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EFFECT OF NORLEUCINE ON GROWTH, PROTEIN AND CATECHOL OXIDASE SYNTHESIS IN TOBACCO SUSPENSION CULTURES

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Key Word Index—*Nicotiana tabacum* Solanaceae; norleucine; cell suspension culture; catechol oxidase; protein synthesis.

Abstract—Tobacco cells were grown in artificial media with defined amino acid composition. In such media, the addition of methionine or norleucine caused increases in the specific activity of the catechol oxidase, while in the normal medium norleucine depressed it. The differences of the effect of norleucine on synthesis of catechol oxidase and on cell growth is demonstrated, as is the reversibility of the norleucine effect by methionine. The incorporation of norleucine into a purified enzyme fraction is shown. The change in the electrophoretic patterns of the enzyme during growth in the absence and presence of norleucine was followed. [^{14}C]-Leucine incorporation by control and norleucine treated cells was examined and it was shown that protein synthesis in the norleucine treated cells was markedly changed and total incorporation reduced. Incorporation into soluble protein was reduced, but increased in the 20000 *g* precipitate fraction. Nevertheless use of autoradiography indicates that some catechol oxidase is apparently synthesised in the presence of norleucine.

INTRODUCTION

In a previous paper [1] we reported that norleucine suppressed catechol oxidase activity in suspension cultures of tobacco. We suggested that the effect could be brought about by incorporation of norleucine into the catechol oxidase, during synthesis and turnover.

The incorporation of amino acid analogues into cell protein is well known and has been reported in many tissues. However differential effects of an analogue or other toxicant on formation of a specific protein, as compared to general protein synthesis has been studied far less [2–5]. This type of study would be of value in investigating protein turnover which is often difficult to follow [6].

Plant cell lines resistant to specific amino acid analogues has been reported [6–8] and their possible use in studying secondary plant metabolism discussed. The nature and genetic basis of changes in suspension cell cultures has been reviewed by Meins [9] and the use of mutants to increase the free amino acid content of plants discussed [10]. A cell line resistant to ethionine has been characterised by Gonzales *et al.* [11], in which no evidence for changed enzyme structure of an ethionine sensitive enzyme was detected. In the case of our studies with norleucine, the effects observed were rapid and not apparently related to selection of resistant lines or of mutants. Thus the demonstration of norleucine incorporation into a specific enzyme, and of a differential effect of the analogue on catechol oxidase synthesis as compared to general protein synthesis would be of interest. In the following we bring evidence to this effect and also analyse in greater detail the difference of the effect of norleucine on cell growth and catechol oxidase synthesis.

RESULTS AND DISCUSSION

In previous experiments [1] norleucine was added to the normal growth media. Under these circumstances the effect of norleucine was not always clear cut, and reversal by methionine was not always complete. In order to study the importance of the effect of methionine on cell growth and enzyme activity we first grew cells for some days in medium devoid of methionine–artificial medium. They were then transferred to media containing either methionine, as control or methionine and norleucine. The results are shown in Fig. 1A.

The artificial medium, in the absence of methionine resulted in very poor growth. Addition of either methionine or norleucine improved growth, while the addition of both together further improved it, indicating that both could promote cell growth. The effect of norleucine on enzyme activity of the cells grown in the normal medium was very marked (Fig. 1B). In the artificial medium, in the absence of methionine, there was initially no increase in specific activity of the enzyme, but this was followed by a rise. Addition of methionine caused a rapid and spectacular increase in enzyme specific activity. When norleucine was added to the artificial medium, in the absence of methionine there was an initial drop in enzyme activity followed by a rise. This effect was almost totally reversed by the addition of methionine. Enzyme activity rose as if no norleucine had been added.

From these results it seems clear that the requirement for methionine is much greater for catechol oxidase activity than for cell growth. It is also clear that the artificial amino acid mixture did not support growth as well as did the normal medium and apparently lacked some other factor or did not correctly represent the ratio

