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TWO OCTOPINE DEHYDROGENASES IN CROWN-GALL TUMOR TISSUE

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Abstract—Extracts from four crown-gall tumor tissue culture lines, originally induced by two octopine-type strains of *Agrobacterium* on three plant species, converted L-arginine-[5-^{3}H] to a compound which co-migrated with octopine on electrophoresis. Synthesis showed dependence on added pyruvate and reduced pyridine nucleotide. Both NADH and NADPH were active and mixtures of the two coenzymes, when tested with *Vinca* strain W1 tumor extracts, were more effective than either coenzyme at comparable concentrations. Addition of an NADH-consuming enzyme system to reaction mixtures containing NADPH had little effect on this activity. Products formed by *Vinca rosea* strain W1 tumor extracts and *Phaseolus vulgaris* strain B6 tumor extracts in reaction mixtures containing pyruvate plus NADH or NADPH co-eluted with unlabeled octopine on ion exchange chromatography. The product from the *Vinca* reaction mixtures co-migrated with an octopine standard in three TLC systems. Permanganate treatment of the enzymatically formed tritiated product and of unlabeled octopine gave compounds with R_f similar to arginine and γ-guanidinobutyric acid, the products expected from permanganate degradation of octopine. The *Vinca* W1 extracts catalyzed the oxidative cleavage of octopine, with the formation of arginine, in the presence of NAD or NADP. Two octopine dehydrogenases were concluded to be present in these tissues, one dependent on NAD, the second on NADP.

INTRODUCTION

The tumors induced by *Agrobacterium tumefaciens* typically contain high levels of either lysopine [N^2-(D-1-carboxyethyl)-L-lysine] and octopine [N^2-(D-1-carboxyethyl)-L-arginine] or nopaline [N^2-(1,3-dicarboxypropyl)-L-arginine], while normal plant tissue contains little or none of these rare amino acids [1–3]. The strain of bacterium determines whether lysopine + octopine or nopaline is produced and this property is correlated with the ability of the bacterium to utilize these compounds [1, 3]. Thus, nopaline-utilizing strains induce tumors which produce nopaline and octopine- and lysopine-utilizing strains induce tumors which produce lysopine and octopine. The genes responsible for bacterial utilization and for bacterial induction of tumors which produce elevated levels of these amino acids are on a large plasmid which is essential for tumor induction by the bacterium [1]. The correspondence between bacterial utilization and bacterial induction of synthesis led to the proposal that bacterial genes for octopine or nopaline synthesis are transmitted to plant cells in the process of tumor formation [3].

Lejeune and Jubier [4], using crown-gall tissue cultures of tobacco, Jerusalem artichoke and *Parthenocarpus tricuspida*, showed that each contained an enzyme, lysopine dehydrogenase, which catalyzed the reaction:

lysine + pyruvate + NADH → lysopine + NAD

The enzyme was dependent on all three substrates and NADPH could not substitute for NADH. Goldmann-Ménagé [5] showed that an octopine-synthesizing enzyme is present in sterile crown-gall tumor cultures, since these cultures converted labeled arginine and pyruvate into octopine. Bomhoff [6] reported that extracts from three crown-gall tissue cultures showed octopine dehydrogenase activity which was dependent on NADH. Because characterization of octopine dehydrogenase in crown-gall tumors of different plants and of tumors induced by different strains of bacteria could resolve the question of the origin of the structural gene for its synthesis, it is important to isolate and describe this enzyme in several crown-gall tumors. In this paper, we report experiments which suggest that octopine producing crown-gall tissues contain two octopine dehydrogenases, one dependent on NADH, the other on NADPH.

RESULTS

Extracts of four crown-gall tissues grown *in vitro*, strain B6 induced *Vinca rosea* tumor, strain W1 induced *Vinca rosea* tumor, strain B6 induced *Nicotiana tabacum* tumor, and strain B6 induced *Phaseolus vulgaris* tumor, all catalyzed the conversion of L-arginine-[5-^{3}H] into a compound with an electrophoretic mobility similar to octopine. Boiled extracts were inactive. The reaction showed dependence on pyruvate (Table 1) and extracts of strain W1 *Vinca* tumor did not form this product when either alanine or lactate was substituted for pyruvate. Reduced pyridine nucleotide, at a concentration of 10 mg/ml in the reaction mixture, increased the apparent

Table 1. Formation of octopine by extracts from four different culture lines of crown-gall tumors

