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ORNITHINE CARBAMOYLTRANSFERASE ACTIVITY AND EMBRYOGENESIS IN A CARROT CELL SUSPENSION CULTURE

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Key Word Index—*Daucus carota*; Umbelliferae; carrot; suspension culture; embryogenesis; ornithine carbamoyltransferase; aspartate carbamoyltransferase; polyamines.

Abstract—In a line of carrot cells with a high level of demonstrable embryogenicity, the activity of ornithine carbamoyltransferase was observed to rise transiently within hours of the induction of embryogenesis by auxin depletion. No such rise was observed for aspartate carbamoyltransferase. When the cells spontaneously lost their embryogenic potential, the transient enzyme pulse vanished concomitantly. The possible involvement of this pulse in polyamine biosynthesis is discussed.

INTRODUCTION

Somatic embryogenesis from totipotent plant cells is assuming increasing importance for the production of genetically-uniform plants of commercial importance and for regenerating genetically-modified plants in the laboratory. Embryogenesis is readily induced in suspension cultures of many plant cells by transferring them from auxin-containing to auxin-free media. One of the most-studied species in this connection is carrot (*Daucus carota* L.). The morphological changes that occur in the transition from cultured cells to carrot embryos have been the subject of several studies and are known in some detail [1, 2]. However, the underlying molecular and physiological processes are still largely unexplored. A number of biochemical changes have been shown to ensue from auxin removal in embryogenic carrot cultures, including qualitative changes in RNA template activity [3, 4], rRNA and poly(A)RNA sequences [5], histone and non-histone chromosomal proteins [3, 6], the appearance of embryo-specific proteins [7], quantitative changes in the polyamines [8] and changes in the activity of the enzyme arginine decarboxylase [9]. Changes in the turnover of RNA and protein [4] and in the polyamines and their associated enzyme arginine decarboxylase [8, 9] were noted within 24 hr of auxin removal; most of the other changes were noted later.

Carbamoyl phosphate is a precursor of, on the one hand, pyrimidine nucleotides for RNA and DNA biosynthesis, and on the other, of the amino acid arginine, required for protein biosynthesis and itself a possible precursor in higher plants of the polyamines putrescine, spermidine and spermine [10], which have been postulated to have a role in the regulation of gene expression [11]. The flux of carbamoyl phosphate into these two pathways is mediated by the enzymes aspartate carbamoyltransferase† (EC 2.1.3.2) and ornithine carbamoyltransferase† (EC 2.1.3.3), respectively. We have examined the specific activities of these key enzymes in the period immediately following the onset of embryogenesis in carrot cell cultures, and report our findings here.

†Abbreviations: ACTase, aspartate carbamoyltransferase; OCTase, ornithine carbamoyltransferase.

RESULTS

Using a suspension culture of high embryogenic potential that had been propagated in an embryo-suppressing medium containing the synthetic auxin 2,4-dichlorophenoxyacetate (2,4-D), embryogenesis was triggered by subculture into fresh, auxin-free medium. An initial result (Fig. 1a), with sampling over a 24-hr period, showed a transient rise in the activity of OCTase peaking at *ca* 6 hr after subculture. Figure 1 (b and c) shows repeated trials of the experiment over the first 8 hr, with controls to confirm that the transient activity pulse was associated with auxin removal and was not merely due to 'subculture shock', and was significantly greater than the random variations to be expected from extraction and assay procedures. Although there were variations in the extractable OCTase activity from one trial to the next, these occurred in both auxin-free and in auxin-containing (i.e. control) experiments with no differences discernable within significance limits. The transient rise in OCTase, however, occurred only in auxin-free medium, and was a consistent feature of all trials with highly-embryogenic cultures. In contrast, the parallel experiments on ACTase (Fig. 2a and 2b) showed no marked differences between auxin-free and auxin-containing experiments.

All of these measurements were made well before the start of widespread cell division within the culture. Cell counts taken over a much longer period after subculture showed a lag of *ca* 50 hr before any significant increase in cell number, with 'mid-log-phase' occurring *ca* 5 days later. The total extractable protein rose slowly during the first 8 hr, but was not significantly different in the presence or absence of 2,4-D.