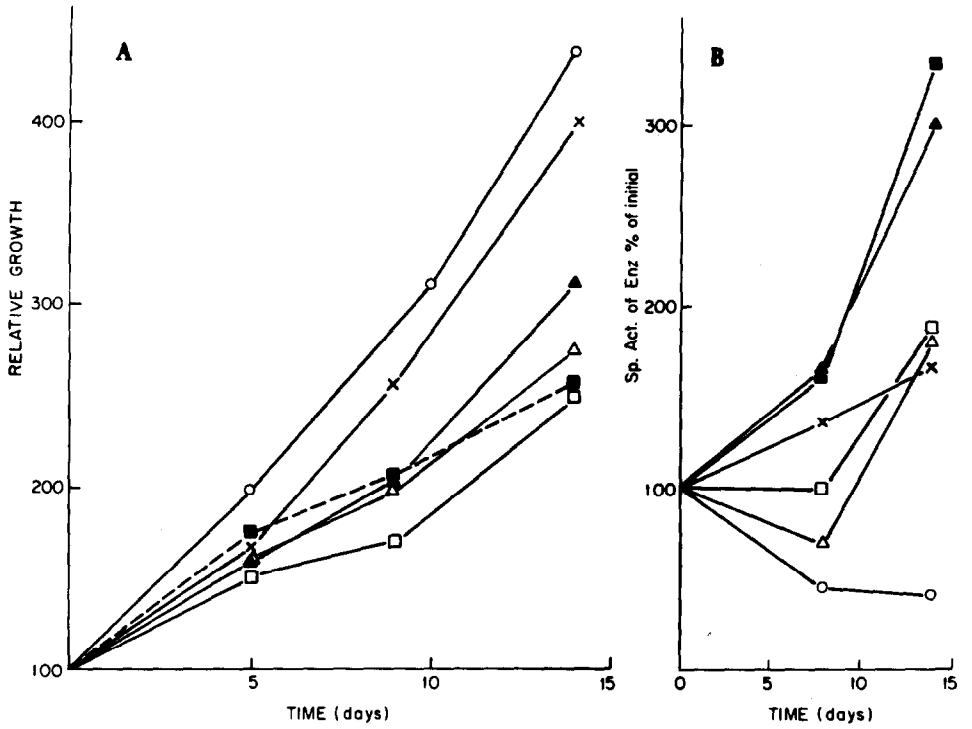

Fig. 1. Growth and enzyme activity of tobacco cell suspension cultures in medium with or without methionine (3 mM) and with or without norleucine (5 mM). A = Relative growth, (% of initial packed cell volume). B = Specific enzyme activity % of initial. X——X, Normal growth medium; O——O, normal medium + norleucine; ■----■, medium with artificial amino acid mixture + methionine; □——□, artificial medium − methionine; △——△, artificial medium + norleucine − methionine; ▲——▲, artificial medium + norleucine + methionine.

of amino acids in casein amino hydrolysate. We therefore purified the enzyme from cells grown in normal medium by Sephadex chromatography (Table 1) and then determined the amino acid composition of enzymes for control and norleucine cultured cells (Table 2). The amino acid composition of the purified enzyme was compared with that of a crude protein fraction, obtained by precipitating protein from the 20 000 *g* supernatant by the addition of TCA. A number of important conclusions emerge from the data in Table 2 which gives both the amino acid content relative to leucine and as percent of the total. Growth of the cells in the presence of norleucine resulted in incorporation of norleucine into the fraction containing the enzyme. This preparation was purified over 100 ×, and contained almost only enzyme protein. Indeed we were unable to obtain further purification. The amount of methionine in the enzyme dropped and it was partly replaced by norleucine. The cysteine content appeared to increase due to norleucine treatment, but since cysteine is labile during hydrolysis of protein this would require further confirmation. The amino acid composition of purified enzyme in other respects was fairly similar in control and norleucine treated cells. On a molar basis norleucine appeared to replace about 9% of the methionine in the enzyme. In contrast, in the crude protein fraction from the control cells, total methionine

Table 1. Purification of catechol oxidase from tobacco suspension cultures (for details see Experimental)

	Specific activity μl O_2/min/mg protein		Purification	
Purification step	Control	Norleucine treated	Control	Norleucine treated
20 000 *g* supernatant	0.4	0.25	1	1
$(NH_4)_2SO_4$ ppt (40–60% satn)	6.45	4.13	16.0	16.8
Eluate from Sephadex G 200 column	48.7	28.2	121	115

Table 2. Amino acid composition of purified catechol oxidase and crude soluble protein in tobacco suspension cell cultures grown with or without norleucine (5 mM) for 10 days

