

Changes in Polyamine Biosynthesis Associated with Postfertilization Growth and Development in Tobacco Ovary Tissues¹

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ABSTRACT

Polyamine (PA) titers and the activities of arginine decarboxylase (ADC, EC 4.1.1.19) and ornithine decarboxylase (ODC, EC 4.1.1.17), enzymes which catalyze rate-limiting steps in PA biosynthesis, were monitored during tobacco ovary maturation. In the period between anthesis and fertilization, the protein content of ovary tissues rapidly increased by about 40% and was accompanied by approximately a 3-fold increase in ODC activity, while ADC activity remained nearly constant. PA titers also remained relatively unchanged until fertilization, at which time they increased dramatically and the DNA content of ovary tissues doubled. This increase in PA biosynthesis was correlated with a further 3-fold increase in ODC activity, reaching a maximum 3 to 4 days after fertilization. During this time, ADC activity increased only slightly and accounted for approximately 1% of the total decarboxylase activity when ODC activity peaked. The postfertilization burst of biosynthetic activities slightly preceded a period of rapid ovary enlargement, presumably due to new cell division. During later stages of ovary development, DNA levels fell precipitously, while PA titers and decarboxylase activities decreased to preanthesis levels more slowly. In this period, growth producing a 300% increase in ovary fresh weight appears to be the result of cell enlargement.

Synchronous changes in PA titers and in the rates of PA biosynthesis, macromolecular synthesis, and growth in the tobacco ovary suggest that PAs may play a role in the regulation of postfertilization growth and development of this reproductive organ.

Changes in polyamine metabolism have been correlated with numerous growth and developmental processes in plants (24). The precise biological roles for these amines are not understood, but it seems clear that PA² titers influence many aspects of cellular metabolism, such as nucleic acid and protein synthesis (28), upon which growth and differentiation are dependent.

In mature, nongrowing plant tissues, both PA levels and the activities of enzymes regulating PA metabolism are low, while the situation in actively growing tissues is reversed (23). During active growth, PA titers appear largely to reflect the relative activities of ADC and ODC. These enzymes directly or indirectly, in the case of ADC, catalyze the formation of Put, the diamine

precursor to the PAs, Spd and Spm. Although ADC and ODC both represent constitutive pathways for Put biosynthesis, functionally, changes in the activity of one or the other appear to regulate PA biosynthesis in a given plant during particular stages of growth and development. It is not clear to what extent this phenomenon reflects tissue or subcellular compartmentation of these enzymes or PA metabolites.

In growing, nondividing tissue, such as expanded cereal leaves, ADC appears to be the primary enzyme regulating PA titers (26). This is also true of carrot tissue undergoing embryogenesis (10). ADC has also been extensively investigated with regard to its induction by various types of stress, light, and several plant hormones in differentiated tissues (*cf.* 24)

In rapidly dividing cells and in many meristematic or reproductive tissues, particularly among solanaceous plants, PA biosynthesis tends to be correlated with changes in ODC activity (6, 7, 15). However, there have been comparatively few studies of the involvement of ODC in regulation of PA biosynthesis in growing tissues, relative to that of ADC.

Recently, Cohen *et al.* (6) have suggested that ODC and ADC regulate Put biosynthesis and growth during early and later stages of tomato ovary development, respectively. This conclusion was supported not only by observed changes in the relative activities of these enzymes, but also by the fact that specific inhibition of ODC during the postfertilization period resulted in a significant inhibition of growth, which was partially reversible by exogenous Put supplied to the tissue. Unfortunately, the authors provided no information on endogenous PA titers in control or inhibitor-treated tissues, or in ovary tissues at different developmental stages. Thus, a causal relationship between changes in PA levels and growth could not be supported.

We have recently been investigating various aspects of PA metabolism in tobacco, which has been extensively studied with regard to nicotine biosynthesis, since the *N*-methylpyrrolidine ring of this alkaloid is derived from Put (22). In addition, PAs in tobacco callus tissue usually occur almost entirely as hydroxycinnamic acid amide conjugates (21), and in suspension cell cultures of certain tobacco mutants, these conjugates represent the majority of phenolic constituents in the cell (2). While the physiological significance of these amide conjugates is not known, it has been suggested that they may play a role in the regulation of flowering (4). Their close association with the reproductive organs of most plants studied to date (19) and reports that several tobacco mutants exhibiting altered PA metabolisms also exhibit abnormal floral and vegetative morphologies (17) further support the possible involvement of PAs in the regulation of growth and developmental processes in this plant.

These observations, together with the previously mentioned studies implicating PAs in the regulation of tomato ovary development, suggested to us that similar investigations of ovary

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² Abbreviations: PA, polyamine; Put, putrescine; Spd, spermidine; Spm, spermine; PLP, pyridoxal-5'-phosphate; ADC, arginine decarboxylase; ODC, ornithine decarboxylase; AS, ammonium sulfate; PCA, perchloric acid; DAPI, 4',6-diamidino-2 phenylindole · 2HCl.

maturation in the tobacco flower might lead to a better understanding of the relationship between PA metabolism and the developmental biology of this reproductive organ. In this study, we have investigated changes in the titers of various classes of PAs (*i.e.*, free and conjugated), as well as the activities of two PA biosynthetic enzymes, ADC and ODC, in relation to pre- and postfertilization changes in macromolecular synthesis and growth in the tobacco ovary. In a companion study (R. D. Slocum, A. W. Galston, unpublished data), we have further examined the *in vivo* effects of several inhibitors of PA metabolism on endogenous PA levels and decarboxylase activities and their consequences for growth in this organ.

