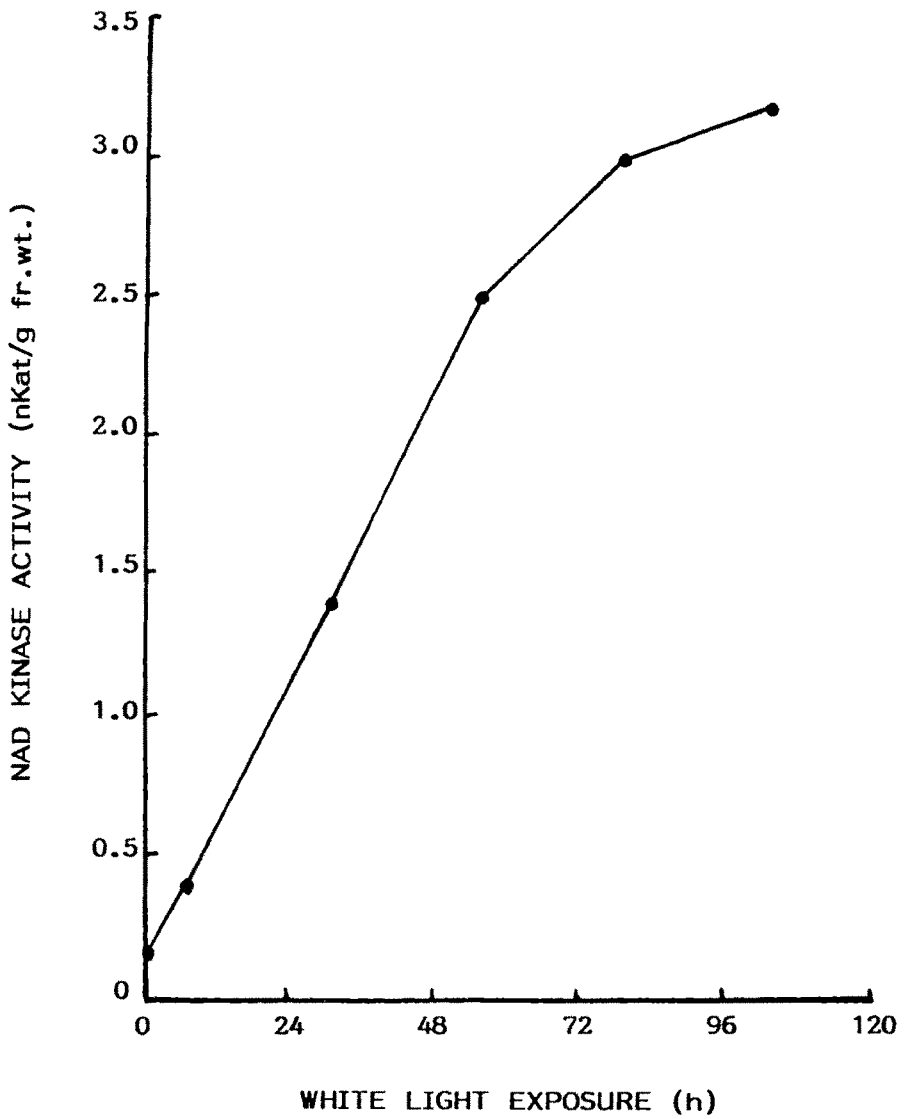

Fig.2 : Effect of white light exposure on NAD kinase activity in etiolated pea terminal buds.

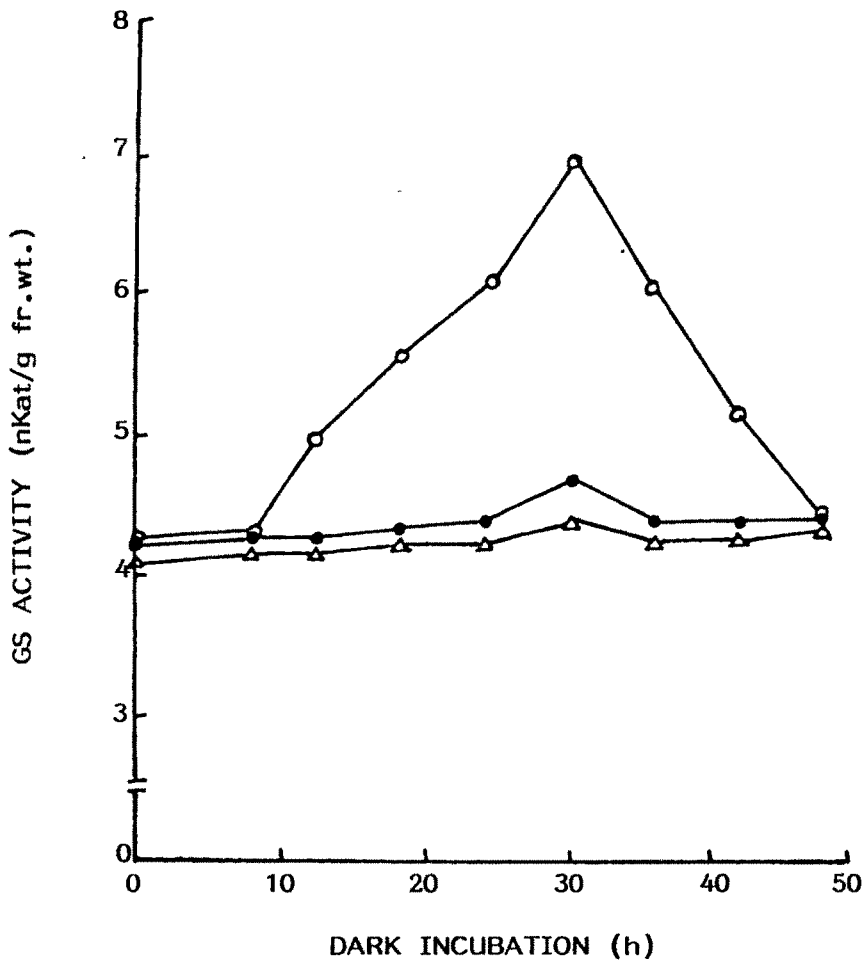

Fig.3 : Effect of red and far-red light pulses given at time zero on GS activity in etiolated pea terminal buds.

(△) Dark Control; (o) 5 min red light;
(•) 5 min red followed by 5 min far-red light.

