Phytochemistry, 1971, Vol. 10, pp. 783 to 788. Pergamon Press. Printed in England.

REGULATION OF HISTIDINE BIOSYNTHESIS IN CULTURED PLANT CELLS: EVIDENCE FROM STUDIES ON AMITROLE TOXICITY

M. E. DAVIES
Department of Botany, University of Birmingham, P.O. Box 363, Birmingham 15

(*Received* 21 *May* 1970)

Abstract—Early exponential phase cells of Paul's Scarlet rose in suspension culture respond to the herbicide amitrole (3-amino-1,2,4-triazole) by accumulating the histidine precursor imidazoleglycerol phosphate and its derivative, imidazoleglycerol. This accumulation is inhibited by L-histidine, which provides evidence for the operation of endproduct inhibition *in vivo*.

INTRODUCTION

THE HERBICIDE amitrole (3-amino-1,2,4,-triazole) selectively inhibits growth of green plants and microorganisms. The first evidence for a metabolically significant site of amitrole inhibition was the demonstration of its ability to interfere with histidine metabolism in yeast[1-3] and *Salmonella*[4] through a specific inhibition of the enzyme imidazoleglycerol phosphate (IGP) dehydratase leading to an accumulation of imidazoleglycerol (IG) in the growth medium. This inhibition is probably the major contributary factor in the growth inhibition of microorganisms since the effects of the herbicide are readily reversed by L-histidine. Furthermore, many bacteria show only transient inhibition in the presence of amitrole and this circumvention of toxicity appears to be associated with derepression of the histidine biosynthetic enzymes.[4]

Somewhat paradoxically, the herbicide has not hitherto been shown to inhibit histidine metabolism in higher plants. Accumulation of IGP and IG could not be detected in treated plants,[5] and histidine alone usually fails to reverse amitrole-mediated growth inhibitions.[6] On the other hand, IGP dehydratases isolated from several higher plant species have been shown to be sensitive to amitrole,[7] and certain green algae respond to the herbicide by accumulating IG, although here histidine does not reverse the growth inhibition.[8] The evidence for multiple amitrole-sensitive sites in autotrophic organisms has been reviewed by Hilton.[6]

If amitrole does in fact inhibit histidine biosynthesis in higher plants in a comparable way to that observed in microorganisms then it could prove a very convenient means of investigating feedback control of that pathway *in vivo*. Previous work with cultured rose cells[9] using isotopic competition methods indicated that exogenously supplied histidine did

[1] J. L. HILTON, *Weeds* **8**, 392 (1960).
[2] T. KLOPOTOWSKI and D. HULANICKA, *Acta Biochim. Polonski* **10**, 209 (1963).
[3] T. KLOPOTOWSKI and A. WIATER, *Arch. Biochem. Biophys.* **112**, 562 (1965).
[4] J. L. HILTON, P. C. KEARNEY and B. N. AMES, *Arch. Biochem. Biophys.* **112**, 544 (1965).
[5] C. G. MCWHORTER and J. L. HILTON, *Physiol. Plantarum* **20**, 30 (1967).
[6] J. L. HILTON, *J. Agri. Food Chem.* **17**, 182 (1969).
[7] A. WIATER, personal communication (1968).
[8] P. CASTELFRANCO and T. BISALPUTRA, *Am. J. Botany* **52**, 222 (1965).
[9] D. K. DOUGALL, *Plant Physiol* **40**, 891 (1965).

indeed regulate its own biosynthesis, although the extended period of these experiments (4–5 days) does not allow one to distinguish between feedback inhibition and repression as the controlling mechanism.

The present paper defines the conditions under which amitrole induces IG accumulation in cultured rose cells and describes the short-term effects of L-histidine on this accumulation.