MATERIALS AND METHODS

Growth of Plants. Cloned tobacco plants (*Nicotiana tabacum* L. cv 'Wisconsin' 38) were regenerated from callus tissue by Dr. N. S. Shekhawat, using standard tissue culture procedures. Plants were grown in plastic pots containing washed vermiculite, subirrigated twice daily with a 1.2 g/L solution of 'Hyponex' (Hydro-

ponics Chemicals Co, Copley, OH), and maintained in controlled environment chambers under a 16L/8D photoperiod at 24°C (9:1 energy mixture of fluorescent and incandescent light at a fluence rate of 17.6 W m⁻²). Under these conditions, the cultivar flowered continuously.

Flower Development. Flower buds tagged at the d 0 stage (Fig. 1) were measured daily, flower and ovary development for numerous flowers of the same plant being recorded over a period of several weeks. Data were pooled for several plants and a developmental series was defined operationally on the basis of ovary fresh weight, flower and ovary (or capsule) morphology. Ovaries were dissected away from other flower parts and most of the pedicel tissue was trimmed away. Dissected ovaries were quickly weighed then prepared for extraction, as described below.

Enzyme Extraction. All enzyme extraction procedures were carried out at 4°C unless otherwise indicated. Ovary tissue was ground with a chilled mortar and pestle at 200 mg fresh weight/ml of either pH 7.5 (4°C), 100 mM Tris·HCl or pH 7.5, 100 mM K-phosphate extraction buffers containing 10 mM DTT, 20 mM

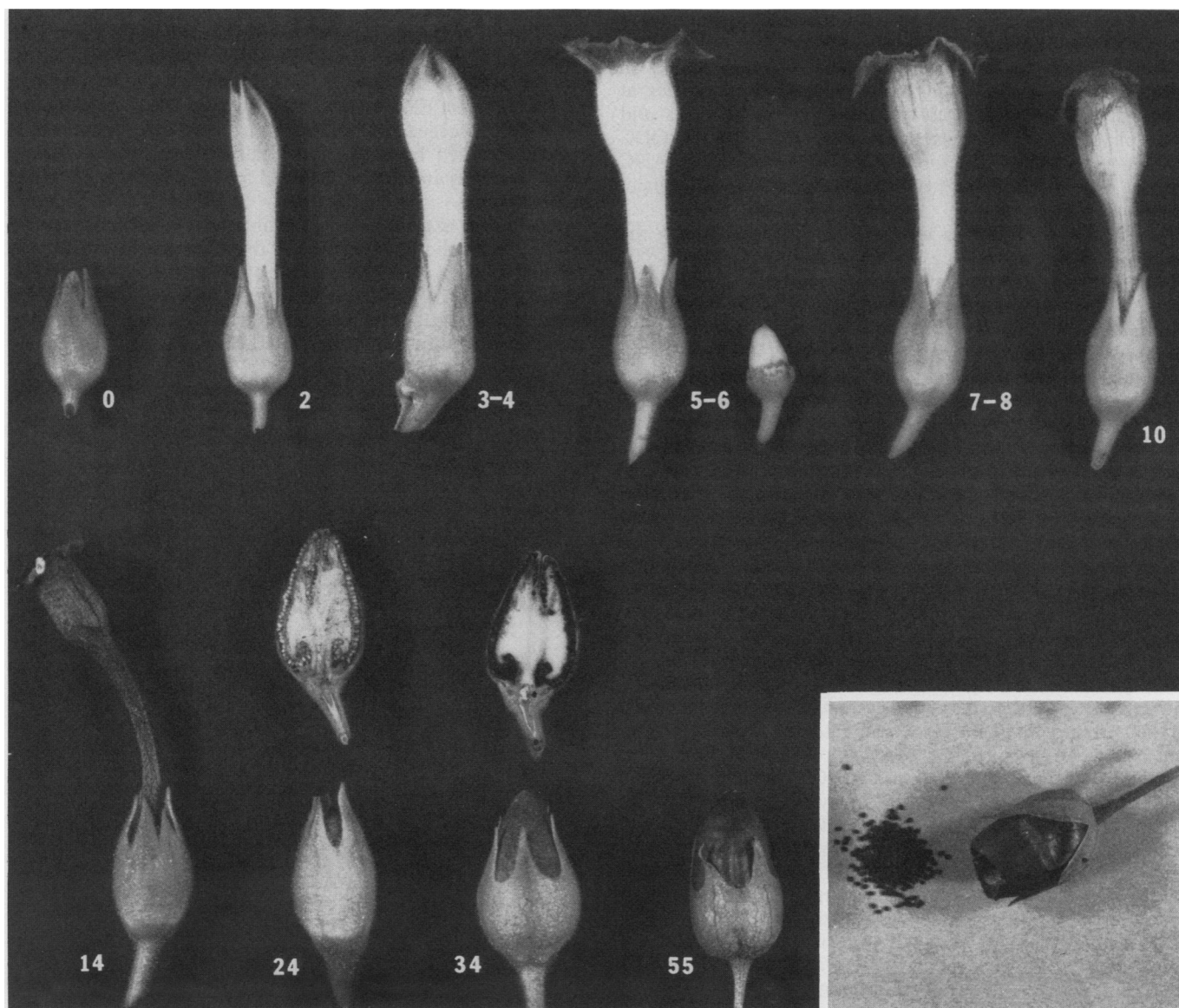


FIG. 1. Illustration of tobacco flower developmental stages operationally defined on the basis of ovary fresh weight and floral morphology. Anthesis begins at d 3 to 4 stage. The d 5 to 6 ovary is shown to right of flower at same stage, at which fertilization occurs. Longitudinally bisected d 34 and d 40 ovaries, showing seed maturation, are positioned above d 24 and d 34 ovaries. Inset: d 55 capsule and liberated seed.

