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Biosynthesis and regulation of polyamines in higher plants

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1. Introduction

The polyamines (PAs), spermidine (SPD) and spermine (SPN) and their diamine precursor, putrescine (PUT) represent a set of evolutionarily highly conserved small molecular weight organic polycations which play vital roles as modulators of a plethora of biological processes from enzyme activation and maintenance of ionic balance, through regulation of growth and development, to mediation of hormone action and progress of cell division cycle. Because of this functional versatility, research on PAs represents one of the most vigorously pursued areas of modern biology. While most of the currently available information on these important classes of bioregulators is derived from microbial and animal systems, interest in their possible participation in various facets of plant biochemistry and physiology is of relatively recent origin. The early pioneering investigations of Smith and others [34, 48, 62, 54, 63] on K^+-deficient barley leaves, set the stage for the elucidation of the general metabolic sequences involved in the biogenesis of these amines in plants and pinpointed the important roles PAs play in maintenance of intracellular pH and ionic balance. However, with the recognition that plant systems also offer tremendous potential for unravelling the riddles concerned with the biological functions of PAs, new vistas have been opened in plant PA research. As a consequence, the general biosynthetic and catabolic routes [59] as well as a myriad of functions PAs perform in the plant systems under various conditions have been identified and extensively investigated [2, 6, 18]. Thus, there is now considerable evidence to support the intimate involvement of PAs in plant growth and development derived from observations correlating alterations in PA titers of metabolism to such diverse processes as normal and abnormal growth [6], pollination [10], sprouting of dormant buds [24], embryogenesis [15, 33], phytochrome-induced photomorphogenesis [19], hormone-induced changes [71] and normal or stress-induced senescence [22]. This review surveys briefly the current status of PA biosynthesis and regulation in higher plants with emphasis on our own contributions to these aspects.

2. Choice of the model systems

Our investigations on plant PA biosynthesis and regulation were initiated with *Lathyrus sativus* (Family, Leguminosae) and later extended to *Cucumis sativus* (Family, Cucurbitaceae) as model systems of higher plants. The choice of *L. sativus* was promoted by our earlier observation [43] that the seeds of this legume are a rich source of L-homoarginine and that neither the metabolic fate nor the physiological significance of this nitrogen-rich unusual guanido amino acid during germination and seedling growth was known at that time. It was attractive to visualize that besides serving as a rich nitrogen source, this unusual free amino acid could also contribute vital metabolites such as di- and polyamines during seed germination/plantlet development. At this juncture, the earlier clear-cut demonstration [54] that in K^+-deficient barley leaves, arginine (ARG) rather than ornithine (ORN) is the major precursor of PUT and hence of PAs provided an impetus to explore the universality of the operation of the postulated pathway of PA synthesis in higher plants and to investigate the relationship between amine elaboration and normal plant growth. From this point of view, the developing *L. sativus* embryo was considered an attractive model system which incidentally provided an opportunity to compare the metabolism of ARG and homoarginine in terms of biogenesis of di- and polyamines. For short-term studies, to elucidate the regulatory aspects of PA metabolism and enzymic basis thereof, particularly to examine amine-hormone-growth inter-relationships, we preferred the *Cucumis sativus* (cucumber) cotyledons in organ culture for the following reasons: this plant tissue can be maintained in organ culture in a viable state for a period of 5–6 days in the absence of any complex medium; in the presence of light it grows and differentiates into a succulent leaf-like structure, simulating the situation during normal intact plant growth. Furthermore, it responds dramatically to exogenous hormones like cytokinins, K^+, amino acids and amines which, when included in the culture buffer, are readily taken up.