Since a high proportion of non-embryogenic cells in the

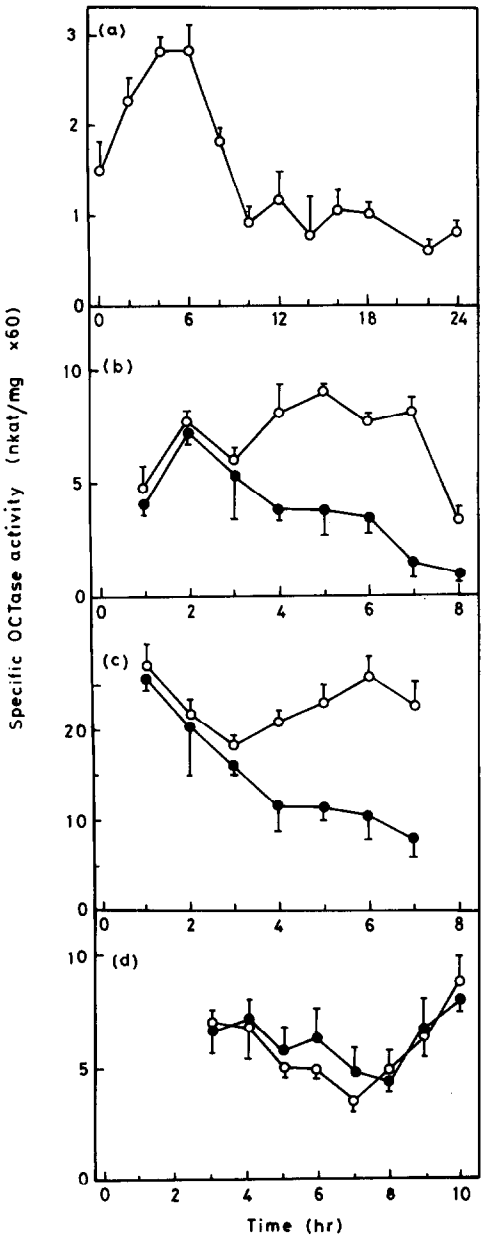

Fig. 1. Specific activity of ornithine carbamoyltransferase after transferring cells into media with (●) and without (○) 2,4-D. Time was measured from the completion of transfer. Trials (a)–(c) used cells of high embryogenic potential. Trial (d) used cells of low embryogenic potential. In (a) is shown the mean and half-range (bar) of a duplicate assay. In (b)–(d) are shown the mean and s.d. (bar) of four or six replicate values (see Experimental).

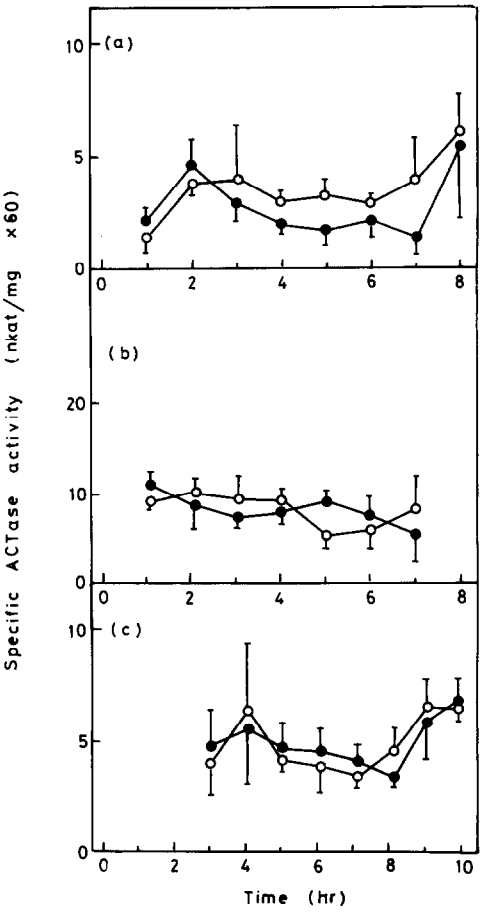

Fig. 2. Specific activity of aspartate carbamoyltransferase after transferring cells into media with (●) and without (○) 2,4-D. Trials (a)–(c) are the same experiments as (b)–(d), respectively, of Fig. 1. For other details see the legend to Fig. 1.

culture would obscure any changes in embryogenic cells, it is essential to use cultures of high demonstrable embryogenicity in this type of experiment. That this condition was satisfied in the experiments described thus far and reported in Figs. 1(a–c) and 2(a and b) was confirmed by microscopical examination of the starting cultures, which showed a very high proportion of globular proembryos and budding proembryonic masses [1], and by examination of the same auxin-free cultures that had been sampled for enzyme activity, after they had been in culture for *ca* 10 days, when they showed large numbers of heart- and torpedo-stage embryos, very young plantlets and very little else.