Na-ascorbate, 5 mM Na₂EDTA, and 1 mM pyridoxal phosphate (PLP) (Note: the final pH of Tris-buffered solutions is for the temperature indicated in parentheses). Insoluble PVP (Polyclar AT, GAF Corp, New York, NY), purified (16) and pre-equilibrated in the appropriate buffer, was quickly added to the crude extract at 0.5 g wet weight/g tissue fresh weight to adsorb phenolics. The extract (+ Polyclar AT) was centrifuged for 10 min at 12,000g, then the supernatant was further disrupted by 5-min sonication (Branson S-75 probe sonicator tuned to resonance at power setting 6; Branson Ultrasonic Corp., Stamford, CT) in a salt/ice bath. This method of homogenization was found to be preferable to the usual Dounce homogenization procedure during which tissue extracts are aerated, facilitating the oxidation of phenolics to chemically reactive quinones. Sonicated extracts were fractionated in 60% saturated (v/v) (NH₄)₂SO₄ (AS, enzyme grade; Schwarz/Mann) for 30 min with gentle stirring. The AS pellet was collected, after a 15 min, 27,000g centrifugation, and resuspended in half the original volume of pH 8.0 (37°C), 100 mM Tris or 100 mM K-phosphate dialysis buffers containing 1 mM DTT, 0.1 mM EDTA, and 50 μM PLP. This fraction was dialyzed against two changes (250 ml each) of the same buffer for 18 h in the dark. The dialyzed extracts were used directly in enzyme assays.

Ornithine and Arginine Decarboxylase Assays. Changes in ODC and ADC activities in dialyzed extracts of ovaries at different developmental stages were monitored in both Tris- and K-phosphate-buffered assay systems, with similar results. ODC activities were slightly higher in Tris-buffered extracts, but ADC activities were as much as 2 to 3.5 times higher in K-phosphate-buffered extracts.

ODC and ADC activities were estimated by measuring the rates of decarboxylation (*i.e.* ¹⁴CO₂ evolution) of DL-[1-¹⁴C] ornithine (Amersham; 54 mCi/mmol) and L-[U-¹⁴C] arginine (ICN; 270 mCi/mmol), respectively. The assay mixture consisted of 180 μl extract in pH 8.0, 100 mM K-phosphate dialysis buffer and 20 μl of aqueous substrate stock solution (10 μCi/ml [¹⁴C] ornithine in 50 mM unlabeled L-ornithine, for ODC; 20 μCi [1-¹⁴C] arginine in 10 mM unlabeled L-arginine, for ADC). In both assay mixtures, the ratio of labeled to unlabeled substrate was identical and enzyme activity was measured at saturating levels of substrate. The rate of decarboxylation was linear for at least 60 min and was directly proportional to the amount of enzyme added. Three replicates of each sample were run for each experiment.

Aliquots of extracts were added to assay tubes containing substrate in a salt/ice bath. The reaction was initiated by incubation of the assay mixture (200 μl) in a 37°C water bath and stopped, after 45 min, by the addition of 200 μl of cold 10% PCA. Assay tubes were then incubated at 37°C for an additional 45-min period to allow equilibration of ¹⁴CO₂ between the KOH-impregnated collection disc, the gas phase and the acidified reaction mixture in the tube. Basal rates of decarboxylation (controls) were estimated by addition of PCA at time zero, followed by 45 min equilibration at 37°C. After equilibration, KOH discs were removed from the assay tubes, allowed to air dry, then added to 4 ml scintillation cocktail (Ready-Solv MP, Beckman) in glass vials. Radioactivity on the discs was quantitated using a Beckman LS-7000 liquid scintillation counter. Pipetting accuracy was monitored by counting 20-μl aliquots of substrate stock solutions applied directly to the discs.

ODC and ADC pH Optima. Day 10 ovary tissue was extracted as described above, but the AS fraction was dialyzed against pH 7.5 (37°C), 10 mM Tris buffer containing 1 mM DTT, 0.1 mM EDTA, and 50 μM PLP for 18 h in the dark. ODC activity in this fraction was assayed as a function of pH in the following manner. Ninety μl of the dialyzed extract were added to 90 μl of a 200 mM Tris buffer, otherwise identical to the dialysis buffer,

adjusted to 0.2 unit intervals between pH 6.2 and 9.0 (37°C). Twenty μl of [¹⁴C]ornithine substrate solution was combined with the extract (180 μl) and decarboxylase activity was measured as described previously. Because the buffering capacity of Tris (pK_a = 8.1, 25°C) is poor below neutral pH, ODC activity was also determined in an identical assay using K-phosphate (pK_a = 7.1, 25°C) between pH 6.2 and 8.0. ODC activities in this buffer were slightly depressed, particularly between pH 7.6 and 8.0, but the activity curve was similar (data not shown). The precise pH optimum for ADC activity was not determined. However, ADC activities in K-phosphate-buffered pH 7.6, 8.0, and 8.4 extracts indicate an optimum close to pH 8.0 for this enzyme, as well.