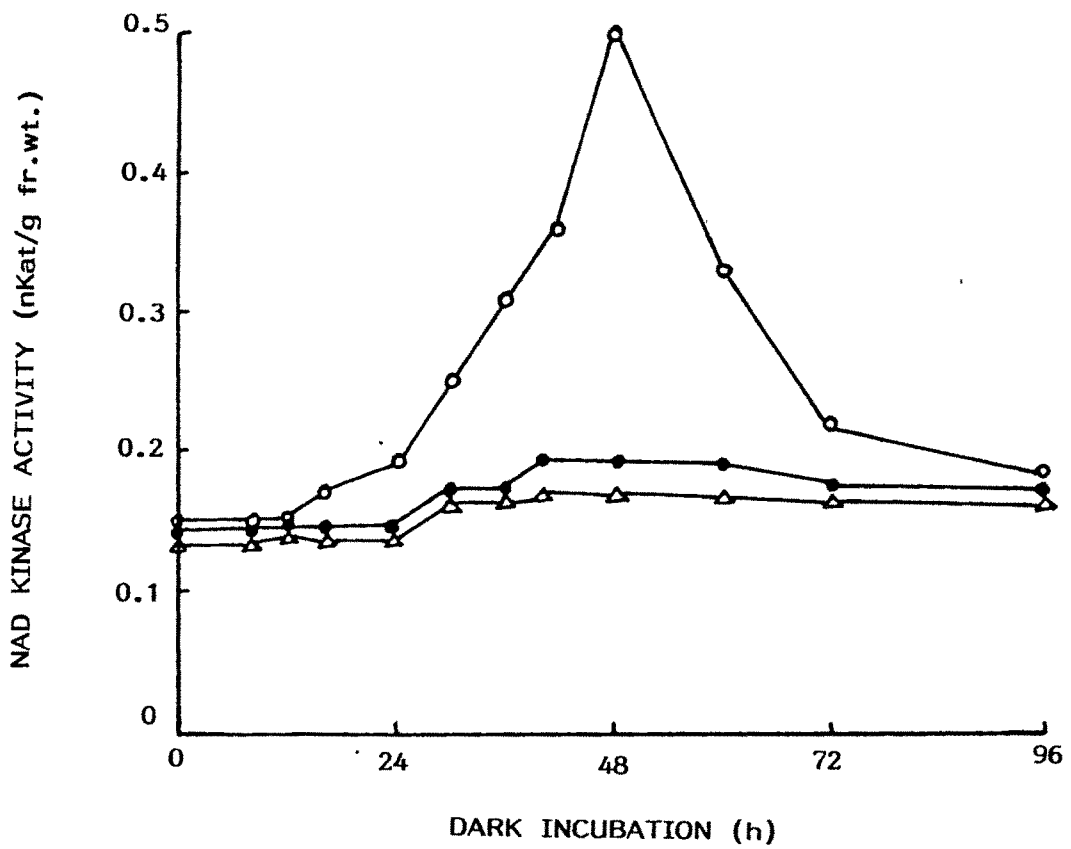

Fig.4 : Effect of red and far-red light pulses given at time zero on NAD kinase activity in etiolated pea terminal buds.

(△) Dark Control; (○) 5 min red light;
(●) 5 min red followed by 5 min far-red light.

Table - 2 : Effect of period of red and far-red light exposure on GS and NAD kinase activity in etiolated pea terminal buds.

Time of light exposure (min)		Enzyme Activity (nKat/g.fr.wt.)	
R	FR	GS	NAD kinase
0	-	4.2	0.14
1	-	4.9	0.20
3	-	5.6	0.32
5	-	6.9	0.51
7	-	6.4	0.48
10	-	6.1	0.40
15	-	5.3	0.25
5	1	6.0	0.45
5	3	5.1	0.25
5	5	4.4	0.16
5	7	4.4	0.14
5	10	4.2	0.15
5	15	4.8	0.20

Five-day-old seedlings were exposed to red (R) and/or far-red (FR) light for different periods and then transferred to darkness.
GS and NAD kinase were assayed at 30 and 48 h respectively after dark incubation.

marked for NAD kinase than for GS. A 5 min far-red light exposure was optimum to abolish the red light mediated increase for both the enzymes. The red-far-red reversibility clearly indicated the involvement of phytochrome in light mediated regulation of both GS and NAD kinase. Age of the seedling between 5-8 day at the time of exposure to lights (Table-3) had no significant effect on the phytochrome regulation of the two enzymes and a complete red-far-red reversibility was obtained irrespective of the seedling age.

The time course of escape from reversibility by far-red light was evident from 30-45 sec onwards and a complete escape occurred at about 2-3 min after red irradiation (Table-4). The escape period of about 2-3 min indicates the time of P_{fr} action.

Since certain phytochrome mediated responses can be induced by repeated short exposures to red light over a period of time, the effect of repeated red and red-far-red light exposures was also studied (Figs. 5 & 6). It was found that increasing the number of red light exposures at intervals of 24 h increased both the enzymes. Increase of 0.7, 1.4, 2.0 and 2.5 fold in case of GS and 1.8, 2.5, 3.7 and 4.7 fold in case of NAD kinase were observed over the dark control levels with 1,2,3 and 4 red light exposures respectively. Repeated red light mediated increases for both the enzymes were also completely reversed to the dark control level when each red light exposure was followed immediately by far-red light. In case of GS a similar phytochrome mediated response was obtained when it was assayed as synthetase rather than as transferase activity (Table-5).

Table - 3 : Effect of seedling age on phytochrome mediated GS and NAD kinase activity in etiolated pea terminal buds.

Age of seedling at the time of exposure to light (days)	Enzyme Activity (nKat/g fr. wt.)			
	GS		NAD kinase	
	R	R-FR	R	R-FR
5	6.9	4.2	0.47	0.17
6	7.2	4.4	0.50	0.19
7	7.8	4.7	0.53	0.19
8	8.3	4.7	0.56	0.17

GS and NAD kinase were assayed at 30 and 48 h respectively of dark incubation after red (R) or red-far-red(R-FR) light exposures.

The dark control values during the period of experiment ranged from 4.2 to 4.7 and 0.15 to 0.19 nKat/g fr. wt. for GS and NAD kinase respectively.

Table - 4 : Time course of escape from phytochrome control on the induction of GS and NAD kinase activity in etiolated pea terminal buds.

Light treatment	Dark period between R and FR light treatment (min)	Enzyme Activity (nKat/g fr.wt.)	
		GS	NAD kinase
-	-	4.7	0.19
R	-	7.7	0.53
R-FR	-	4.7	0.19
	0.25	4.7	0.19
	0.50	4.7	0.22
	0.75	4.7	0.25
	1.00	5.3	0.33
	1.50	6.1	0.42
	2.00	7.8	0.47
	3.00	8.0	0.55

Seven-day -old seedlings were exposed to red (R) and red followed by far-red (R-FR) light for 5 min each and then transferred to darkness.

GS and NAD kinase were assayed at 30 and 48 h respectively after dark incubation.

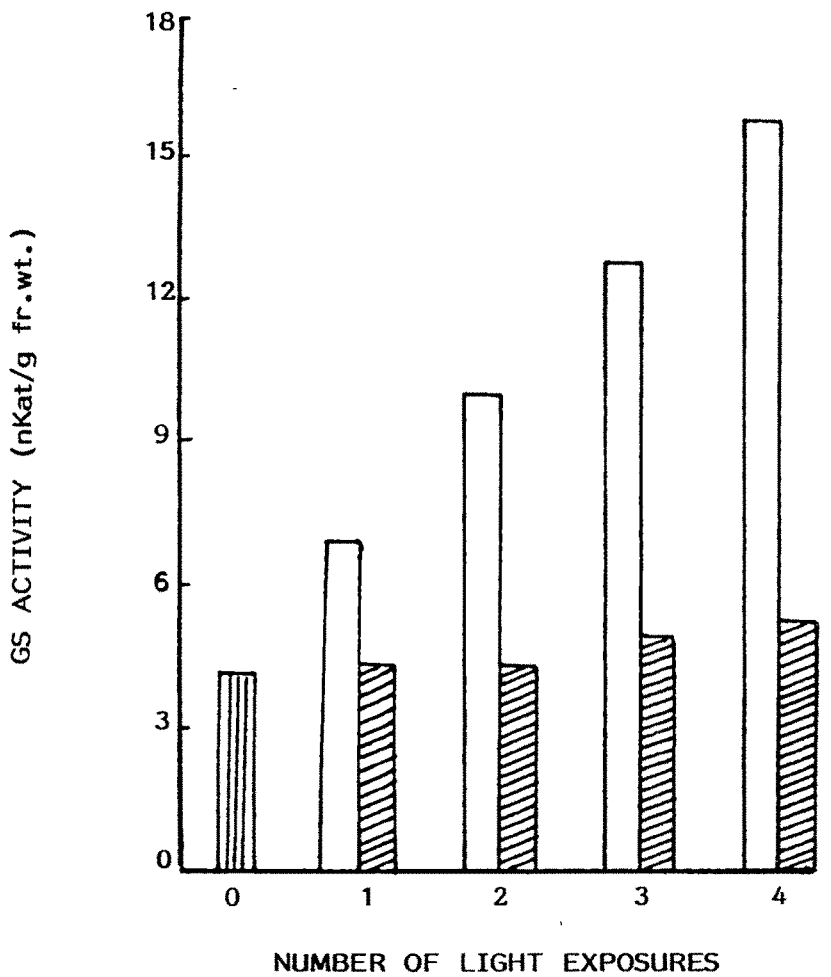

Fig.5 : Effect of repeated red and far-red light exposures spaced by 24 h of darkness on GS activity in etiolated pea terminal buds.