Reaction mixtures	Octopine formation (μmoles g^{-1} hr^{-1})			
	P. vulgaris, B6	*N. tabacum*, B6	*V. rosea*, B6	*V. rosea*, W1
Minus pyruvate (NADH)	9.1	4.5	2.7	2.3
Minus pyruvate (NADPH)	8.5	5.2	3.1	2.8
Complete (NADH)	13.2	15.1	8.1	16.1
Complete (NADPH)	26.0	16.7	6.6	29.2

yield of octopine by two to four fold, depending on the extract, and both NADH and NADPH were active. The NADH-dependent and NADPH-dependent octopine dehydrogenase was determined in 12 *Vinca* W1 tumor extracts. The ratio of NADH to NADPH octopine dehydrogenase activity ranged from 0.4 to 3.4 with an average of 1.4 and a S. D. of 0.8.

To establish that octopine was produced in these reactions, the products of four reactions (*Vinca* W1 tumor extract + NADH + pyruvate; *Vinca* W1 tumor extract + NADPH + pyruvate; *P. vulgaris* tumor extract + NADH + pyruvate: and *P. vulgaris* tumor extract + NADPH + pyruvate) were each mixed with unlabeled octopine and chromatographed on a column of SP-Sephadex. In each case the major radioactive product co-eluted with octopine. The two *P. vulgaris* B6 tumor extract reactions also contained a radioactive product whose formation was not dependent on pyruvate and which appeared to be more acidic than octopine, as it eluted before octopine.

The octopine-like products formed by the two *Vinca* W1 tumor extracts were further examined on silica gel plates developed with *iso*-propanol–water, (7:3) and *n*-butanol–17.6 M acetic acid–water (4:1:1) and on Chrom AR plates developed with 95% ethanol–15 M NH_4OH–water (50:7:7). The tritiated octopine-like product from both reactions co-migrated with unlabeled octopine in all three systems. Octopine and the NADPH-dependent octopine-like product were treated for 90 min at room temperature in an aqueous solution of 0.15% $KMnO_4$ and the products resolved by thin layer electrophoresis. Both yielded two products which had electrophoretic mobilities similar to arginine and to γ-guanidinobutyric acid, the breakdown products expected from this oxidation [5].

Octopine dehydrogenase activity was determined in a *Vinca* W1 tumor extract using several concentrations of NADH, NADPH or a mixture of the two nucleotides (Table 2). At each concentration tested, NADPH was more effective than NADH and the combination of the two nucleotides gave activity approximately equal to the sum of the individual activities, except at the highest concentration where the mixture appeared to be somewhat inhibitory. The activity obtained with the mixture of nucleotides was as great or greater than that obtained with the next higher (3-fold higher) concentration of either coenzyme.

Table 3. Activity of NADPH-dependent octopine dehydrogenase in the presence and absence of an NADH utilizing enzyme system

Reaction mixtures	Octopine formation (μmoles $g^{-1}hr^{-1}$)
Arginine, pyruvate, NADPH	22.0
Above + ADH* (2.5 units/ml)	19.0
Above + acetaldehyde (67 mM)	9.7
Above + ADH + acetaldehyde	13.4

* ADH = yeast alcohol dehydrogenase

To determine if conversion of NADPH to NADH contributed to the apparent NADPH-dependent octopine dehydrogenase activity, an extract of *Vinca* W1 tumor tissue was tested for NADPH-dependent octopine dehydrogenase activity in the presence of an NADH-utilizing system. Table 3 shows that addition of NADH-dependent alcohol dehydrogenase with its substrate, acetaldehyde, to the reaction mixture reduced the NADPH-dependent octopine dehydrogenase activity *ca* 40% but was less effective in this regard than acetaldehyde alone. Thus, NADPH must participate as the direct reductant in the formation of octopine by these extracts.

Vinca W1 tumor extracts in the presence of either NAD or NADP also catalyzed the conversion of octopine-[^{3}H] to a tritiated product with the electrophoretic mobility of arginine. The arginine-like product co-eluted with unlabeled arginine from a column of SP-Sephadex.