RESULTS AND DISCUSSION

The Effect of Amitrole on Growth and IG Accumulation

Lag phase cells treated with 1 mM amitrole accumulated IG in both the medium and cells. The identity of the accumulated product as IG is based on two characteristics; (a) it is oxidized by periodate to give a substance with u.v. characteristics of the expected product, imidazoleformaldehyde, and (b) it is chromatographically indistinguishable from synthetic IG in the 'formic' and 'formix' solvents of Ames and Mitchell.[10] Traces of a compound with R_f characteristics of IGP and giving a positive reaction with both Pauly's reagent and the molybdate reagent of Bandurski and Axelrod[11] were also noted on chromatograms of cell extracts. That cultured rose cells produce IG in response to amitrole application is not unexpected in view of the reported effects of this herbicide on histidine metabolism in a wide variety of other organisms.[6] However, the present observation does constitute the first conclusive evidence that amitrole affects *in vivo* histidine metabolism in higher plants in a similar manner, and is further supported by the observation that amitrole inhibits *in vitro* the activity of IGP dehydratases from cereal seedlings.[7]

The accumulation pattern observed with rose cells differed from that observed with most other organisms in that the IG was largely retained within the tissue with relatively little leakage into the growth medium (Table 1). This point is of some interest since it indicates that plant cells, growing as suspensions in a highly artificial environment, still retain the capacity to sequester unwanted materials internally.

TABLE 1. IMIDAZOLE GLYCEROL (IG). ACCUMULATION IN AMITROLE TREATED CULTURES

Duration of amitrole treatment (days)	IG content (μmole/culture)	
	Medium	Cells
1	0·5	3·1
2	0·8	7·6
3	1·1	13·4
4	1·2	15·0

2-day old cultures were supplemented with amitrole (1 mM). Samples of cells and media were removed daily and analysed for IG.

Figure 1 shows the effect of amitrole concentration on growth and IG accumulation. The two phenomena differed somewhat in their sensitivity to the inhibitor, 50 per cent inhibition of growth occurring at 5×10^{-5} M amitrole, whereas a higher concentration of 2×10^{-4} M was required to achieve half-maximal accumulation of IG. This suggests that

[10] N. B. AMES and H. K. MITCHELL, *J. Biol. Chem.* **212**, 687 (1955).
[11] R. S. BANDURSKI and B. AXELROD, *J. Biol. Chem.* **193**, 405 (1951).

amitrole inhibits at more than one site, which is also in agreement with the finding that L-histidine fails to reverse amitrole inhibition of cell growth. A similar inability of histidine to reverse amitrole growth inhibition has been reported for other higher plants[5] and some green algae.[8]

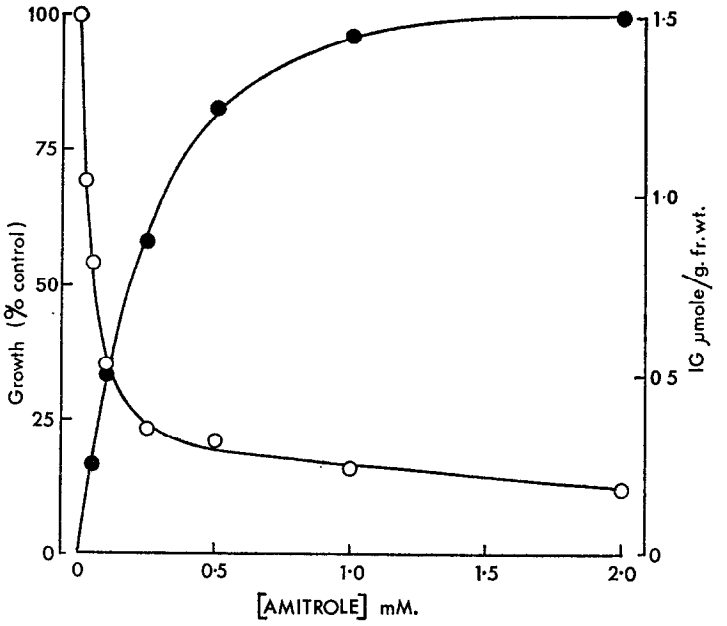

FIG. 1. EFFECT OF AMITROLE CONCENTRATION ON GROWTH AND IG ACCUMULATION. For effects on growth (○—○) cells were inoculated into fresh media containing the indicated concentration of amitrole and fresh weights determined after 7 days. For effects on IG accumulation (●—●) 3-day-old cultures were supplemented with amitrole and IG contents determined after a further 24 hr incubation.