(▥) Dark Control; (□) 5 min red light;
(▨) 5 min red followed by 5 min far-red light.

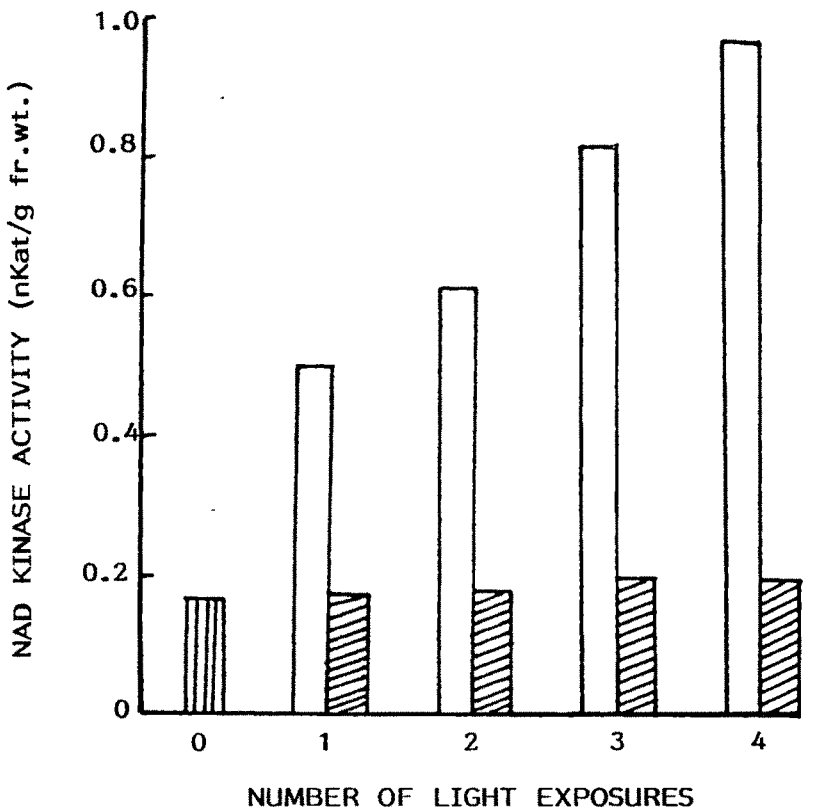

Fig.6 : Effect of repeated red and far-red light exposures spaced by 24 h of darkness on NAD kinase activity in etiolated pea terminal buds.

(▥) Dark Control; (□) 5 min red light;
(▨) 5 min red followed by 5 min far-red light.

Table - 5 : Effect of red and far-red light on GS activity measured as transferase or synthetase in etiolated pea terminal buds.

Light treatment	GS (nKat/g fr.wt.) as	
	Transferase	Synthetase
-	4.7	4.4
$R_{(7)}$*	7.5	7.8
$R\text{-}FR_{(7)}$	5.0	4.7
$R_{(6,7)}$	10.5	10.0
$R\text{-}FR_{(6,7)}$	5.3	5.3
$R_{(5,6,7)}$	13.6	12.5
$R\text{-}FR_{(5,6,7)}$	5.3	5.0

* Numbers in the parentheses indicate the age of the seedlings when a single or repeated exposure to R or R-FR light was given.

GS was assayed at 30 h of dark incubation after the last light exposure.

Effect of protein synthesis inhibitors :

The effect of phytochrome on GS and NAD kinase could be either by affecting the synthesis of the enzyme or by an activation of the preexisting enzyme. To investigate this the effect of various protein synthesis inhibitors was studied. Plants were sprayed with cycloheximide, chloramphenicol or a mixture of two immediately after each red light treatment. The data reported in Table-6 show that the level of both the enzymes from dark grown terminal buds was not affected by any of the protein synthesis inhibitors. However, in case of single or repeated red light exposed seedlings cycloheximide almost completely inhibited the increase in both the enzyme activities. Chloramphenicol did not show a significant inhibition in either case. Mixture of cycloheximide and chloramphenicol also showed inhibition identical to cycloheximide treatment. Thus, increase in both GS and NAD kinase activity by phytochrome appears to be due to de novo synthesis of the enzymes. Actinomycin D also inhibited the red light mediated increase in GS and NAD kinase activity though in case of repeated red light treated seedlings the inhibition of GS activity was only 70%. These results suggest that the de novo synthesis of the enzyme by phytochrome is controlled at the level of transcription.

Effect of calcium and calmodulin on GS and NAD kinase activity in vitro :

It is very well known that the enzyme NAD kinase is dependent on calcium and calmodulin for its activity. Similar role for calcium and calmodulin has not been reported for GS. To investigate the calcium-calmodulin dependency of these enzymes, both GS and NAD kinase activities

Table - 6 : Effect of protein-synthesis inhibitors on phytochrome mediated increase of GS and NAD kinase activity in etiolated pea terminal buds.

Compounds	Enzyme Activity (nKat/g fr.wt.)					
	GS			NAD kinase		
	Dark	$R_{(7)}$	$R_{(5,6,7)}$	Dark	$R_{(7)}$	$R_{(5,6,7)}$
-	4.4	7.0	12.8	0.19	0.53	0.83
Cycloheximide	4.2	4.7	4.4	0.17	0.25	0.25
Chloramphenicol	4.7	7.0	12.2	0.17	0.47	0.77
Cycloheximide + Chloramphenicol	4.2	4.7	4.7	0.17	0.22	0.27
Actinomycin-D	4.4	5.0	6.9	0.19	0.22	0.22

Etiolated seedlings were exposed to single or repeated red light treatment (as per Table-5) followed immediately by a spray with cycloheximide and/or chloramphenicol (20 µg/ml) or actinomycin-D (2 µg/ml) on each day.

GS and NAD kinase were assayed at 30 & 48 h respectively of dark incubation after last light exposure.

from 8-day old light grown pea terminal buds were assayed in the presence of $CaCl_2$, EGTA and calmodulin antagonists (TFP, R_{24571} and W_7). The data reported in Table-7 show that both the enzymes were inhibited by EGTA indicating a possible calcium requirement. However, addition of $CaCl_2$ had no effect on either enzymes. Calmodulin antagonists had no effect on GS but inhibited NAD kinase activity suggesting that NAD kinase is calcium-calmodulin dependent. The inhibition of GS by EGTA was concentration dependent (Table-8). A complete inhibition was obtained at 5mM concentration. $CaCl_2$ had no effect on GS activity. Inhibition of GS by EGTA was not reversed by $CaCl_2$ but was reversed by $MnCl_2$, which is routinely added to the asssay system, indicating that the inhibition by EGTA was due to the unavailability of Mn^{+2} and not of Ca^{+2} .

Role of calcium and 5-hydroxytryptamine on phytochrome regulation of GS and NAD kinase in vivo :

To investigate the role of calcium ions, the effect of calcium and its various antagonists was studied. Effect of 5-hydroxytryptamine (5-HT) was also studied since it has been shown to mimic the phytochrome responses (Das and Sopory, 1985; Reddy et al, 1987). The compounds were sprayed an hour before giving light treatment on each day to three sets of seedlings (i) dark grown (ii) red light treated on day 5, 6 and 7 (iii) red-far-red light treated on day 5, 6 and 7. After 30 h of last light exposure the terminal buds were excised, washed with water and then used for the assay of GS and NAD kinase activity. The data reported in Table-9 show that in dark, $CaCl_2$ caused an increase of about 60 and 125% respectively in GS and NAD kinase whereas 5-HT increased both the enzymes by about 100% over dark control values.