DNA Quantitation. DNA contents of tobacco tissues were quantitated using the procedure of Baer *et al.* (1). Tissues were homogenized at 100 mg fresh weight/5 ml extraction buffer (pH 7.0 [20°C], 10 mM Tris, 10 mM EDTA, 2.0 M NaCl), allowed to stand on ice for 1 h, then centrifuged for 20 min at 27,000g. The supernatant was vortexed at high speed for 1 min with 1.5 volumes of chloroform to remove Chl and other pigments and to aid in the precipitation of protein dissociated from the nucleic acids at high ionic strength. The DNA content of the upper aqueous phase was assayed by monitoring changes in fluorescence (λ_{ex} = 360 nm, λ_{em} = 450 nm) of DAPI (Sigma), which binds specifically to A-T base pairs of DNA. Fluorescence intensity changes in 3 ml assay buffer (pH 7.0, 10 mM Tris, 10 mM EDTA, 100 mM NaCl) containing 100 ng/ml DAPI were recorded after serial additions of 10 μl aliquots of the extract, followed by a 20 μg/ml calf thymus DNA solution, which served as an internal standard for fluorescence quenching by the sample.

Chloroform extraction of the homogenates did not remove oxidized phenolic constituents of the tobacco tissues, as was evidenced by the clear brown color of the aqueous phase. Addition of Polyclar AT (pre-equilibrated in assay buffer; 0.5 g wet weight/g fresh weight tissues) to the homogenate prior to chloroform extraction resulted in a marked clarification of the su-

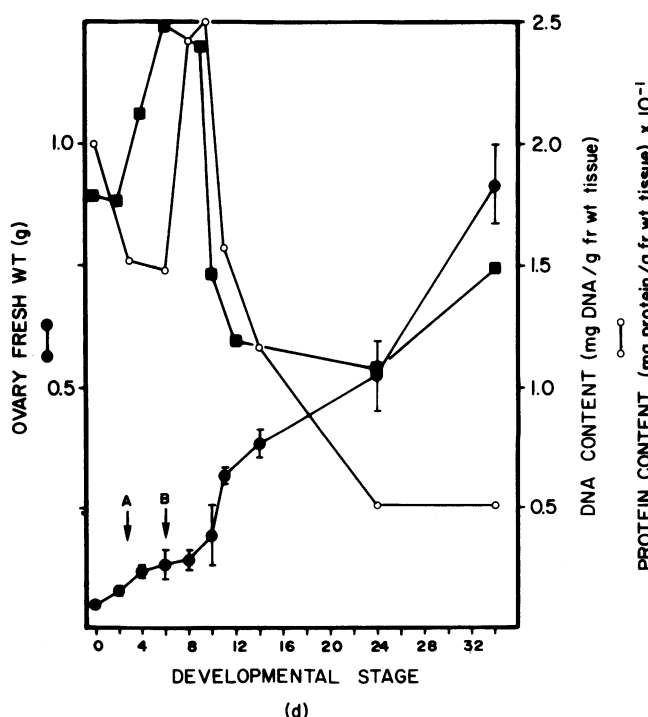


FIG. 2. Changes in ovary fresh weight ($\bar{x} \pm SD$), protein and DNA contents at different stages of tobacco flower development. Approximate times for anthesis (arrow A) and fertilization (arrow B) are indicated.

Table I. Polyamine Titrers in d 9 Tobacco Ovary Tissue

Polyamines were extracted from ovary tissue in 10% PCA. Polyamine titers were quantitated in both the PCA-soluble (27,000g supernatant) and PCA-insoluble (pellet) fractions. Unconjugated ('free') PAs in the supernatant fraction were dansylated directly; PA conjugates (bound forms) in the supernatant and pellet fractions were hydrolyzed to release the free PAs prior to dansylation and spectrophotofluorimetric quantitation. These PA fractions are designated as S (supernatant, nonhydrolyzed), SH (supernatant, hydrolyzed), and PH (pellet, hydrolyzed). Percentage of the total PA pool represented by a given fraction is indicated in parentheses.

Fraction	Polyamine Titer		
	Put	Spd	Spm
	<i>nmol/g fresh wt tissue</i>		
S	183 (3.6)	226 (0.8)	51 (8.0)
SH	4766 (94.1)	27,690 (98.8)	562 (88.4)
PH	117 (2.3)	98 (0.4)	23 (3.7)

pernatant. However, changes in DAPI fluorescence in phenol-containing versus clarified extracts were minimal (<5%) and routine assays were performed on nonclarified extracts.

Protein Determination. Protein contents of tissues were estimated using the protein-dye binding assay of Bradford (3). Bovine γ -globulin (Cohn's Fraction II; Sigma) was used as the