2.1 Biosynthesis of diamines

As stated earlier, the first indication that higher plants, unlike many microorganisms and animals, prefer ARG as the major precursor of PUT and hence of PAs, came from K^+-deficient barley leaves [54]. It was shown that ARG is decarboxylated to agmatine (AGM) by arginine decarboxylase (ADC); AGM is then converted to PUT via *N*-carbamylputrescine (NCP)(Figure 1). Later studies, however, have shown that PUT can also be derived through alternative minor pathways in some plant systems. Under certain special circumstances, the diamine could also arise either via direct decarboxylation of ornithine [64] or by decarboxylation of citrulline via NCP [12, 30]. Using *L. sativus* seedlings [44] we have been able to demonstrate clearly that ARG rather than ORN is the primary precursor of PUT and hence of

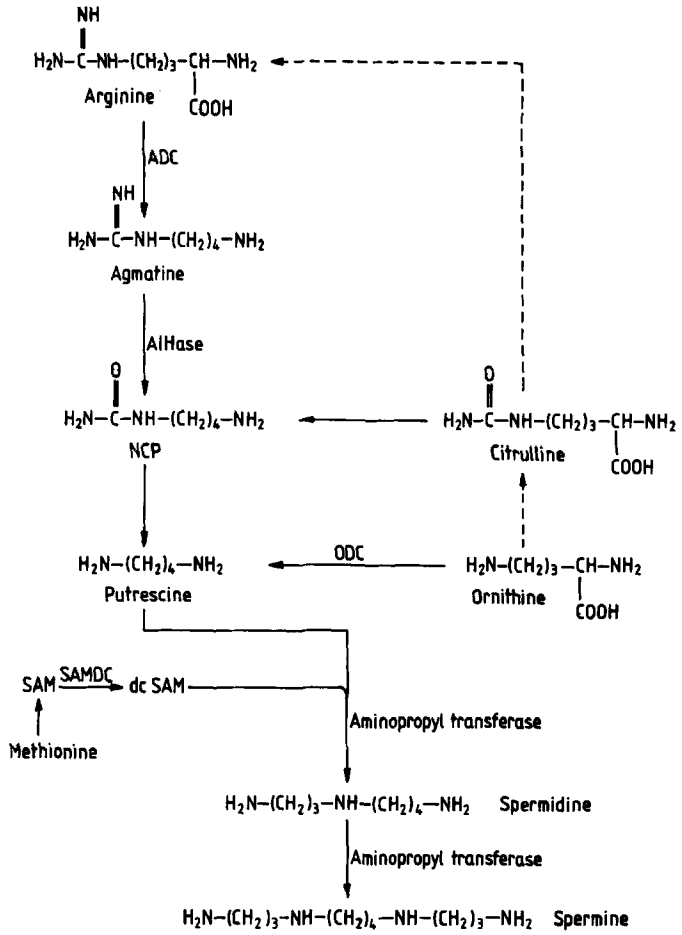

Figure 1. Biosynthesis of polyamines in plants.

PA. Based on isotope infiltration experiments, the postulated sequence of metabolic reactions involved in ARG → PUT sequence in K^+-deficient barley [56] could be largely confirmed in *L. sativus* seedlings during normal growth. In addition, a parallel set of similar reactions involving L-homoarginine as the starting point and giving rise to cadaverine could also be demonstrated (Figure 2). However, it was noteworthy that lysine rather than homoarginine was more efficient as the precursor of the latter diamine. Clearly, the contribution of ORN towards PUT production in this plant system was of minor significance. Recently, however, a considerable body of evidence favours ORN serving as PUT precursor through participation of ODC in some specialized tissues of higher plants under certain conditions of growth and development; e.g. crown gall tissues of *Scorzonera hispanica* [64], tobacco cells in culture [57], tomato ovaries [10], potato tubers [24], oats [16] and carrot