The above experiments leave open the question of whether the early transient rise in OCTase activity and the development of embryos are independent effects of the same cause (auxin removal) or whether both events are parts of a single temporal sequence, the OCTase pulse necessarily preceding embryo formation. Some months after the above experiments were performed, the cell line spontaneously lost its high embryogenic potential, a phenomenon well-known to occur in plant cell suspension cultures [12]. The resulting cells, being descendents of those used previously, provided a useful control for testing the development of OCTase and ACTase activity in the absence of embryo formation. As shown in Figs. 1(d) and 2(c), no differences in the activity of either enzyme could be detected upon omission of auxin from the medium. We suggest tentatively, therefore, that the transient rise in OCTase and the development of embryos are connected events.

DISCUSSION

An increase in the activity of OCTase implies an increased through-put of ornithine to citrulline and, hence, presumably to argininosuccinate and arginine (the well-known ornithine pathway). Arginine could be used in three ways: (1) in protein biosynthesis; (2) in the production of urea and the recycling of ornithine via the enzyme arginase; (3) in the production of agmatine via the enzyme arginine decarboxylase and, thence, in the production of the polyamine series putrescine, spermidine and spermine. A substantial body of evidence, mainly relating to animal cells, has implicated the polyamines in the control of gene expression during differentiation [11]. There is evidence that, in embryogenic carrot cells, arginine is the main source of polyamines [9], contrasting with animal cells which decarboxylate ornithine directly to form putrescine. Using a carrot cell culture system very similar to ours, Montague *et al.* [8, 9] have demonstrated transient rises in polyamine levels and in arginine decarboxylase activity shortly after transfer to auxin-free medium. It is an attractive hypothesis, therefore, that the rise in OCTase activity seen at 3–8 hr after transfer is a requirement for these increases in polyamine levels. In support of a polyamine-synthetic role for OCTase, a specific OCTase has been demonstrated as a component activity of a multifunctional enzyme committed to putrescine synthesis in seedlings of *Lathyrus sativa* [13]. However, other roles for OCTase cannot be ruled out; these may include a catabolic role in energy metabolism [14], or the provision of arginine for transient proteins (the turnover of RNA and protein has been detected as early as 2 hr after transfer into auxin-depleted medium [4]).

EXPERIMENTAL

Suspension cultures of carrot (*D. carota* L. var. Nantes) were established from phloem explants [15] and propagated by serial subculturing every 18–21 days into fresh Murashige and Skoog [16] medium containing 2% sucrose, 0.1 mg/l 2,4-D and 0.2 mg/l kinetin. Growth was in turretted flasks rotated at *ca* 2 rpm. at 26° and in a 16-hr light/8-hr dark regime. At subculture, the flasks were left to settle for 30 sec before transfers were made, to ensure that only fine particles were transferred. This is reported to select against rhizogenesis and favour the propagation of pro-embryonic material [17]. In preparation for an expt, two flasks, each containing 250 ml fresh culture medium, were heavily inoculated (to *ca* 15% vol. of packed cells) and allowed to grow for 5 days (to *ca* mid log-phase). The cells were harvested by centrifugation, pooled and divided into two aliquots. One of these was washed (twice) and resuspended in 250 ml fresh culture medium lacking 2,4-D. The second (control) was washed and resuspended in 250 ml fresh, normal (+ 2,4,-D) medium. For each flask this operation took 45–60 min and aseptic conditions were maintained throughout. The flasks were returned to slow rotation and aseptically sampled (10 ml samples) every 60 min thereafter. The sample cells were immediately pelleted by centrifugation and frozen for later extraction and analysis. Extraction and assay procedures were rigorously standardized to minimize variation from these sources. Extraction was carried out at 4°. Cell pellets were thawed, ground with washed sand in cold 0.1 M Tris–HOAc buffer (pH 7.5) and the extract filtered through coarse Miracloth. The filtrate was immediately homogenized (10 strokes) in a precooled Dounce homogenizer, centrifuged at 20000 *g* for 20 min and the lipid layer discarded. An aliquot (1 ml) of the supernatant was immediately passed through a small column (10 ml) of Sephadex G25 (equilibrated in the previous buffer) to remove endogenous small molecules. It was then assayed for the activities of ACTase [18], OCTase [19] and for total extractable protein [20]. Assays were always performed in duplicate or triplicate. In some expts the extraction was also duplicated, giving four or six replicate values.

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