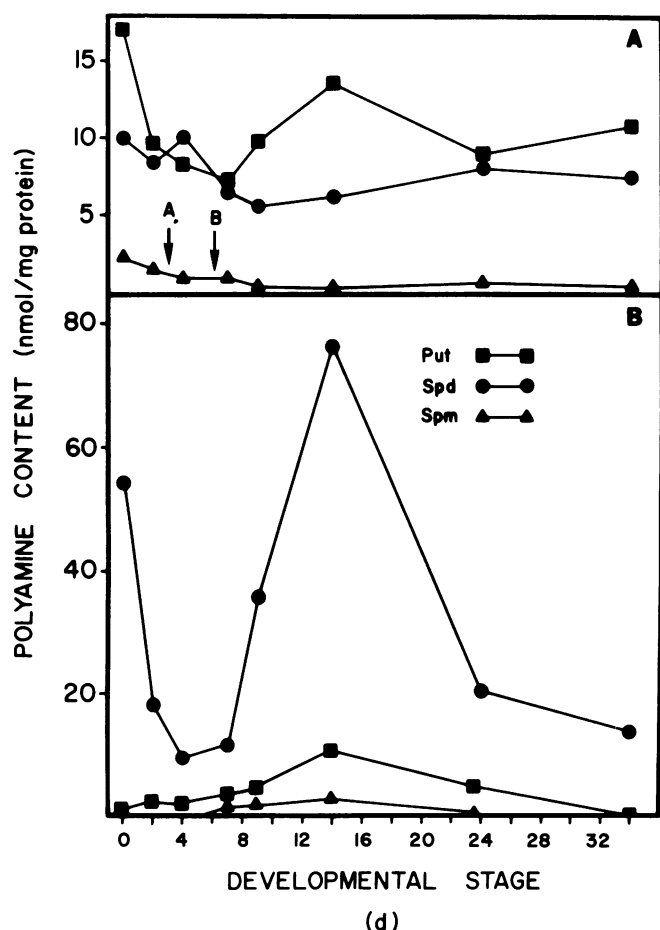


FIG. 3. Titrers of free (A) and PCA-insoluble conjugate (B) PAs in tobacco ovary tissue during flower development. Anthesis (arrow A) and fertilization (arrow B) are indicated. PA titers shown here and in Figure 4 are from a single experiment, but are representative of data obtained in at least three separate determinations.

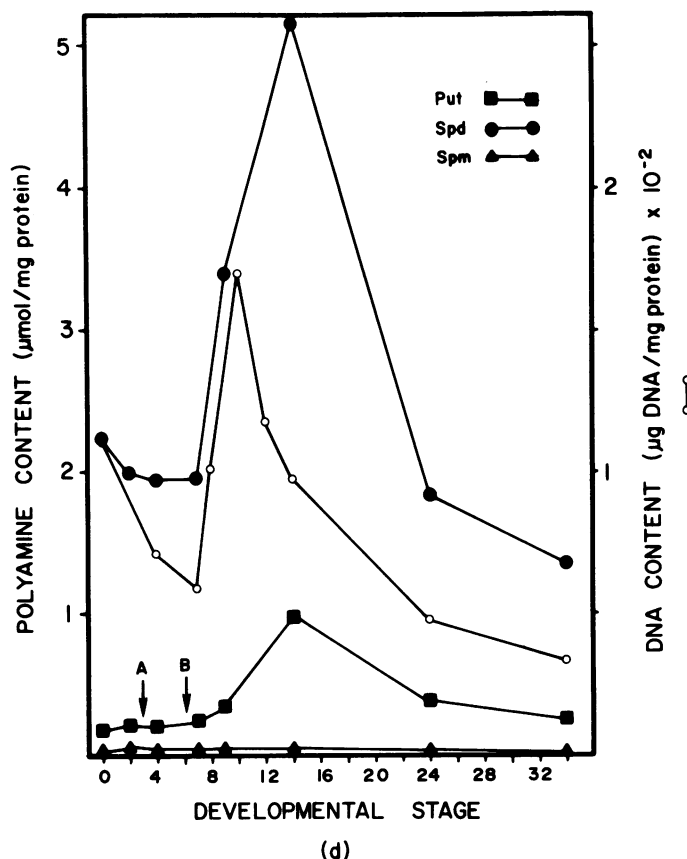


FIG. 4. DNA content and titers of PCA-soluble amide conjugates of PAs in tobacco ovary tissues during successive stages of flower maturation.

protein standard.

Extraction of Polyamines. Ovary tissues were homogenized at 100 mg fresh weight/ml cold 10% PCA on ice. After 30 min, the extracts were centrifuged for 10 min at 27,000g. The supernatant fraction was set aside; the pellet was washed in two 5-ml volumes of PCA, then resuspended in the original volume of PCA by vortexing at high speed for 1 min. Three replicates (200 μ l) each of this pellet suspension and of the original supernatant were added to glass ampoules together with 200 μ l of 12 N HCl. The samples were then hydrolyzed for 18 h at 110°C in flame-sealed ampoules. Following filtration through glass wool to remove charred debris, the hydrolysates were dried under a stream of air at 80°C, then resuspended in 200 μ l PCA.

Polyamine Analysis. Replicates (200 μ l) of both the nonhydro-

Table II. Polyamine Titrers in Unripened Tobacco Seed (d 34 Stage Ovary) and Fully Ripened Seed (d 55 Capsule)

Polyamine fractions are as described in Table I.

Fraction	Polyamine Titer		
	Put	Spd	Spm
<i>nmol/g fresh wt tissue</i>			
d 34 seed			
S	62.4	308	97.5
SH	905	585	133
PH	78.0	74.1	42.9
d 55 seed			
S	35.1	41.0	9.8
SH	62.4	39.0	9.8
PH	95.6	89.7	19.5

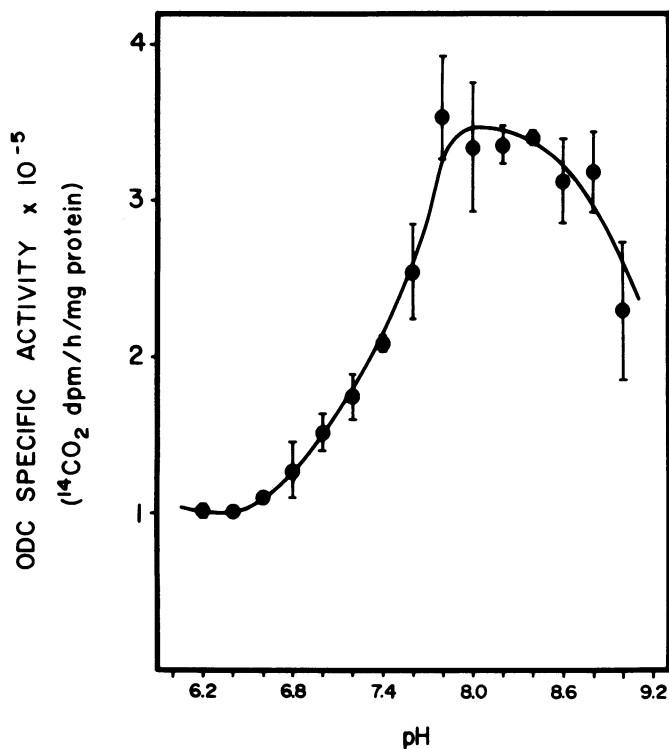


FIG. 5. ODC specific activity in d 10 tobacco ovary tissue extract as a function of pH ($\bar{x} \pm \text{SD}$).