Table - 7 : Effect of EGTA, $CaCl_2$ and calmodulin antagonists on GS and NAD kinase activity *in vitro* .

Compounds	Concentration (mM)	Enzyme Activity (nKat/g fr.wt.)	
		GS	NAD kinase
-	-	19.7	3.20
EGTA	1.0	8.0	1.70
$CaCl_2$	1.0	19.4	3.20
TFP	0.01	19.7	0.22
R_{24571}	0.01	19.7	0.30
W_7	0.01	19.7	0.28

Enzyme from 8-day-old light grown pea terminal buds was used.

Table - 8 : Effect of EGTA on GS activity in vitro.

Treatment	Concentration (mM)	GS (nKat/g fr.wt.)
-		19.7
EGTA	0.1	19.7
	0.5	19.7
	1.0	8.0
	5.0	0.0
$CaCl_2$	1.0	19.4
	5.0	16.3
$MnCl_2$	2.0	19.7
	4.0	19.4
EGTA + $CaCl_2$	1.0 + 1.0	8.3
EGTA + $CaCl_2$	1.0 + 5.0	6.6
EGTA + $MnCl_2$	1.0 + 4.0	19.7

Enzyme from 8-day-old light grown pea terminal buds was used.

Table - 9 : Effect of calcium and 5-HT on phytochrome mediated regulation of GS and NAD kinase activity in pea terminal buds.

Treatment *	GS (nKat/g fr.wt.)			NAD kinase		
	Dark	$R_{(5,6,7)}$	$R\text{-}FR_{(5,6,7)}$	Dark	$R_{(5,6,7)}$	$R\text{-}FR_{(5,6,7)}$
-	4.4	13.3	4.7	0.17	0.72	0.22
$CaCl_2$	7.2	14.1	8.0	0.39	0.75	0.42
5-HT	8.6	14.1	8.9	0.36	0.72	0.39
EGTA	4.4	5.5	5.0	0.17	0.22	0.19
5-HT + $CaCl_2$	8.8	14.1	8.9	0.39	0.78	0.39
5-HT + EGTA	5.5	5.8	5.5	0.17	0.25	0.17
$CaCl_2$ + CH	5.3	5.8	5.0	0.17	0.22	0.19
5-HT + CH	5.5	6.1	5.3	0.19	0.17	0.17

* Five-day-old dark grown seedlings were sprayed with the compounds 1 h before light treatment on each day. The concentrations of $CaCl_2$, 5-HT and EGTA were 5 mM whereas that of cycloheximide (CH) was 20 µg/ml.

GS and NAD kinase was assayed at 30 h of dark incubation after last light exposure.

Light Treatment was same as described in Table-5.

In case of repeated red light treated seedlings $CaCl_2$ and 5-HT did not show any additional response than that obtained with red light alone. The red light mediated increase was, however, more than that observed with $CaCl_2$ or 5-HT treatment in dark. In case of repeated red-far-red light treated seedlings the level of enzymes was decreased to the corresponding dark control group.

Spraying of seedlings with EGTA, a calcium chelator, had no effect on the dark control level but almost completely abolished the red light mediated increase of both the enzymes. A combination of 5-HT and $CaCl_2$ did not show any additive response in dark or any further increase in GS and NAD kinase activity in red light treated group over that obtained with red light alone. However, spraying of 5-HT with EGTA completely abolished the 5-HT mediated increase of both the enzymes in dark. The increase in the activity of the two enzymes in dark by $CaCl_2$ or 5-HT was also completely abolished by cycloheximide treatment.

These results suggest that the phytochrome induction of both the enzymes is mediated through calcium, 5-HT, which plays a role in calcium release via phosphoïnositide pathway also indicated that calcium is involved in the process. Further, calcium appears to control the <u>de novo</u> synthesis of the enzymes since the effect of calcium or 5-HT was abolished by cycloheximide.

The role of calcium ions in phytochrome induction was further confirmed by spraying the seedlings with various calcium channel blockers. The data reported in Table-10 show that $LaCl_3$ and flunarizine were

Table - 10 : Effect of calcium channel blockers on phytochrome mediated regulation of GS and NAD kinase activity in etiolated pea terminal buds.

Treatment *	GS (nKat /g fr.wt.)			NAD Kinase (nKat /g fr.wt.)		
	Dark	$R_{(5,6,7)}$	$R\text{-}FR_{(5,6,7)}$	Dark	$R_{(5,6,7)}$	$R\text{-}FR_{(5,6,7)}$
-	4.4	13.3	4.7	0.17	0.72	0.22
Verapamil	4.4	10.0	4.7	0.17	0.44	0.22
Diltiazem	4.2	9.1	4.4	0.19	0.32	0.22
Flunarizine	3.6	5.8	4.2	0.14	0.28	0.19
$LaCl_3$	4.2	5.3	4.4	0.16	0.23	0.15

The concentration of verapamil, diltiazem and flunarizine was 2mM whereas that of $LaCl_3$ was 5 mM. Other conditions were same as in Table-9.

quite effective in abolishing the red light mediated increase in both the enzymes but the effect of verapamil and diltiazem was only partial at the concentration used.

Effect of polyamines and guanidines on phytochrome mediated regulation of GS and NAD kinase :

To study the effect of polyamines and guanidines on phytochrome mediated regulation of GS and NAD kinase, the seedlings were sprayed with di- and polyamines (1,3-diaminopropane, putrescine, cadaverine, spermidine and spermine) at a concentration of 5 mM an hour before giving red light treatment on day 5,6 and 7. Both GS and NAD kinase were assayed 30 h after the last light exposure. The data reported in Table-11 show that all the di- and polyamines tested almost completely abolished the red light mediated increase in both the enzymes. The compounds, however, had no effect on the dark control level of the enzymes.

A concentration and time course study of spermine spraying on the two enzymes showed (Table-12) that the inhibitory effect was concentration dependent and spraying of seedlings with spermine 1 h before the light treatment was essential for the inhibition of phytochrome mediated increase in activity. Spraying of spermine either immediately or 1 h after light treatment had no effect.

Studies with spraying of guanidino compounds (agmatine, creatine, creatinine, guanidino acetic acid, guanidino butyric acid) showed (Table-13) that all the comounds, except creatinine, increased GS and NAD kinase activity over the dark control values but had no additional

Table - 11 : Effect of di-and polyamines on phytochrome mediated regulation of GS and NAD kinase in etiolated pea terminal buds.

Treatment *	GS (nKat/g fr.wt.)			NAD kinase (nKat/g fr.wt.)		
	Dark	$R_{(5,6,7)}$	$R\text{-}FR_{(5,6,7)}$	Dark	$R_{(5,6,7)}$	$R\text{-}FR_{(5,6,7)}$
-	4.4	13.3	4.7	0.17	0.72	0.22
1,3-Diaminopropane	4.2	5.3	5.0	0.17	0.19	0.19
Putrescine	4.2	5.5	4.4	0.14	0.19	0.17
Cadaverine	4.2	5.3	4.7	0.17	0.19	0.17
Spermidine	4.2	5.0	4.4	0.14	0.22	0.14
Spermine	3.9	5.0	4.4	0.14	0.22	0.17

* The concentration of di- and polyamines was 5 mM. Other conditions were same as in Table-9.

Table - 12 : Effect of concentration and time course of spermine spraying on phytochrome mediated regulation of GS and NAD kinase in etiolated pea terminal buds.