The Effect of L-*Histidine on IG Synthesis*

Accumulation of IG in amitrole-treated 3-day-old cultures was markedly influenced by the histidine content of the medium (Table 2). L-Histidine at $1{\cdot}8 \times 10^{-5}$ M gave a 50 per cent inhibition of IG synthesis and accumulation virtually ceased at concentrations in excess of 1×10^{-4} M. L-Histidinol at 5×10^{-4} M also strongly inhibited IG synthesis but since there was chromatographic evidence for extensive accumulation of histidine in treated cells (and cultured rose cells have also been shown to contain an active L-histidinol dehydrogenase)[12] it is doubtful whether L-histidinol itself was responsible for the observed inhibition. One possible explanation for the histidine effect is that when added simultaneously with amitrole it could inhibit uptake of the herbicide thereby leading to a diminished inhibition of histidine biosynthesis. This possibility was excluded by the results shown in Fig. 2, where the addition of L-histidine to cells which had been pretreated with amitrole for 12 hr resulted in an almost immediate inhibition of IG synthesis.

[12] M. E. DAVIES, unpublished results.

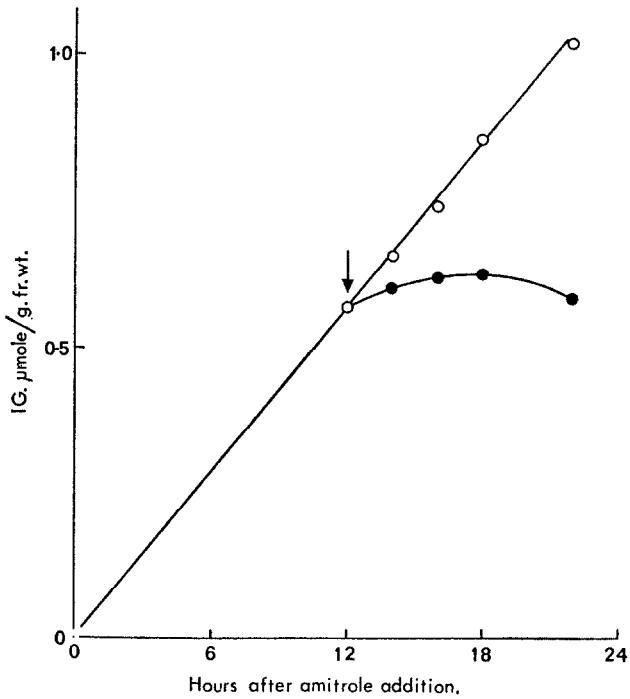

FIG. 2. TIME COURSE OF HISTIDINE EFFECT ON IG ACCUMULATION. 3-day-old cultures were supplemented with amitrole (2 mM) and incubated for a further 12 hr. L-Histidine was then added to half the flasks at a final concentration of 2×10^{-4} M (●—●); the remaining cultures received a similar volume of H_2O (○—○). Cells were then harvested from both series at 2 hr intervals and assayed for IG.

TABLE 2. THE EFFECT OF L-HISTIDINE CONCENTRATION ON IG ACCUMULATION IN AMITROLE TREATED CULTURES

L-Histidine concentration (mM)	IG accumulation (μmole/g. fr.wt./24 hr)
0	1·28
0·01	0·85
0·025	0·46
0·05	0·25
0·1	0·08
0·2	0·05

3-day-old cultures were supplemented with amitrole (1 mM) together with the indicated concentration of L-histidine. After a further 24 hr incubation the cells were harvested and IG contents determined.