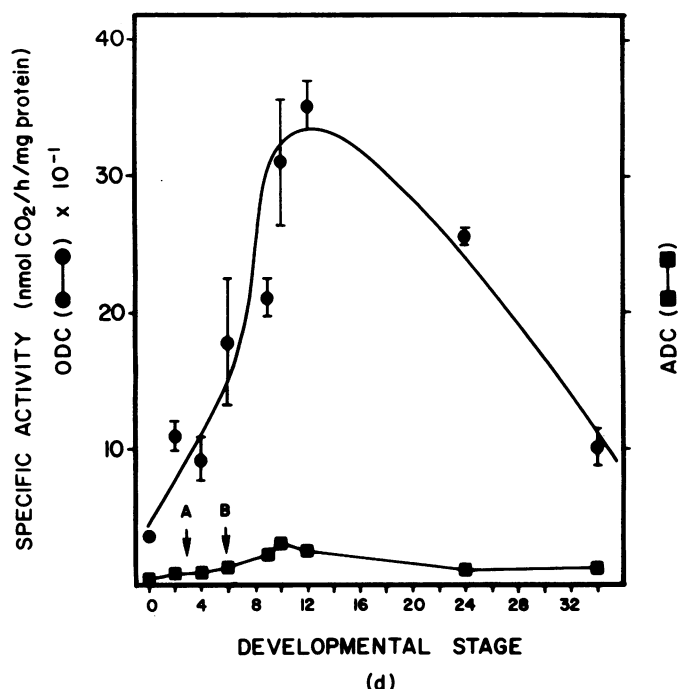


FIG. 6. Changes in ODC and ADC activities during tobacco ovary development. Values represent means, with $\pm \text{SD}$ bars indicated for those points where not covered by symbol. Anthesis (arrow A) and fertilization (arrow B).

lyzed PCA supernatant, containing the unconjugated ('free') PAs, and the hydrolyzed supernatant and pellet fractions, containing PAs liberated from various types of conjugates, were dansylated and chromatographed according to the procedure of Flores (11). Polyamine standards (Spm, Spd, Put, cadaverine,

diaminopropane; 20 nmol each) were prepared in the same way. Dansyl-PAs in the extracts were separated on high-resolution silica gel TLC plates (Whatman LK6D) and identified by comparison of R_f values for unknowns and standards. Chromatograms were developed in a cyclohexane:ethyl acetate (5:4, v/v) solvent system. Dansyl-PA bands were visualized by UV fluorescence, collected from the TLC plate, eluted in 2 ml ethyl acetate and quantitated by their relative fluorescence intensities ($\lambda_{ex} = 350 \text{ nm}$, $\lambda_{em} = 495 \text{ nm}$), as compared with standards, using an Aminco-Bowman spectrophotofluorimeter. Previous experiments, in which ^{14}C -labeled PAs were added directly to tissue extracts of several species of plants and carried through dansylation and chromatographic separation procedures (11), indicate good, but somewhat variable recovery for Spm ($75.6 \pm 5.4\%$), Spd ($8.30 \pm 3.9\%$), and Put ($95.5 \pm 3.1\%$). Since the recoveries for standards do not differ significantly in a given experiment, however, quantitation of PAs by this method is quite reproducible.

RESULTS

A chronological series of tobacco flower and ovary developmental stages examined in this study is illustrated in Figure 1. Various stages of ovary development were identified both on the basis of floral morphology and ovary fresh weight. This was necessary because during certain periods of development, ovary fresh weights alone were indistinguishable, although floral morphology clearly identified the stage (d 3–9), or vice versa (d 9 versus 10).

As the tobacco flower bud (d 0) opened and the corolla tube emerged and opened (anthesis, d 4), the ovary fresh weight nearly tripled, then remained more or less constant for several days (Fig. 2). Between d 0 and 6 stages, the protein content of the ovary tissues increased approximately 40%, while DNA content decreased rapidly ($\sim 25\%$) between d 0 and 3, then leveled off prior to fertilization (d 6). This suggests that ovary enlargement during this period was due primarily to cell enlargement, rather than cell division. Following fertilization, between d 6 and 10, a large ($\sim 80\%$) and rapid increase in DNA content of this tissue was recorded and protein levels remained elevated. Ovary weight, however, increased only marginally, indicating that this period was characterized by new cell division. These events slightly preceded a 2-d (d 10–11) period in which ovary weight again nearly doubled, presumably due to rapid enlargement of newly divided cells. DNA fell to prefertilization levels and protein content decreased by 50% on a fresh weight basis. The rate of ovary enlargement was then constant over the next 2 weeks (to d/24) and was associated with a reciprocal decrease in DNA content to approximately 20% of the postfertilization levels, while protein levels remained constant. Between d 24 and 34, where maturing seeds accounted for a fourth of the ovary fresh weight, a slight increase in the rate of ovary enlargement and protein content of this organ was seen. DNA content of this tissue remained constant, thus later stages of ovary maturation appear to be characterized principally by further cell enlargement.