Treatment		Concentration (mM)	GS (nKat/g fr.wt.)			NAD kinase (nKat/g fr.wt.)		
			Dark	$R_{(5,6,7)}$	$R\text{-}FR_{(5,6,7)}$	Dark	$R_{(5,6,7)}$	$R\text{-}FR_{(5,6,7)}$
--			4.4	13.3	4.7	0.17	0.72	0.22
Spermine								
(i)	1 h before light treatment	2	4.4	8.3	4.9	0.18	0.38	0.22
		5	4.2	5.5	4.4	0.17	0.25	0.17
(ii)	immediately after light treatment	2	4.6	13.6	5.0	0.19	0.69	0.18
		5	4.4	11.3	5.0	0.19	0.64	0.22
(iii)	1 h after light treatment	2	4.6	13.6	4.7	0.18	0.76	0.22
		5	4.4	13.0	4.7	0.17	0.78	0.22

GS and NAD kinase was assayed at 30 h of dark incubation after last light exposure.

Light Treatment was same as described in Table-5.

Table - 13 : Effect of guanidines on phytochrome mediated regulation of GS and NAD kinase activity in etiolated pea terminal buds.

Treatment	GS (nKat/g fr.wt.)			NAD kinase		
	Dark	$R_{(5,6,7)}$	$R\text{-}FR_{(5,6,7)}$	Dark	$R_{(5,6,7)}$	$R\text{-}FR_{(5,6,7)}$
-	4.4	13.3	4.7	0.17	0.72	0.22
Agmatine	6.7	13.0	9.7	0.34	0.74	0.58
Creatine	6.5	13.2	10.5	0.32	0.70	0.53
Creatinine	4.2	12.7	5.0	0.17	0.75	0.17
GBA	6.5	13.5	10.0	0.29	0.74	0.49
GAA	6.9	13.6	10.5	0.36	0.75	0.59

The concentration of guanidino compounds was 5 mM. Other conditions were same as in Table-9.

GBA = Guanidino butyric acid

GAA = Guanidino acetic acid

effect on red light mediated increase in enzyme activity. However, the reversal by far-red light was only about 35% in both cases in presence of guanidino compounds. Creatinine, which does not have a free guanidino group, had no effect on either enzymes.

Since phytochrome mediated increase of both the enzymes was modulated by calcium, an attempt was made to study the interaction between the effect of polyamines, guanidines, 5-HT and calcium on the phytochrome regulation of GS and NAD kinse (Table-14). When $CaCl_2$ or 5-HT were sprayed along with spermine, the increase in GS and NAD kinase activity by either calcium or 5-HT in dark was abolished. $CaCl_2$ or 5-HT given together with GAA did not show any additive response for the two enzymes. The activating effect of guanidino acetic acid (GAA) was, however, abolished by spermine, EGTA or cycloheximide in case of both the enzymes. These results confirm that phytochrome mediated increase in case of both the enzymes is modulated through calcium and spermine antagonizes the phytochrome response since it can abolish the effect of red light, calcium and 5-HT. GAA, however, appears to mimic the phytochrome response since it does not show any additive response with red light and the effect is abolished by spermine.

Partial purifiction of NAD kinase and determination of calmodulin levels :

The results of Table-6 showed that de novo protein synthesis is involved in the increase of the two enzymes by phytochrome. In the case of NAD kinase this increase could be due to new synthesis of the enzyme or its activator protein, calmodulin. To investigate this, calmodulin level in terminal buds from seedlings treated with different

Table - 14 : Interaction between the effects of calcium, 5-HT, EGTA, spermine and GAA on phytochrome mediated regulation of GS and NAD kinase activity in etiolated pea terminal buds.

Treatment	GS (nKat/g fr.wt.)			NAD kinase (nKat/g fr.wt.)		
	Dark	$R_{(5,6,7)}$	$R\text{-}FR_{(5,6,7)}$	Dark	$R_{(5,6,7)}$	$R\text{-}FR_{(5,6,7)}$
-	4.4	13.3	4.7	0.17	0.72	0.22
$CaCl_2$	6.9	13.6	7.5	0.39	0.75	0.42
5-HT	8.9	13.9	8.6	0.36	0.72	0.39
EGTA	4.4	5.8	5.0	0.17	0.22	0.19
SPM	4.2	5.2	4.2	0.17	0.22	0.17
GAA	7.2	13.0	10.5	0.36	0.76	0.61
$CaCl_2$ + SPM	4.4	5.5	4.7	0.17	0.19	0.14
5-HT + SPM	4.7	5.0	4.7	0.19	0.25	0.22
$CaCl_2$ + GAA	7.5	13.9	11.1	0.36	0.78	0.53
5-HT + GAA	8.9	13.6	11.1	0.39	0.75	0.58
GAA + SPM	4.4	5.0	5.6	0.17	0.19	0.17
GAA + EGTA	5.0	5.5	5.3	0.22	0.25	0.25
GAA + CH	5.0	5.5	4.7	0.17	0.19	0.22

The concentration of all the compounds was 5 mM except for cycloheximide (CH) it was 20 µg/ml. Other conditions were same in Table-9. SPM = Spermine

lights was measured on the basis of its ability to activate calmodulin-deficient NAD kinase as this enzyme is more sensitive to calmodulin than other mammalian enzymes such as cyclic-nucleotide phosphodiesterase or Ca^{+2} -ATPase (Harmon et al, 1984). It was, therefore, necessary to first purify calmodulin-deficient NAD kinase.

Calmodulin-deficient NAD kinase was isolated from terminal buds of light grown pea seedlings by blue-sepharose and DEAE-sephadex chromatography giving 55 fold purification with 95% recovery (Table-15). A partial separation of the enzyme from calmodulin was achieved at blue-sepharose stage since exogenous addition of calmodulin activated the enzyme by 7 fold. After DEAE-sephadex step the enzyme was completely free from calmodulin since it showed negligible activity without added calmodulin. NAD kinase has been purified to a very large extent from pea seedlings using various purification procedures including calmodulin -sepharose affinity chromatography (Jarrett et al, 1983; Harmon et al, 1984). However, the preparations were not completely free from calmodulin.

Calmodulin content of the terminal buds from seedlings treated with different lights was estimated from crude calmodulin preparation. Estimation of calmodulin content from crude homogenates has earlier been reported to be consistent with estimates obtained after various purification procedures (Stinemetz et al, 1987). Tissue calmodulin content was calculated by comparing it with pure bovine brain calmodulin since calmodulin from different sources can activate pea NAD kinase (Harmon et al, 1984). Fig.7 shows the calibration curve of pure bovine brain calmodulin with calmodulin-deficient NAD kinase. An almost linear increase in activation

Table - 15 : Partial purifiction of NAD kinase from pea terminal buds.

Fraction	Total volume (ml)	Total enzyme units (nKat)		Total protein (mg)	Specific activity (nKat/mg protein)		Purification (fold)	Yield (%)
		-CaM	+CaM*		-CaM	+CaM		
Supernatant	12	13.87	13.87	67.8	0.20	0.20	0	100
Blue-sepharose eluate	42	2.0	13.44	30.5	0.07	0.44	2.2	97
Unadsorbed fraction from DEAE-sephadex	47	0.026	13.24	1.2	0.02	11.03	55	95

* 1.0 ml of crude calmodulin (CaM) was added to the assay system.