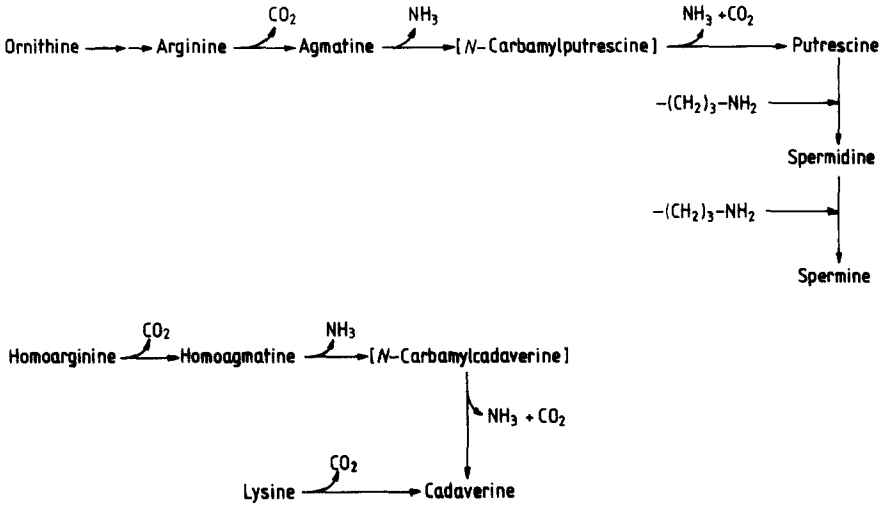

Figure 2. Biosynthetic pathways of amines in *Lathyrus sativus*.

cells on transfer to embryogenic medium [33]. The relative participation of these two amino acids viz., ARG and ORN, which are directly connected by a biosynthetic pathway via citrulline, apparently depends upon the type of the plant tissue, relative concentrations of the substrates and the competing demands for their utilization through other pathways and the physiological trigger for growth etc. For example, in mung bean roots, NaCl stress results in greater utilization of ARG than of ORN. In senescing French beans [^{14}C]-ORN is more efficiently utilized for PUT production than [^{14}C]-ARG [3]. The reasons as to why higher plants generally prefer ARG for putrescine synthesis may be related to such factors as (1) its relative abundance in storage proteins and involvement in long distance transport and (2) its position in the plant economy with high N/C ratio [3].

2.2 Correlation with growth during seedling development

In the past, there have been numerous reports in microbial and animal systems intimately linking enhanced production of PUT and PAs with normal and abnormal growth processes, hormone-induced rapid cellular proliferation etc, but information about similar correlations of PUT and PA levels with plant growth and development was relatively meagre. One of the first indications that plant systems may not be an exception to this general rule was provided by investigations on *Phaseolus vulgaris* [5] which showed that during growth, SPN and SPD decrease in cotyledons and simultaneously increase in shoots in association with similar alterations in RNA and protein levels. In *L. sativus* also [46], during seedling development, amine levels fluctuated in parallel with general concomitants of growth viz., enhanced RNA, DNA and protein contents, thus further supporting the view that the

amine elaboration is closely associated with growth phenomenon. In fact, by day 10 of development, AGM, PUT, SPD, SPN, cadaverine and homoagmatine increased greatly, representing 54, 76, 4, 12, 140 and 40 fold enhancement respectively over those on day 1. Significantly, SPD and SPN occurred initially at high levels in the cotyledons, despite them being primarily storage tissues.

Although underlying reasons were unclear, it was attractive to imagine that the large scale degradation of macromolecules in the cotyledons as a measure of nutrient mobilization to the growing embryo axis might cause localized imbalance in the intracellular pH and hence concomitant accumulation of the amines might represent a compensatory mechanism to offset this disturbance.

More significantly a close relationship was observed between the site and magnitude of cadaverine accumulation and the contents of nucleic acids and proteins in the rapidly proliferating embryo axis, which was suggestive of some important function which cadaverine might fulfil in the vital processes concerned with plant development. In this context it is noteworthy that this diamine can replace PUT, SPD and SPN in some bacterial and mammalian cells in supporting growth [1, 37] and other possible cellular functions ascribed to PAs. More recent data supporting such a close association between amine levels and plant development are provided by experiments with a number of other plant systems such as *Nicotiana glauca* [4], *Phaseolus vulgaris* [35], *Phaseolus mungo*, and *Pisum sativum* [75].