Analyses of PA titers in ovary tissues at all stages of development, for example, at d 9 (Table I), indicated that $>90\%$ of the three major PAs assayed (Put, Spd, Spm) occurred as PCA-soluble conjugates (of which caffeoyl-Put and caffeoyl-Spd have been identified as the major constituents in this tissue; 9). In the case of Spd, $<1\%$ of this PA was present in the free (unconjugated) amine form, along with an even smaller pool of pelletable conjugates, which probably represent PAs covalently bound to cell wall constituents or other macromolecules, such as nucleic acids or proteins.

Changes in free Spd and Spm levels at different stages of ovary development were negligible, but Put titers nearly doubled over

several days following fertilization (Fig. 3A). In the pellet hydrolysates, Put and Spm showed little change, while Spd titers in this fraction (Fig. 3B) followed the much larger changes in soluble Spd conjugate levels (Fig. 4). The levels of soluble Put conjugates exhibited changes similar to those of free Put, although the tissue concentrations of the free diamine were considerably lower.

Changes in Spd conjugate titers in the tobacco ovary appeared to be well-correlated with the biphasic growth and postfertilization DNA synthesis in this organ (Figs. 2 and 4). Prior to fertilization (d 0–6), DNA levels declined, relative to Spd titers, then both showed coincident increases immediately following fertilization and preceding the period of rapid ovary enlargement. The DNA content of the tissue then gradually declined, but Spd and Put titers increased for several more days, reaching a maximum at d 14. At this stage, Spd titers, expressed on a fresh weight basis, were approximately 52 $\mu\text{mol/g}$ fresh weight tissue. Assuming an 80 to 90% water content, the average tissue concentration for this conjugate would be something in excess of 50 mM, with local concentrations possibly being much higher. After d 14, a marked decrease in Spd and Put conjugate titers corresponded to a period in which the rate of ovary enlargement leveled off.

From data shown in Table II, it is obvious that the developing seed does not constitute the major site of PA accumulation in the maturing tobacco ovary. In fact, PA titers continue to decline during seed ripening and, notably, the levels of soluble PA conjugates are very low.

Polyamine biosynthesis during ovary development was also investigated by assaying changes in the activities of ADC and ODC. Preliminary studies indicated that ODC activity in these tissues was much higher than ADC activity at all stages of development, and that maximal activity was reached near d 10. Therefore, extracts of ovaries at this stage were used in the initial characterization of these enzymes.

Tobacco ODC exhibited a rather broad activity optimum around pH 8 (Fig. 5), as was also reported for this enzyme from suspension cultures of the XD tobacco cell line (14). ADC activity was optimal near pH 8, as well (data not shown).

ADC and ODC activities throughout ovary development are shown in Figure 6. ADC activity appeared to increase more or less linearly through anthesis and fertilization, peaking 3 to 4 d later. The activity of this enzyme then declined to prefertilization levels over the next several days, then remained constant during further ovary development. ODC activity, however, nearly tripled in the days preceding fertilization then, following fertilization, increased dramatically, in synchrony with new PA and DNA synthesis preceding the period of rapid ovary enlargement. ODC activity, like that of ADC, peaked about 4 d after fertilization, but unlike ADC, remained elevated above prefertilization levels during the remaining period of ovary development, although decreasing linearly with time. At its peak, ODC activity was approximately 140 times higher than that of ADC.

DISCUSSION

Changes in PA metabolism during tobacco ovary development were generally similar to those reported for maturing tomato fruits (6). In both organs, decarboxylase activities peaked about 3 to 5 d after fertilization, with ODC predominating as the major activity. In tomato, ADC activity constituted approximately one-fourth of the total decarboxylase activity at this time, while in tobacco, it represented less than 1% of the total. This suggests that ODC functions as the primary enzyme regulating Put biosynthesis in tobacco ovary tissues. Unlike tomato, in which ODC activity rapidly declines following a postfertilization period of growth characterized by high rates of cell division and macromolecular synthesis, and where ADC assumes the primary role in PA biosynthesis, tobacco ODC levels are considerably higher than ADC levels at all stages of ovary development. This is true

even during the later period of growth, in which ovary enlargement appears to result from cell enlargement, rather than new cell division. This situation differs from several other studies of root and shoot growing axes in which high ODC activities have been shown to be associated with meristematic tissues, while in older, maturing tissues behind the meristem, ODC activity rapidly declines in relation to that of ADC (6, 9).

Polyamine titers during tobacco ovary maturation closely reflect the relative ODC activity in this tissue. Unexpected, however, was the observation that the major changes in PA levels occurred in the amide conjugate, rather than in free, PA pools. In the present study, we investigated changes not only in free PA titers, but also in two classes of PA conjugates, namely, those released from the PCA-soluble and insoluble fractions by acid hydrolysis of the amide bond. The conjugates in the insoluble (pellet) fraction have not been characterized extensively but, undoubtedly, a large portion of these are bound to uronic acid (13) or lignin (31) constituents of the cell wall, or other macromolecules. In tobacco, it is known that the majority of the acid-soluble amines are represented by hydroxycinnamic acid amide conjugates of PAs and two aromatic monamines, phenethylamine and tyramine (5). While we have not attempted to identify the conjugate moieties associated with PAs, it has been shown (4) that in tobacco ovary tissues, caffeoyl-Put and caffeoyl-Spd are the major PA conjugates.