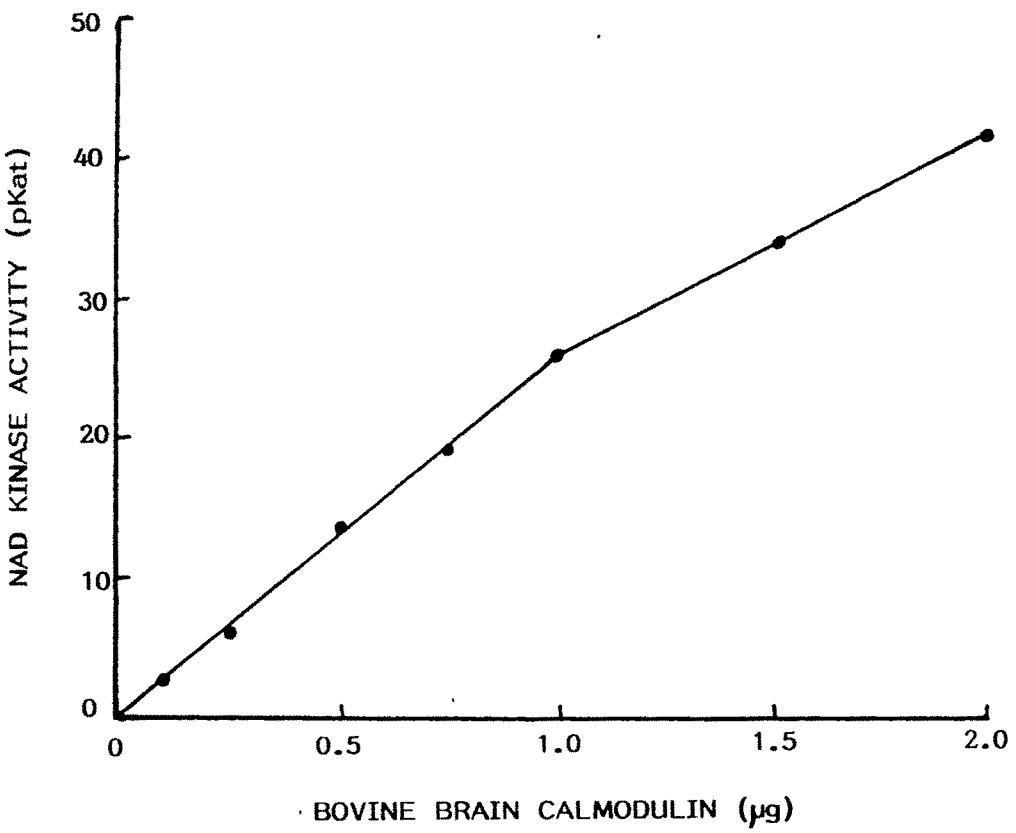

Fig.7 : Activation of pea terminal bud NAD kinase by bovine brain calmodulin.

was obtained upto 1 µg of pure bovine brain calmodulin. Data reported in Table-16 show that calmodulin levels in the terminal buds treated with either red, red-far-red or continuous white light were not significantly different from the dark control level. The light mediated increase in NAD kinase was thus not due to change in the level of calmodulin but due to the de novo synthesis of the enzyme.

Kinetic properties of partially purified NAD kinase :

The maximal activity of the enzyme requires the presence of $MgCl_2$, $CaCl_2$, ATP, NAD and calmodulin (Table-17). To study the kinetic properties of the purified enzyme, crude calmodulin preparation was made in 0.05 M Tris buffer (pH 7.5) containing 5 mM EGTA and was dialyzed for 36 h to remove most of the free and bound calcium of the tissue. Fig.8 shows that 0.25 ml of crude calmodulin preparation gives optimum activity but a saturating level of 1.0 ml was used in the experiments reported hereafter. The enzyme activity increased proportionately with increasing enzyme concentration (Fig.9) and 12.5 nM concentration of $CaCl_2$ was sufficient to give optimal enzyme activity (Fig.10). Lineweaver and Burk plot for the substrates NAD and ATP (Figs.11 & 12) gave a Km of 0.11 mM and 0.28 mM respectively.

A 1.5 mM EGTA concentration was sufficient to almost completely abolish the NAD kinase activity in presence of 1 mM $CaCl_2$ (Table-18) and the inhibition could be reversed by increasing the $CaCl_2$ concentration. Table-19 shows the effect of various concentrations of calmodulin antagonists (TFP, R_{24571} and W_7) on purified NAD kinase. The inhibition was found to increase with increasing concentration of antagonists. The

Table - 16 : Calmodulin content in pea terminal buds from seedlings treated with different lights.

Light Treatment	Calmodulin (mg/g fr.wt.)
Dark	3.5
$R_{(5,6,7)}$	3.1
$R\text{-}FR_{(5,6,7)}$	3.3
WL	3.0

Calmodulin levels were measured at 30 h of dark incubation after last R or R-FR light exposure.

WL, refers to the seedlings grown in continuous white light for 8 days.

Other conditions were same as given in Table - 5.

Table - 17 : Effect of omission of assay components on purified NAD kinase activity.

Omission	NAD kinase (pKat)
None	127.5
- $MgCl_2$	41.2
- $CaCl_2$	2.2
- NAD	2.2
- ATP	2.2
- CaM	0.3

The complete assay medium contained Tris-HCl buffer (pH 8.0), 100 μmol; NAD, 8 μmol; ATP, 12 μmol; $CaCl_2$, 4 μmol, $MgCl_2$, 40 μmol and 12.5 μg protein of purified enzyme.

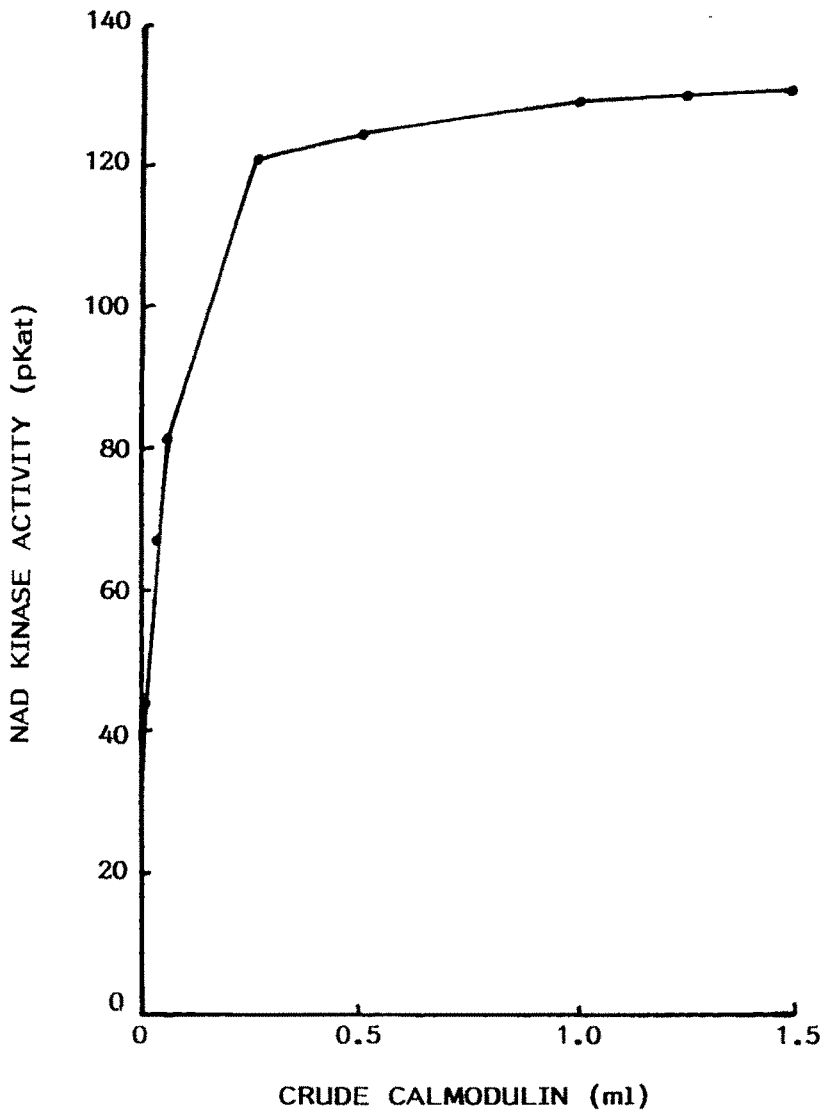

Fig.8 : Activation of NAD kinase by crude calmodulin preparation of light grown pea terminal buds.