2.3 Biosynthesis of polyamines

It is now well established that in animals and microorganisms, PA biosynthesis requires primarily the participation of two precursors viz., PUT and *S*-methyladenosylhomocysteamine (decarboxylated *S*-adenosylmethionine, dSAM)[73]. As depicted in Figure 1, the dSAM donates an aminopropyl group to PUT in a reaction catalysed by SPD synthase while the condensation of another molecule of dSAM results in SPN formation (mediated by SPN synthase). Apparently, SPD and SPN are formed in plants from PUT by the same fundamental mechanism, although most of the data available hitherto in support of this contention are largely based on labelled precursor amino acid incorporation in PA. Recently, however, supporting evidence has been adduced for SPD synthase active in *L. sativus* [70] and *Vinca* [7], although SPN synthase has not hitherto been demonstrated unequivocally in higher plants. More recently, we have been able to provide evidence for the operation of an alternate pathway of SPD synthesis in *L. sativus* [65] on lines suggested for SPD synthesis in bacteria like *R. spheroides* and *M. denitrificans* [74]. According to the proposed scheme (Figure 3) the Schiff base generated by the condensation of aspartic-β-semialdehyde with PUT is reduced by a NADPH-dependent dehydrogenase; the carboxyspermidine thus produced is decarboxylated in a pyridoxal phosphate-dependent reaction

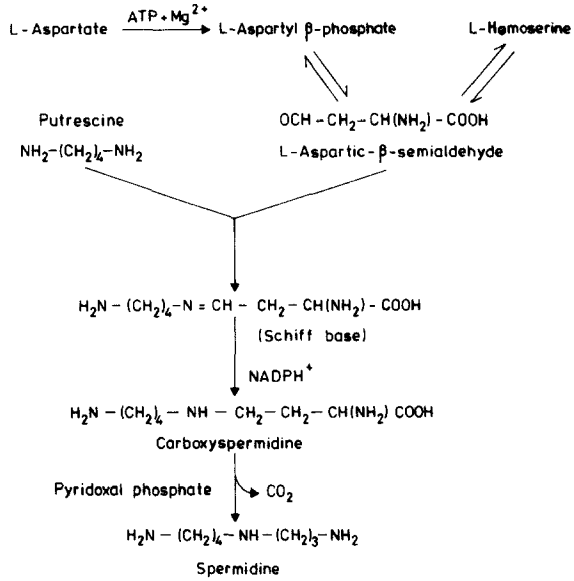

Figure 3. Alternate pathway of spermidine biosynthesis.

to yield SPD. When [U-^{14}C] aspartic acid was infiltrated into *L. sativus* embryo axes, about 72% of the radioactivity recovered in the total amine fraction was associated with SPD, while SPN accounted for only 7%. On the other hand, with [G-^{3}H]-methionine as the precursor 70% and 30% of the radioactivity was recovered in SPD and SPN fractions respectively. It was postulated that those plants endowed with a prokaryotic type of SAMDC lack the versatile built-in mechanism of PUT activation of SAMDC to drive their biosynthetic machinery towards PA synthesis to meet physiological demand as in animals, and hence have retained this additional mechanism of SPD synthesis during evolution to meet the enhanced demand of PA during growth. However, these investigations in no way claimed the universality of the operation of this alternate SPD biosynthetic pathway in higher plants. In fact, a recent preliminary investigation with *Cucumis sativus* seedlings reveals that such a pathway may not be operative in this plant to a discernible extent during normal growth (GL Prasad and PR Adiga, unpublished observations).

3. Enzymes of amine biosynthesis

Among the various enzymes involved in plant amine biogenesis, those responsible for PUT biogenesis have been investigated in greatest detail in terms of their molecular characteristics and regulatory features.

3.1 Arginine decarboxylase (ADC) (ARG → AGM)

As already stated, ADC is the first and rate-limiting step in the PA biosynthetic pathway originating from ARG. This enzyme has been partially purified from barley [54] and purified to homogeneity from *L. sativus* [46], oat seedlings [60], rice embryos [9], K^+-deficient leaves [17] and cucumber seedlings (GL Prasad and PR Adiga, in preparation). The levels of this enzyme in *L. sativus* have been shown to increase progressively with the age of the plant embryo in whole seedlings, cotyledons and in embryo axes indicating that the production of PUT and hence PAs are linked to growth. Interestingly, the specific activities of both ADC and ODC are highest in rapidly growing tissues such as root apices, hypocotyls etc, of *Phaseolus vulgaris* during development [35]. Such correlation of both PA titers and their biosynthetic enzymes with growth has been observed in several mammalian model systems [21].