All the evidence indicates that L-histidine can inhibit *in vivo* some enzyme(s) of the biosynthetic pathway preceding IGP-dehydratase, the most likely site of inhibition being 5-phosphoribosyl-ATP pyrophosphorylase which has been shown *in vitro* to be endproduct

inhibited in bacteria[13] and higher plants.[14] Moreover, the rapidity of the response to L-histidine in cultured rose cells clearly indicates endproduct inhibition rather than repression of component enzymes of the pathway.

The Response to Amitrole over the Growth Cycle

The ability of the cells to accumulate IG in response to amitrole varied considerably with the age of the culture as shown in Fig. 3. Maximal rates of accumulation occurred during the late-lag and early-exponential phases of growth (days 2–4) followed by a dramatic decrease between days 4 and 5. Accumulation was negligible during the remainder of the

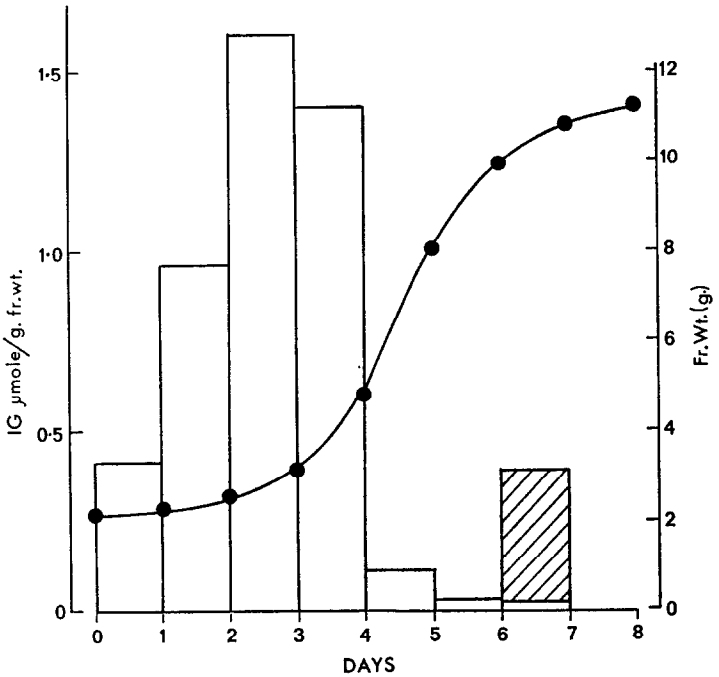

FIG. 3. EFFECT OF AGE OF CULTURE ON IG ACCUMULATION. On day-0 and on each successive day duplicate cultures were supplemented with amitrole (1 mM) and incubated for a further 24 hr. These treated cells were then harvested for fr. wt. (●—●) and IG determinations (histogram). The shaded portion of the histogram represents IG accumulation in day-6 cells supplemented with adenine (0·5 mM) simultaneously with amitrole.

passage. This pattern of development closely parallels that observed for the rate of protein synthesis.[12] In establishing the observed pattern of IG accumulation, at least three factors are involved: (a) the levels of constituent enzymes; (b) the concentration of L-histidine, and (c) the availability of substrates (ATP and 5-phosphoribosyl pyrophosphate). This latter factor is clearly important since, as shown in Fig. 3, the addition of adenine to day-6 cells restores their ability to synthesize IG. Moreover, the rapid decline in the ability to the cells to accumulate IG coincides exactly with the period during which the medium is completely depleted of both nitrate and phosphate.[15]

[13] B. N. AMES, R. G. MARTIN and B. J. GARRY, *J. Biol. Chem.* **236**, 2019 (1961).
[14] K. KRAJEWSKA, personal communication (1968).
[15] D. T. NASH, Ph.D. Thesis, University of Birmingham (1968).