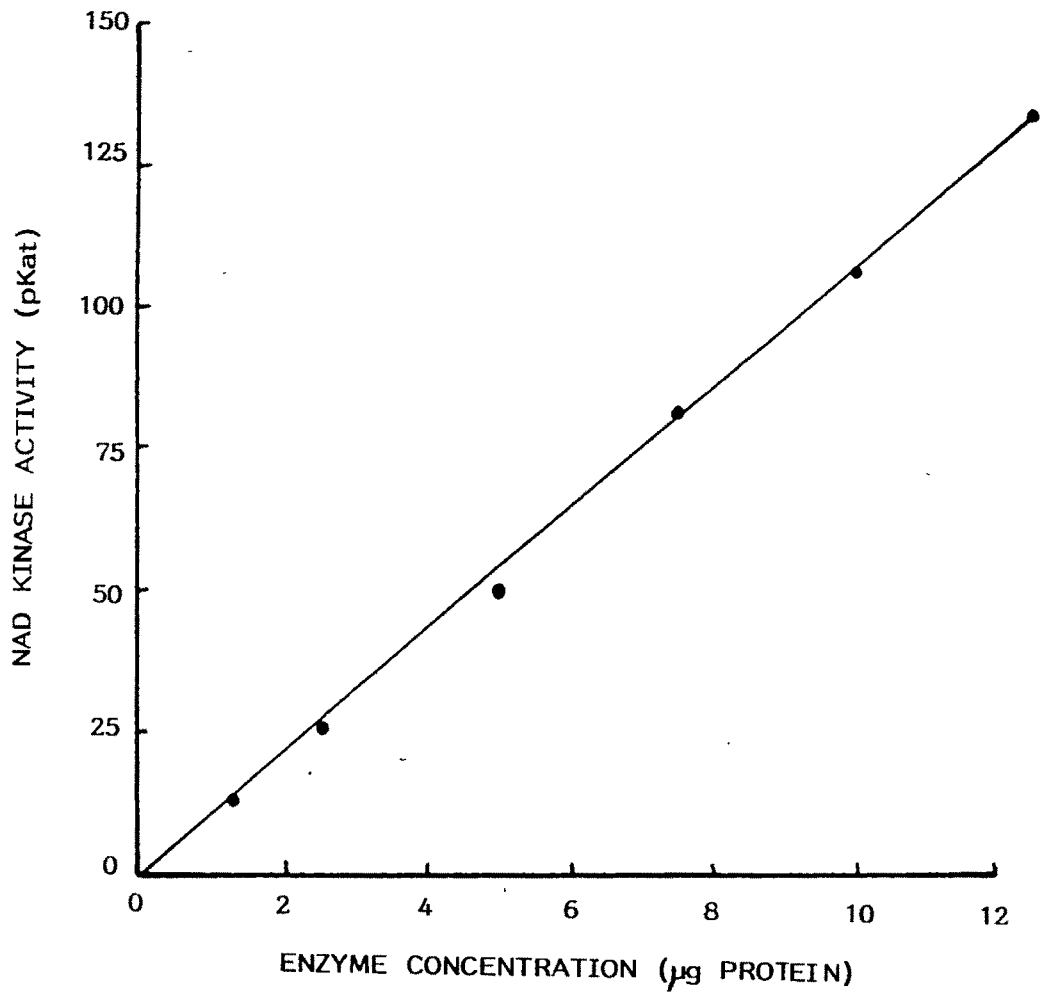

Fig.9 : Enzyme concentration curve for purified NAD kinase.

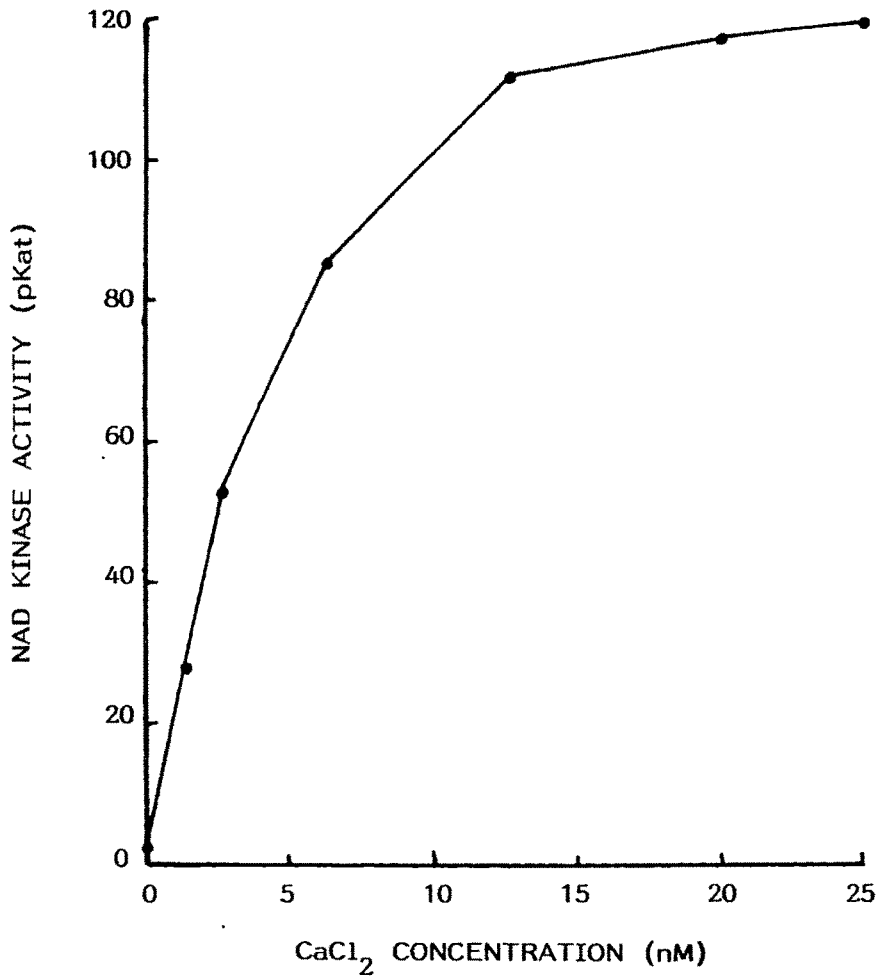

Fig.10 : Effect of $CaCl_2$ concentration on purified NAD kinase.