That ADCs purified from different plant sources differ considerably from each other and with that from *L. sativus* in terms of molecular structure is now accepted. The purified *L. sativus* enzyme exists as homohexamer of subunits of M_r 36 k while the enzyme from oat seedlings occurs as two different and separable molecular entities of M_r 195 k and 118 k, significantly differing in their specific activity [60]. Similarly ADC from rice embryos [9] could be separated into two fractions of M_r 88 k and 174 k. The higher M_r species from both oat seedlings and rice embryos were relatively more active. While the exact inter-relationship between the two species of ADC in the oat seedlings or their relative contribution towards PUT elaboration under either normal or stress conditions is not known at this juncture, it is conceivable that these may be connected by a precursor-product relationship. However, it appears that the two enzyme species of rice embryos are a dimer and a monomer of the same basic unit. Interestingly, the enzyme isolated from K^+-deficient oat leaves [17] seems to differ significantly from that described by Smith [60]. This enzyme resembles its counterpart in *L. sativus* in that it migrates as a single protein species of M_r 39 k upon SDS-polyacrylamide gel electrophoresis (PAGE). These two preparations have comparable K_m values (30 μM) and their activity is stimulated by both pyridoxal phosphate and thiol reagents.

In contrast, the enzyme from *Cucumis sativus* seedlings exhibited an entirely different molecular organization (GL Prasad and PR Adiga, in preparation). Cucumber enzyme purified by a simple three-step procedure resolves into three different polypeptides of M_r 48 k, 44 k and 15 k on SDS-PAGE under reducing conditions. Although the exact M_r of the protein could not be obtained due to its propensity to aggregate, its native size could be gauged by its $S_{20°w}$ value (16 S), with a minimal M_r of 150 k. All the three polypeptides are apparently held together by disulphide bridges and they share many common tryptic peptides. It is quite intriguing to find that purified

ADC of cucumber possesses intrinsic ODC activity also, although the latter activity could not be detected in crude extracts. That the two decarboxylase activities are located on two distinct, yet interacting sites is suggested by their differential response to inorganic phosphate, AGM, PUT and α-difluoromethylornithine (DFMO). It is pertinent to mention that in mammalian systems lysine decarboxylase activity is mediated by ODC and responds identically to DFMO [40, 42]. A summary of the general properties of different plant ADCs is presented in Table 1.

3.1.2 In vivo regulation of ADC. In vivo levels of ADC are known to be regulated by a variety of plant growth effectors and stress conditions. Using cucumber cotyledons in organ culture, we could show that ADC activity and PUT titers are enhanced in concert on treatment of the plant tissues with cytokinins [71]. Among the various plant hormones administered to cotyledons, benzyladenine (BA) and its riboside are most effective in this regard, peak values (4-fold stimulation) being recorded at around 60 h of culture with BA. Although the enzyme activity is elevated by treatment with acid, cotyledon growth is clearly inhibited. Similarly KCl treatment lowers ADC activity but promotes growth, whereas abscisic acid curtails both ADC activity and growth. The most significant aspect of these studies is the intriguing finding that K^+ overrides the effect of other modulators on amine metabolism. Since PUT levels are modulated in concert with ADC levels, these observations show that growth phenomena and fluctuation in PA levels need not necessarily run parallel in the same direction and that the two can be uncoupled. Similar conclusions may be drawn from studies with inhibitors in mammalian systems [49] and these run counter to the dogma regarding an obligatory positive relationship between cell growth and polyamine levels.