	Amount relative to leucine				% of total			
	Purified enzyme		Crude protein		Purified enzyme		Crude protein	
	Control	+Norleucine	Control	+Norleucine	Control	+Norleucine	Control	+Norleucine
Glutamic acid +glutamine	1.16	1.17	4.30	1.20	10.3	11.1	16.0	10.0
Aspartic acid +asparagine	1.13	1.07	2.58	1.40	10.0	10.2	14.4	11.8
Threonine	0.65	0.57	0.88	0.68	5.7	5.4	4.9	5.7
Serine	0.87	0.70	1.00	0.80	7.6	6.7	5.5	6.6
Proline	0.73	0.58	0.92	0.72	6.4	5.5	5.2	6.0
Glycine	0.88	0.85	1.35	1.10	7.8	8.0	7.5	9.3
Alanine	0.97	0.96	1.36	0.99	8.6	9.0	7.6	8.3
Cysteine	0.06	0.11	0.07	0.40	0.6	1.1	0.4	3.0
Valine	0.70	0.64	0.80	0.70	6.2	6.0	4.5	5.9
Isoleucine	0.44	0.45	0.52	0.53	3.9	4.3	2.9	4.5
Leucine	(1)	(1)	(1)	(1)	8.8	9.4	5.6	8.4
Phenylalanine	0.44	0.41	0.70	0.52	3.9	3.2	3.8	4.3
Cysteic acid	0.04	0.004	0.10	—	0.3	0.04	0.6	—
Norleucine	—	0.025	—	0.005	—	0.2	—	0.04
Methionine	0.14	0.03	0.12	0.26	1.3	0.3	0.7	2.2
Tyrosine	0.45	0.34	0.31	0.37	3.9	3.2	2.1	3.1
Histidine	0.28	0.22	0.34	0.22	2.4	2.2	1.9	1.9
Lysine	0.90	0.76	0.96	0.80	8.0	7.2	5.4	6.5
Arginine	0.52	0.54	0.53	0.52	4.5	5.0	3.0	4.4

content was lower than in the purified catechol oxidase but norleucine incorporation was only about one tenth of that in the enzyme on a molar basis (data not shown in Table 2). Norleucine is apparently incorporated to a much greater degree into enzyme than into total protein. The methionine content of crude protein rose as a result of norleucine treatment, while it dropped in the control. It is worth noting that norleucine changed the overall amino acid composition of this crude fraction, especially by reducing acidic and somewhat increasing basic amino acid content.

In our previous report we indicated that the isoelectric focussing pattern of catechol oxidase was changed by treatment with norleucine. At that time only one culture age was investigated. The isoelectric focussing pattern was therefore followed in control and norleucine treated cells during growth (Fig. 2). It can be seen that in control cells two new bands appear during growth, at pI 5.0 and 4.6, and the intensity of the band at pI 4.3 changes. In the norleucine treated cells the band at pI 5.0 does not appear at all, and that at pI 4.6 is transient. The band at pI 4.3 is absent in the treated cells. In addition, in treated cells the band at pI 4.45 slowly becomes more intense while in the controls the band has considerable activity already early on during growth. The changes in isoelectric point are clear cut, but do not suggest major changes in acidic and basic amino acid content, a point confirmed by the results in Table 2 already discussed.

These results led us to study the incorporation of [^{14}C]-leucine into protein in control and norleucine treated cells. Cells were cultured for 6 days. Half were then treated with norleucine for 3 days, while the remainder continued growth in normal medium for 3 days. Both lots were then incubated with [^{14}C]-leucine for 7 and 24 hr (Table 3). Treatment with norleucine induces profound changes in the behaviour of the cells, despite the relatively small effect on cell growth. Leucine uptake from the medium in a 24 hr period is reduced by about 75%. Respiration of leucine taken up is reduced from 4% to about 0.6%, the reduction being much greater in the initial 7 hr of incubation. Incorporation of leucine into the soluble fraction was reduced by treatment with norleucine. This was also the case for incorporation into the TCA ppt from the soluble fraction. The counts in the soluble fraction represent total protein and non-protein forms. However counts in the TCA ppt represent soluble protein. Clearly norleucine treatment drastically reduces incorporation of leucine into soluble protein. In contrast, in the norleucine treated cells much more of the leucine was incorporated into the 20000 *g* ppt fraction. This result was unexpected but may well be related to the enormous increase of amyloplasts which resulted from norleucine treatment [1].