The possibility that changes in the histidine content of the cells also contribute to the observed response to amitrole cannot be excluded, but it is rendered somewhat unlikely in view of the fact that changes in the free amino acid content of the tissue conform closely to the pattern observed for IG accumulation.[15] Furthermore, subjecting lag phase cells to treatments designed to reduce endogenous histidine concentrations, specifically by exposing them to a step-up nutritional shift in a medium supplemented with a mixture of all protein amino acids except histidine, failed to increase the rate of IG accumulation.

EXPERIMENTAL

Tissue Culture

The culture of Paul's Scarlet rose was obtained from Dr. W. Tulecke, and was grown in a synthetic medium of the following composition (all concentrations in mg/l.): $MgSO_4 \cdot 7H_2O$, 250; KCl, 750; $NaNO_3$, 850; $CaCl_2$, 110; KH_2PO_4, 140; H_3BO_3, 0·2; $MnSO_4 \cdot 2H_2O$, 1·0; $ZnSO_4 \cdot 7H_2O$, 0·5; KI, 0·1; $CuSO_4 \cdot 5H_2O$, 0·02; $CoCl_2 \cdot 6H_2O$, 0·01; $NaMoO_4 \cdot 2H_2O$, 0·02; ferric citrate, 5·0; Ca-pantothenate, 1·0; pyridoxine-HCl, 0·5; thiamine-HCl, 0·5; nicotinic acid, 1·0; myoinositol, 100; sodium glutamate, 187; 2,4-D, 1·1; kinetin, 0·5; sucrose, 20,000. Cultures were initiated by pipetting 12 ml of a 14-day-old suspension into 60 ml of fresh medium in a 250 ml Erlenmeyer flask. Cells were incubated at 28° on a rotary shaker (80 rev/min with 1 in. eccentric) under continuous low intensity illumination. All inhibitor and amino acid supplements were autoclaved separately and added to the medium when cold.

Fresh Weight Determination

Cells were harvested in preweighed filtration tubes consisting of 30 ml polypropylene syringe barrels fitted with discs of porous polythene. The contents of a single flask, with washings, were filtered through such tubes which were then centrifuged at 325 *g* for 10 min and again weighed.

Extraction and Estimation of IG

Washed and centrifuged cells (about 5 g) were added to 5 vol. of boiling H_2O, boiled for a further 5 min and allowed to stand for 15 min. The slurry was then filtered and the residue washed twice with a further 5 vol. of hot H_2O. The combined filtrates were evaporated to dryness *in vacuo* at 45° in a rotary evaporator and the residue resuspended in 10 ml of H_2O. Insoluble residues were removed by centrifugation and the clear solutions stored at −15° until used.

IG and IGP were determined by the method of Ames[16] as modified by Casselton.[17] O.D. measurements of the periodate oxidation product in *n*-butanol were made at 310 nm against a reagent blank and subsequently corrected for endogenous interfering substances by similarly assaying extracts of untreated cells. Since it was found that histidine increased blank values, appropriate controls were carried out for each histidine concentration. The absorption spectrum of each extract was also recorded over the range 220–350 nm and where aberrant spectra were encountered, as for example in cells treated with L-histidinol, the extracts were analysed by paper chromatography.

Materials

Imidazoleglycerol phosphate (IGP) was synthesized by the method of Ames.[16] Imidazoleglycerol (IG) was prepared from synthetic IGP by the action of a commercial phosphatase preparation and it was also isolated from culture filtrates of a *his* 1 mutant of *Neurospora crassa*.

Acknowledgements—This investigation was generously supported by the Science Research Council. I wish to thank Dr. T. Klopotowski, Dr. A. Wiater and Dr. K. Krajewska of the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, for generously supplying information in advance of publication.

[16] B. N. AMES, *J. Biol. Chem.* **228**, 131 (1957).
[17] P. J. CASSELTON, *Physiol. Plantarum* **19**, 411 (1966).