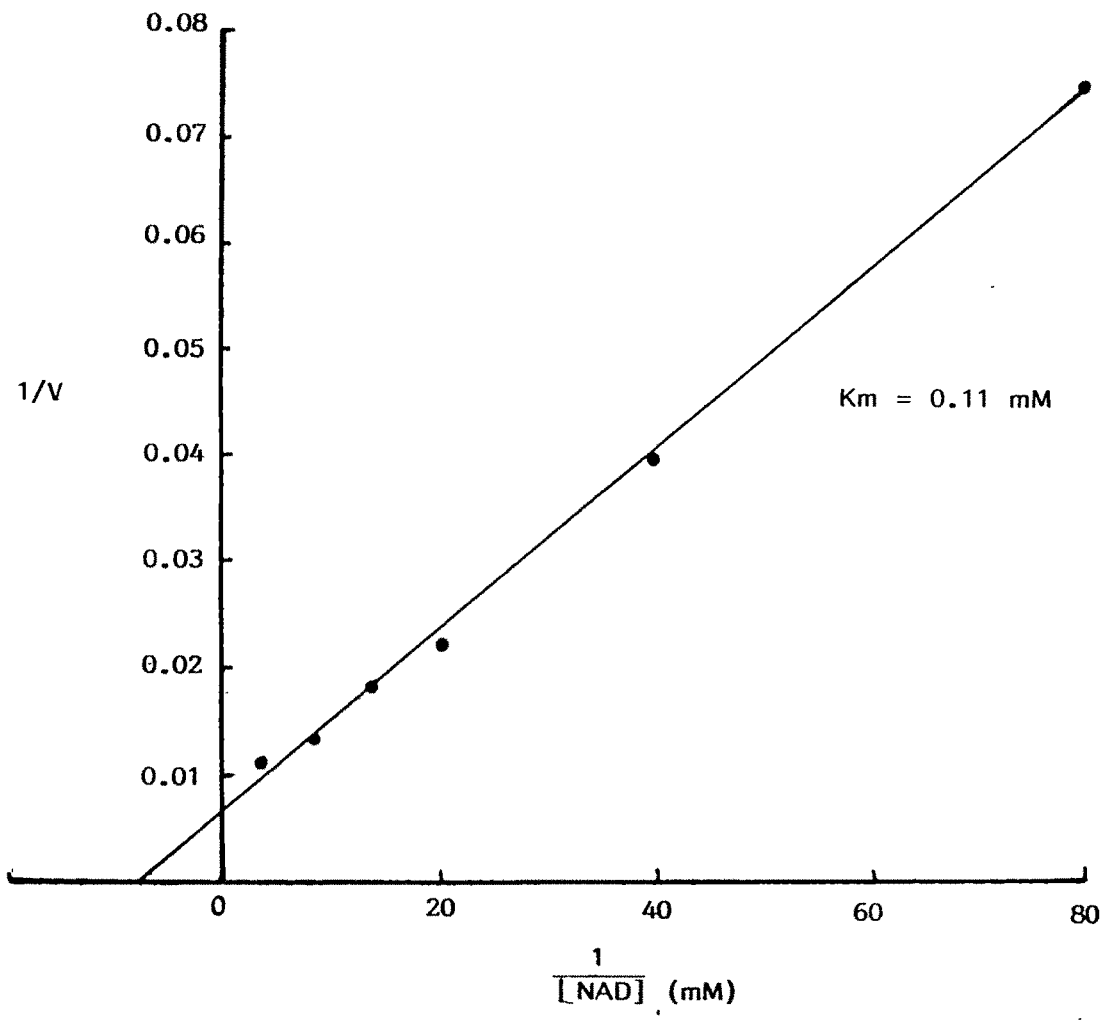

Fig. 11 : Lineweaver - Burk plot of NAD kinase for NAD.

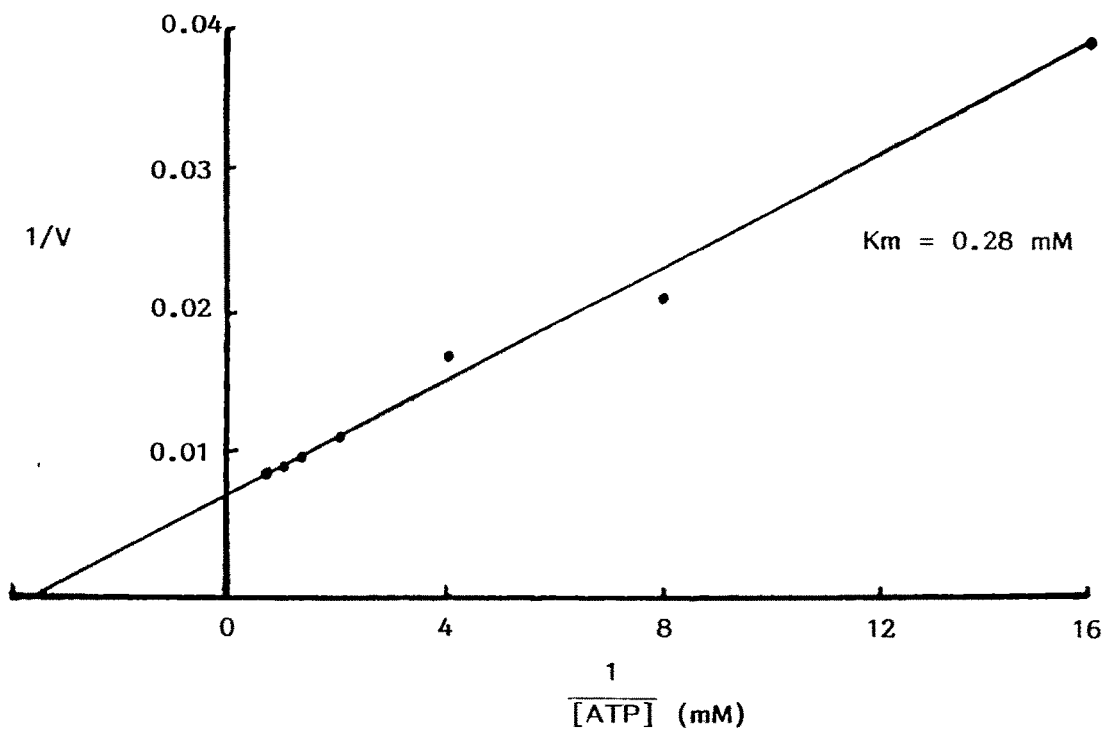

Fig. 12 : Lineweaver- Burk plot of NAD kinase for ATP.

Table - 18 : Effect of different concentrations of EGTA on purified NAD kinase activity.

EGTA concentration (mM)	NAD kinase (pKat)
0	128.0
0.5	126.3
1.0	120.8
1.5	4.4
2.0	2.2
5.0	2.2
EGTA + $CaCl_2$ (1.5 mM)+(2.0 mM)	115.6

Table - 19 : Effect of different concentrations of calmodulin antagonists on purified NAD kinase activity.

Concentration (μM)	NAD kinase (pKat)		
	TFP	R_{24571}	W_7
0	128.0	128.0	128.0
6.25	108.6	116.3	116.3
12.5	85.3	106.4	118.5
25.0	42.1	53.2	109.7
50.0	7.8	12.2	105.3
100.0	5.5	4.4	89.7
200.0	5.5	4.4	29.9

inhibition by all the three antagonists could be reversed by increasing the concentrations of calmodulin (Table-20).

Polyamines and guanidines had no significant effect on the enzyme activity at 2.5 mM concentration in the assay system (Table- 21).

Table - 20 : Effect of calmodulin concentration on the inhibition of purified NAD kinase by calmodulin antagonists.

Calmodulin (ml)	NAD kinase (pKat) with						
	Control	TFP		R_{24571}		W_7	
		at concentration(μM)					
		3	6	3	6	25	100
0.005	44.4	29.6	19.2	14.8	11.8	25.6	6.6
0.025	68.0	39.2	33.4	35.4	28.0	51.0	13.2
0.05	81.2	53.8	46.7	56.8	43.8	65.8	20.0
0.25	121.2	130.0	124.0	124.0	106.4	109.2	65.8
0.50	124.3	125.0	122.0	125.0	120.0	115.4	100.4
1.00	129.6	128.0	125.0	127.0	129.0	129.0	128.6

Table - 21 : Effect of polyamines and guanidines on purified NAD kinase activity.

Compound	NAD kinase (pKat)
-	133.0
1,3-Diaminopropane	135.7
Putrescine	137.1
Spermidine	124.6
Spermine	125.6
Agmatine	135.7
Creatine	134.6
GAA	124.6
GBA	133.9

Concentration of all the compounds used was 2.5 mM in the assay system.