The physiological significance of the amide conjugates of PAs is not known. These compounds do not normally occur in leaves or other vegetative tissues of tobacco, but they accumulate in shoot apices upon floral initiation, leading to a proposal that they may be involved in the physiology of flowering (4). This notion is strengthened by the finding that in the nonflowering mutant, RMB₇, these amides are not produced under conditions leading to floral initiation in the wild type plant (*cf.* 28). It has been observed, however, that these conjugates accumulate in shoot apices of induced plants maintained at 30°C, a temperature which inhibits flowering (5); therefore, their precise role in floral differentiation is unresolved. It is known that certain classes of PA conjugates are specifically associated with different floral tissues (4) and in one corn mutant, low levels of PA conjugates and the complete absence of feruloyl-Put appear to constitute a biochemical marker for male sterility in seeds (20). In addition, amide conjugates of PAs have been reported to be among the major phenolic compounds of most higher plant reproductive tissues examined to date (19), which would suggest that they do indeed play some functional role in reproductive physiology or development. They may confer virus resistance to the seed or other reproductive tissues (27), since it has been shown that infection of leaves with tobacco mosaic virus results in increased production of these amides and topical application of the conjugates to infected leaves reduces the number of tobacco mosaic virus lesions (18).

The functions of the conjugates in cellular PA metabolism similarly are poorly understood. Berlin (2) has found that in suspension cell cultures of a tobacco mutant (TX4) which overproduces the enzyme phenylalanine ammonia lyase, resulting in a greatly increased production of phenolics, there is a corresponding increase in PA biosynthetic activity. In these cells, caffeoyl- and feruloyl-Put conjugates may represent 85% of the phenolic constituents, accounting for approximately 10% of the cell dry weight. Radiolabeled arginine or ornithine are largely incorporated into Put conjugates in this mutant but, following specific inhibition of phenylalanine ammonia lyase, label accumulates in free Put pools. The latter situation is also noted for the wild-type TX1 cells, which do not have elevated levels of phenolics. These studies seem to indicate that PAs may play a role in regulating the levels of free phenolics in this plant, with PA conjugate levels reflecting the relative tissue concentration of

phenolics.

Another factor which may determine the levels of PA conjugates is the availability of free PAs themselves. For example, we have shown that in tobacco callus maintained on high levels of exogenous auxin, PAs occur primarily as conjugates, while in callus grown on low levels of auxin, a condition which promotes alkaloid biosynthesis, titers of PA conjugates decrease (29). It is well known that the *N*-methylpyrrolidine ring of nicotine is derived from Put (22); thus, it seems likely that PA conjugate formation in the callus was decreased as a result of Put utilization in pyrrolidine alkaloid biosynthesis. Flores and Filner (12) have also shown that in a variant tobacco XD cell line selected for growth on Put as the sole nitrogen source, [1,4-¹⁴C]Put is metabolized primarily to γ -aminobutyric acid, presumably generating NH₃ via amine oxidase catabolism of this diamine. In normal XD cells grown on NH₃, however, most of the [¹⁴C]Put is metabolized to various PA conjugates, again suggesting that Put availability may be the limiting factor in conjugate formation.

We and others have considered the possibility that PA conjugates may serve as a storage form of PAs which, upon enzymic hydrolysis, could supply the cell with additional PA reserves. Relatively little is known, however, about the formation, turnover, or even the subcellular compartmentation of the amide conjugates of PAs, although Smith *et al.* (27) have suggested that these amides are probably synthesized by enzymes similar to those involved in hordatine biosynthesis. Recently, we have investigated the effects of simultaneous inhibition of both ADC and ODC in excised tobacco flowers and have observed that free and conjugated Put pools were depleted by 20 and 40%, respectively, while exogenous Put restored Put titers in both pools to control levels (R. D. Slocum and A. W. Galston, unpublished data). Although such evidence is indirect, it suggests that PA conjugates may exhibit limited exchange with free PA pools, maintaining free PA titers at more or less constant levels.

One might further consider PA conjugates as a storage form of nitrogen in the tobacco plant. Nearly a third of the entire plant's supply of nitrogen is stored in ripened seed, in which there is a large accumulation of bound amino acids (30). It occurred to us that PAs might represent another pool of stored nitrogenous compounds in the seed. Analyses of seeds removed from the capsule at different stages of ripening, however, show that PA titers are relatively low, as compared with the ovary tissue as a whole, and they decrease with ripening. Unlike the ovary tissue, PA conjugate levels in seed are also reduced to approximately the levels of free PAs.

In conclusion, we have characterized PA metabolism in tobacco ovary tissues during developmental stages encompassing anthesis and postfertilization maturation. These studies have demonstrated that: (a) increases in PA titers closely reflect increase in ODC activity, rather than that of ADC—suggesting that ornithine decarboxylation is the rate-limiting step in PA biosynthesis; and (b) increases in PA titers parallel increased rates of macromolecular (DNA, protein) synthesis preceding a period of rapid ovary enlargement resulting from new cell division, while decreasing PA titers characterize later stages of growth involving cell expansion.

The latter observation supports several previous studies documenting increased rates of PA biosynthesis and cell division growth in tobacco (15) and other related plants (7). However, the fact that cell expansion growth, and even protein synthesis, continue unabated during ovary maturation, while PA biosynthesis and titers are declining, suggest that there is no simple correlation between PA titers, *per se*, and "growth." This may be the result of PA accumulation to levels which sustain growth for extended periods of time, perhaps due to some storage form of PAs, such as the amide conjugates in tobacco. This situation

might explain why there have been so few reports in which significant growth inhibition has been shown to result from markedly decreased PA titers accomplished through the use of specific inhibitors of PA biosynthesis *in vivo*. Indeed, it has been reported that in log phase cultures of *Chlorella* grown in the presence of difluoromethylornithine, a significant increase in generation time is not observed until the second cell cycle, suggesting that endogenous PA titers are sufficient to support cell division and related biosynthetic activities for a considerable time (8).

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