In order to gain some insight into the effect of norleucine on leucine incorporation into the catechol oxidase, we fed treated and control cells [^{14}C]-leucine, extracted and partially purified the enzyme fraction and examined it for radioactivity using autoradiography. In parallel, gels were studied for protein and for enzyme activity (Fig. 3). It is clear that our preparation, which was at least 15 fold purified, contained a number of protein bands, most of which were associated with enzyme activity. At pI 6.0 a band is clearly labelled with [^{14}C]-leucine. The ratio of labelling of control versus norleucine treatment was 1.15:1, as determined by densitometric measurements. This band accounted for only a part, not more than 10%, of the total protein in the extract and corresponded with a zone having marked enzyme activity. It seems unlikely that this band constituted a major soluble protein with minor catechol oxidase activity accompanying it, since enzyme activity was very strong. No major protein such as Rubisco would be expected in cells grown in dim light, almost lacking chlorophyll and with few chloroplasts. Thus it seems likely that the band labelled corresponded with newly synthesised catechol oxidase. Apparently norleucine does not prevent enzyme synthesis, but as we previously suggested acts by altering enzyme structure.

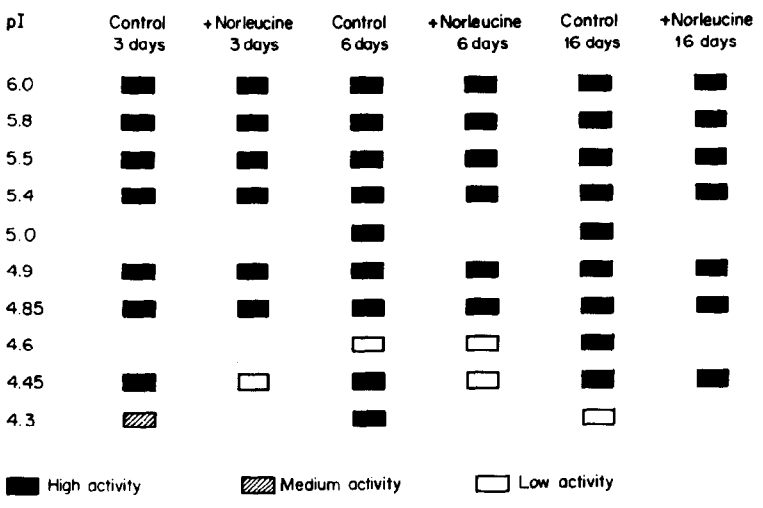

Fig. 2. Isoelectric focussing pattern of catechol oxidase from tobacco cell suspension cultures during growth. Enzyme after ammonium sulphate fractionation was applied to the gels at approximately equal total activity. ■, High activity; ▨, medium activity; □, low activity.

Table 3. ^{14}C balance in 9 day old tobacco suspension cultures incubated with ^{14}C-leucine for 7 hr and 24 hr

	Control 7 hr cpm	Control 24 hr cpm	+ Norleucine 7 hr cpm	+ Norleucine 24 hr cpm
(A) Appl. radioactivity	548 000	548 000	548 000	548 000
(B) External solution	62 000	50 000	438 000	424 000
(C) Uptake	485 000	498 000	110 000	124 000
(D) Released as CO_2	17 060	21 800	240	770
Ratio D/C (%)	3.5	4.3	0.22	0.63
(E) Incorporation into soluble fraction	429 000	326 200	45 000	57 400
Ratio E/C (%)	88.3	65.4	41.0	46.4
(F) TCA precipitate	19 700	10 640	750	1 250
Ratio F/C (%)	4.0	2.1	0.68	1.0
Ratio F/E (%)	4.59	3.26	1.66	2.2
(G) 20 000 *g* precipitate	27 100	125 000	44 000	61 000
Ratio G/C (%)	5.58	25.0	40.0	49.0