Recently, we have demonstrated that ADC activity in cucumber cotyledons during short-term culture can be modulated by various amines and amino acids connected with the PA biosynthetic pathway (GL Prasad and PR Adiga, submitted to J. Plant Growth Regulation). Among the various amines tested, AGM inhibits ADC activity maximally at 10 m*M* concentration and at 12 h of culture, while under these conditions PUT curtails ADC activity to 65% and NCP is ineffective. Thus in vivo modulation of plant ADC by amine intermediates of the biosynthetic pathway exhibits a close similarity to that of ODC in animal and microbial systems [8]. The most notable feature of this study has been the remarkable stimulation of ADC activity by homoarginine; the substrate analogue (also a competitive inhibitor of ADC in *L. sativus*) augmented the enzyme activity up to 300–400% of control value by 12 h of culture. This observation is reminiscent of the situation in mammalian systems where SAMDC activity assayed is significantly enhanced following in vivo treatment with its inhibitor viz., methylglyoxal *bis* (guanylhydrazone)(MGBG)[38]. In these studies, the involvement of macromolecular effectors in the free-state has been ruled out.

Table 1. Comparison of properties of ADCs from different plants

Property	Barley [54]	*L. sativus* [46]	Oat seedlings [60]	Rice embryos [9]	*Cucumis sativus*[a]
Purification stage	Partially purified	Homogeneous	Homogeneous	Homogeneous	Homogeneous
Number of catalytically active species	ND[b]	One	Two (A more active than B)	Two (A more active than B)	One
Subunits	ND	6 (36 k) native M_r 220 k	A 195 k B 118 k	A 174 k B 88 k	Three polypeptides of M_r 48 k, 44 k and 15 k as detected by SDS-PAGE
Optimal pH	6.5–9.0	8.5	7–7.5	8.0	8.2
Optimal temperature	37 °C	45 °C	32 °C	45 °C	40 °C
K_m (mM)	0.75	1.75	0.03	0.28	0.5
Metal requirements	ND	Nil	Nil	Nil	Nil
Stimulated by thiol reagents	ND	Yes	Yes	ND	Yes
pyridoxal phosphate	ND	Yes	Yes	ND	Yes
Amine inhibitors	ND	SPN, arcain, SPD, AGM, PUT	AGM and other amines	SPN, AGM, SPD, PUT	PUT, AGM

[a] GL Prasad and PR Adiga, in preparation.
[b] ND = not determined.

Interestingly, ADC activity is known to be elevated in embryonic cells of *Daucus carota* [33]. Recently, enhanced ADC and PA levels have been shown as essential concomitants for embryogenesis in wild carrot [15]. It has been claimed that gibberellic acid treatment promotes PA biosynthesis in light grown dwarf peas as a consequence of ADC stimulation [14]. Furthermore the enzyme activity seems to be elevated in crown-gall tissues of *Scorzonera hispanica* [64]. Phytochrome is also apparently involved in modulation of ADC activity in etiolated peas [13]. Recently, Goren et al. [19] were able to show that effects of phytochrome on ADC activity in etiolated pea are not a simple consequence of the altered growth rate and may be causally linked to growth.

Another remarkable feature of ADC is that its activity is greatly altered under various stress conditions (including K^+ feeding to cucumber cotyledons described above). As early as 1952, Richards and Coleman [48] demonstrated that K^+-deficiency causes accumulation of PUT. Later it was firmly established that K^+-deficiency results in a marked elevation of ADC in barley [54]. Recently, Flores et al. [17] have shown that K^+-deficiency results in dramatic enhancement (30-fold) of ADC accompanied by marked accumulation of PUT in oat seedlings. Several monovalent cations could partially replace K^+ in this system, both in terms of restoring growth and ADC activity.

Cereal leaves under osmotic stress (e.g. feeding of sorbitol) also exhibit elevated ADC (2- to 3-fold) and putrescine (30-fold) over control values [16]. It has also been observed that in oat leaves, the enhanced ADC and PUT levels could be curtailed by inclusion of difluoromethylarginine (DFMA). Different results were obtained with difluoromethylornithine (DFMO); interestingly this suicide inhibitor of DFMO potentiates the stress response of the tissue in terms of PUT elaboration and ADC levels. Similarly, as earlier shown with cucumber cotyledons [71], oat leaf segments and pea leaf discs [78] also synthesize large quantities of PUT in response to low pH conditions and exhibit increased ADC activity. Another important aspect of plant physiology with which PAs are believed to be intimately connected is senescence. It has now been well-documented that either PAs or their precursors effectively retard senescence; in fact, a suggestion has been made that changes in SPD levels would probably serve as the single best biochemical indicator of senescence. As logically expected from PA reversal of the degenerative phenomenon, ADC levels decline progressively in senescing oat leaves [23].