DISCUSSION

Extracts of four octopine-type crown-gall tissue

Table 2. Dependence of octopine snythesis on reduced pyridine nucleotide

Coenzyme concentration	Octopine formation (μmoles g^{-1} hr^{-1})			
	NADH (1)	NADPH (2)	NADH + NADPH*	Sum of 1 & 2
0.37 mg/ml	3.5	7.6	12.1	11.1
1.1 mg/ml	2.3	12.7	19.0	15.0
3.3 mg/ml	10.8	17.8	25.4	28.6
10.0 mg/ml	13.9	21.1	15.8	35.0

* Each nucleotide present at concentration indicated.

cultures have been found to synthesize octopine. The reaction shows dependence on pyruvate and reduced pyridine nucleotide. Unlike lysopine dehydrogenase [4] or the octopine dehydrogenase (EC 1.5.1.11) of animals [7], which are NADH specific, these extracts formed octopine with either NADH or NADPH. Three possibilities could account for this difference: (1) the octopine dehydrogenase is non-specific with regard to the coenzymes; (2) there is an enzyme in these extracts which catalyzes the interconversion of NADPH and NADH, in addition to a coenzyme specific octopine dehydrogenase; and (3) there are two octopine dehydrogenase enzymes present, one requiring NADH, the other NADPH.

The 8-fold variation observed in the ratio of NADH to NADPH-dependent activity with different *Vinca* W1 tumor extracts and the enhanced octopine dehydrogenase activity obtained with mixtures of the two coenzymes is inconsistent with possibility (1) above, that the octopine dehydrogenase is non-specific with regard to coenzyme requirement. The second possibility is improbable because in some extracts NADPH activity was greater, in others NADH activity was greater. If a pyridine nucleotide transhydrogenase or a pyridine nucleotide kinase were responsible for these results, the activity obtained with one of the coenzymes would always be equal to or less than that obtained with the other coenzyme. Also, the octopine dehydrogenase activity observed with mixtures of the two coenzymes would be less than that obtained with *ca* 50% greater total concentration of either of the individual coenzymes. This was not the case (Table 2), as the equal weight mixtures of coenzymes at 2.2 and 6.6 mg/ml total coenzyme gave more activity respectively than 3.3 and 10 mg/ml of NADPH, the more active of the individual coenzymes in this experiment.

The third possibility, that there are two octopine dehydrogenase enzymes present in these extracts, is consistent with the activity observed with the two coenzymes, and the mixtures of coenzymes. Because NADPH-dependent octopine dehydrogenase activity was obtained in the presence of an NADH-consuming enzyme system, it is clear that a NADPH-dependent octopine dehydrogenase is present. Based on the results of Lejeune and Jubier [4] and Bomhoff [6], an NADH-dependent octopine dehydrogenase should also be present in these extracts, as our results indicate. We conclude, therefore, that two octopine dehydrogenase enzymes are present in the four tumor tissues thus far examined.

The correlations between bacterial utilization of octopine, bacterial induction of octopine-producing tumors, and the presence on the *Agrobacterium* virulence plasmid of genes governing octopine utilization have suggested that a transfer of plasmid genes to the plant host occurs in the process of crown-gall tumor formation [3, 8]. The presence of two octopine dehydrogenase enzymes in these tumors increases the possible sources of the tumor genes which determine octopine synthesis to four: both genes are bacterial; both genes are plant; only the gene for NADPH-dependent octopine dehydrogenase is bacterial; only the gene for NADH-dependent octopine dehydrogenase is bacterial.

EXPERIMENTAL

Tumor tissues. Crown-gall tissues of *Vinca rosea* induced by *A. tumefaciens* strain B6 and strain W1 were obtained from A. Braun and R. Manasse and cultured on 10 × White's medium without hormones, as described by Manasse [9]. Strain B6-induced *Nicotiana tabzcum* cv Havana crown-gall tumor tissue was obtained from C. Kado and cultured on hormoné-free Murashige and Skoog medium [10]. *Phaseolus vulgaris* cv Pinto crown-gall tissue was isolated from tumors on strain B6 inoculated leaves [11] and cultured on a hormone-free defined medium consisting of salts plus organic supplements as described by Liau and Boll [12].

Tissue extracts. Tumor tissue extracts were prepared by grinding tissues in a mortar and pestle with glass beads and an equal weight of buffer (0.01 M Tris–HCl, pH 8.0, containing 10 μM dithiothreitol and 10 μM pyridoxal phosphate). The homogenate was centrifuged at 12 000 *g* for 30 min at 4° and the supernatant used for enzyme assays. Me_2CO powders of tumor tissues were also prepared as described by Umbreit *et al.* [13] and the lyophilized powders extracted by incubating for 30 min at 0° in the above buffer, using 1.0 ml buffer per 50 mg of dry powder. The buffer extract was centrifuged at 20 000 *g* for 30 min at 4° and the supernatant used in enzyme assays. The activity of enzymes prepared by these 2 methods was similar.