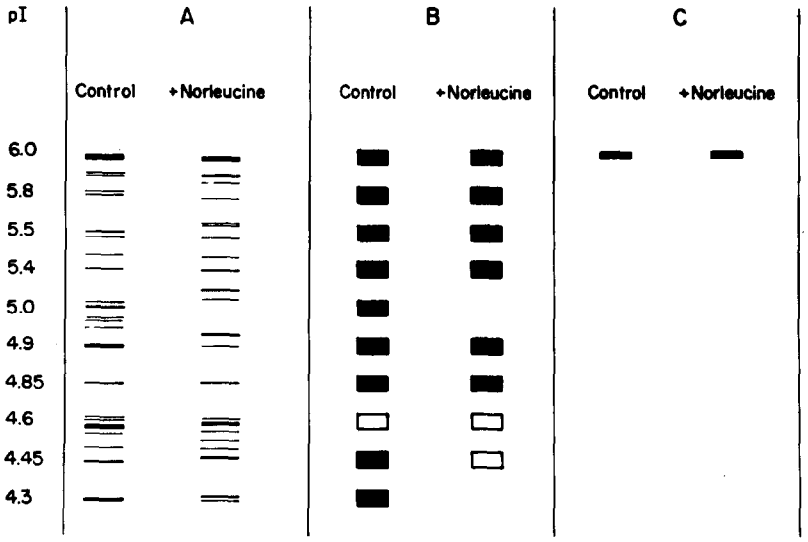

Fig. 3. Comparison of protein bands (A), enzyme activity (B) and radioactive labelling of partially purified enzyme (C) extracted from cells of tobacco suspension culture, treated with or without norleucine. Enzyme activity as in Fig. 2. Protein bands drawn to show intensity of staining. Labelling directly from X-ray film. (Cells were grown for 3 days. Half were then treated with norleucine (3 mM) for 5 days, while the remainder continued growth in the normal media for 5 days. To both cultures [^{14}C]-leucine (1 μCi) was added on the 7th day and the cells harvested on the 8th day. The cells were extracted and the ammonium sulphate fraction 40–60% saturation was analysed.)

CONCLUSIONS

The amino acid analogue norleucine when supplied together with methionine is able to profoundly alter the metabolism of plant cells in suspension culture without appreciably changing overall growth. In particular it appears that the incorporation of norleucine into different proteins differs considerably, catechol oxidase being particularly sensitive to its incorporation. The structure of catechol oxidase is altered due to incorporation of norleucine. This work has implications at two levels. On the one hand it may open up a way to regulate the level of catechol oxidase in plants. However in a more general way, the use of norleucine may serve to study the metabolism and involvement of methionine and methionine derived compounds in plant cells. The system described could be used as a model for such investigations.

EXPERIMENTAL

The cells of tobacco, *Nicotiana tabacum* cv Xanthi were grown as previously described [1] on the medium of ref. [12]. In detailed experiments on the effect of methionine, the hydrolysate of casein

(casamino acid mixture) was replaced by an artificial mixture of amino acid prepared according to the presumed amino acid content of whole casein from milk according to ref. [13] from which methionine was omitted or added as required.

Enzyme activity was determined as previously described and isoelectric focussing was carried out by standard procedures using a Multiphore apparatus. For routine assays the enzyme was extracted by disrupting cells in KP_i buffer pH 7.2 containing sucrose (0.4 M) and ascorbate (0.1 M). The homogenate was centrifuged at 20000 *g* for 30 min and the supernatant used as enzyme source or further purified. Purification was with $(NH_4)_2SO_4$; the ppt between 40–60% saturation being collected. This was resuspended in 0.1 M KP_i, pH 7.2 and applied to a Sephadex G 200 column (30 × 1 cm). The column was eluted with 0.1 M KP_i buffer, pH 6.8. The most active fractions were collected, pooled and used as source of purified enzyme. Purification was more than 100×. Incorporation studies were made by adding 0.25 μCi [^{14}C]-L-leucine (sp ac 336 mCi/mmol) to the growing cultures at a suitable stage. CO_2 evolution was followed by absorption on fluted filter paper containing 0.4 ml 20% KOH placed in a center well of the experimental flask. The cells were then treated in the usual way and radioactivity in the cell fractions determined by scintillation counting, after suitable times of incubation. TCA ppts were prepared by addition of TCA to a final concn of 15% to the supernatant, the ppt being collected after 20 min at 90°.

In order to determine incorporation of leucine into the enzyme fraction, control and norleucine treated cells were fed 1 μCi labelled L-leucine, the cells collected after a further 24 hr and enzyme extracted and partially purified with $(NH_4)_2SO_4$. The extracts were dialysed, and applied to gels for isoelectric focussing. The gels were dried on Whatman 3 MM paper and autoradiographed at −70° using Kodak 5× film, with 6 weeks exposure time. In parallel, proteins in gels were fixed with 12% TCA for 30 min, stained overnight with 0.1% Coomassie Brilliant Blue R250 and destained by repeated washings in 25% EtOH + 8% HOAc. Enzyme activity was determined as previously described [1] in parallel samples.

Amino acid composition was determined in suitable samples of TCA ppts of supernatant or purified enzyme after hydrolysis at 115° in 6 M HCl for 24 hr. The amino acid composition was determined with a Beckman amino acid analyser. Protein was determined according to ref. [14].

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