Octopine dehydrogenase assay. Assays for octopine formation utilized 50 μl of enzyme extract, 10 μl of NADH or NADPH (100 mg/ml in reaction buffer), 10 μl of Na pyruvate (0.1 M in reaction buffer), 10 μl of reaction buffer and 20 μl of L-arginine-[5-^{3}H] (*ca* 4 × 10^5 cpm of arginine with a specific activity of 16.7 Ci/mmole). Reaction buffer was either 0.1 M Na phosphate, pH 7.0, containing 0.1 mM EDTA and 20 mM β-mercaptoethanol; or 0.1 M Tris–maleate, pH 7.0. No significant difference in enzyme activity was observed with these two buffers. Reaction mixtures were incubated for 60 min at room temp. (*ca* 24°), the reaction stopped by addition of 200 μl of cold redistilled 95% EtOH and the mixture placed at −35° for *ca* 15 min. The mixtures were centrifuged at 12 000 *g* at 4° for 12 min, a 10 μl aliquot of the supernatant removed and spotted on a Si gel (Eastman #6061) or cellulose (EM #5502) plastic-backed TLC plate. The components of these spots were separated by electrophoresis (40 to 50 min at 1000 volts) using 5.3% HOAc in H_2O, which fully resolves arginine and octopine. The electrophoretograms were cut into 1 cm sections and the individual sections placed in 5 ml vials with 3 ml of a scintillation fluid consisting of toluene + 0.4% premixed dry fluor (Research Products International). Radioactivity was determined in a liquid scintillation spectrometer. About 2000 cpm as octopine was obtained in typical experiments. Enzyme activity (expressed as μmoles of octopine per g tissue per hr) was estimated from the number of cpm in the position corresponding to that of octopine. The reaction was linear to about 2 hr. The reverse reaction, oxidative cleavage of octopine to arginine plus pyruvate, was determined in the following mixture: 25 μl enzyme extract, 5 μl of NAD or NADP (100 mg/ml), 5 μl of water, 20 μl of buffer (0.2 M glycine–NaOH, pH 9.55), and 5 μl of octopine-[^{3}H] (*ca* 2.5 × 10^5 cpm of octopine with specific activity of 16.7 Ci/mmole). The reaction mixture was incubated at room temp. for 60 min, the reaction stopped by addition of 100 μl of cold 95% EtOH and the mixture kept at −35° for *ca* 15 min. The mixture was centrifuged for 12 min at 12 000 g at 4° and supernatant aliquots of 30 μl removed and spotted on Whatman 3 MM paper. The sample was separated by electrophoresis for 60 min at 500 volts using a buffer consisting of 10% pyridine, 0.35% HOAc and 90% H_2O. Arginine and octopine were well resolved in this system and portions of the electrophoretogram at the expected position of arginine were cut in 1 cm segments and placed in 5 ml vials containing 0.5 ml water and extracted for 30 min. Then 3 ml of scintillation fluid (toluene–Triton X-100, 2:1 plus 0.4% dry fluor) was added and the sample counted.

Chemicals. L-Arginine-[5-^{3}H] was purified before use on a Dowex 50 column (0.5 × 5.0 cm) which separated the arginine from neutral and weakly basic compounds. Octopine-[^{3}H] was prepared enzymatically from this arginine using octopine dehydrogenase isolated from scallop muscle. Quick-frozen

Nova Scotia scallops obtained from a local market were homogenized and octopine dehydrogenase extracted and purified through step B as described by Thoai *et al.* [14] and dialyzed against 0.005 M Na phosphate buffer, pH 6.0, containing 0.1 mM EDTA. 30 μl of this enzyme preparation was reacted in pH 6.6 Pi buffer with L-arginine-[^{3}H], 0.01 M pyruvate and 0.01 M NADH for 24 hr at room temp. and the octopine-[^{3}H] formed was purified by chromatography on SP-Sephadex columns. Alcohol dehydrogenase was added to some octopine dehydrogenase assays at a concentration of 2.5 units per ml of reaction mixture. Octopine, NADH, NADPH, NAD and NADP were from Sigma Chemical Co., Inc.,

Column chromatography of octopine and arginine. SP-Sephadex C25 was equilibrated in pH 3.25 ammonium formate buffer (12 vols of 0.2 M formic acid + 5 vols of 0.2 M ammonium formate) and a 1.1 cm × 120 cm column of resin prepared. Samples of 1 to 5 ml were adjusted to pH 2.5 with 0.1 N HCl, applied to the column, and eluted with the above buffer. Arginine remained bound to the column and was subsequently eluted with 0.5 M NH_4OH. The elution of unlabelled octopine or arginine from these columns was determined by the diacetyl colorimetric method described by Lippincott *et al.* [15].

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