Althouth it is now well-recognized that ADC levels may be affected by many external factors/physiological conditions, the exact molecular mechanisms that operate intracellularly and which are responsible for the observed alterations may be multifarious and remain to be elucidated. Under conditions of K^+ feeding to cucumber cotyledons, the observed decrease in ADC appears to be ascribable to enhanced degradation of the enzyme (GL Prasad and PR Adiga, unpublished observations). Another factor which should be

carefully considered is the role of endogenous PAs and their pool sizes which may determine the direction of alteration in enzyme levels by feed-back and/or repression mechanisms.

3.2 Ornithine decarboxylase (ODC)

Until recently, the participation of ODC, the enzyme responsible for PUT biogenesis directly from ornithine in plants, was considered to be of minor significance. However, recent studies have shown that this is not always the case, and that the ODC pathway could be important as well as the ADC route. ODC has been detected in many plant systems including *Nicotiana*, normal and crown gall tissues of *Scorzonera hispanica* [64], oats [16], tomato ovary cells in culture [10], peas [14, 35], potatoes [24] and barley [28]. Kyriakidis et al. [28] have purified ODC from barley seedlings and found that 90% of the enzyme activity is localized in the nuclear fractions. The enzyme activities from both cytosolic and nuclear fractions seem to be superinducible by actinomycin-D [36]. The nuclear localization of this enzyme may be compatible with its presumed role as a labile subunit of RNA polymerase [50, 51]. As stated earlier, ODC activity is held responsible for PUT biogenesis and cell growth in tomato ovary and XD-cells of tobacco [10, 20] and during profuse sprouting of potato tubers [24]. During germination of barley seeds, ODC activity enhanced by plant hormones is curtailed by PUT and SPD; an antizyme acting on lines suggested for the modulation of the mammalian ODC is considered responsible for this inhibition [27]. Although ODC activity could be demonstrated in the purified ADC preparations from cucumber seedlings, the former activity could not be detected in crude extracts or partially purified preparations. Since in mammalian systems it has been demonstrated that ODC is regulated by a number of factors, including both small M_r and macromolecular effectors, it is conceivable that the 'cryptic' nature of this enzyme in a less purified form is revealed after removal of 'an inhibitor' during purification (GL Prasad and PR Adiga, unpublished observations). Both the nature of this putative inhibitor and the factors governing its association-dissociation with the ODC active site at present remain unexplored. These observations clearly contrast with those made with the mung bean tissue crude extracts where both the decarboxylase activities with similar kinetic constants and pH optima could be assayed [3].

3.3 Enzymology of agmatine to putrescine conversion

According to the early hypotheses of Smith [54–56], AGM to PUT transformation in plants occurs in a two step conversion in which the AGM → NCP conversion is mediated by agmatine iminohydrolase (AIHase) and NCP is cleaved to PUT by the action of NCP amidohydrolase. Since in general it is tacitly assumed that ADC is the rate-limiting regulatory step in PUT biosynthesis, relatively less importance was attached to reactions distal to ADC.

AIHase activity has been detected in a number of plants [40] and partially purified from groundnut cotyledons [53] and corn [77]. Groundnut enzyme is apparently a dimer of identical subunits of M_r 43 k. NCP amidohydrolase has been detected in K^+-deficient barley leaves [55].

Contrary to the above presumed two-step hydrolytic scheme involving two distinct enzyme proteins, Srivenugopal and Adiga [68, 69] have provided clear evidence for the participation of a single multifunctional protein, viz., 'putrescine synthase' in the above conversion in *L. sativus* and advanced the concept of an agmatine cycle (Figure 4). The proposed scheme of reactions catalysed by putrescine synthase, besides explaining the non-accumulation of NCP in vivo satisfactorily, also envisages the conservation of energy inherent in the carbamyl group of NCP in the form of production of either ATP or citrulline. The various assayable component activities of the multifunctional enzyme are shown in Figure 4.

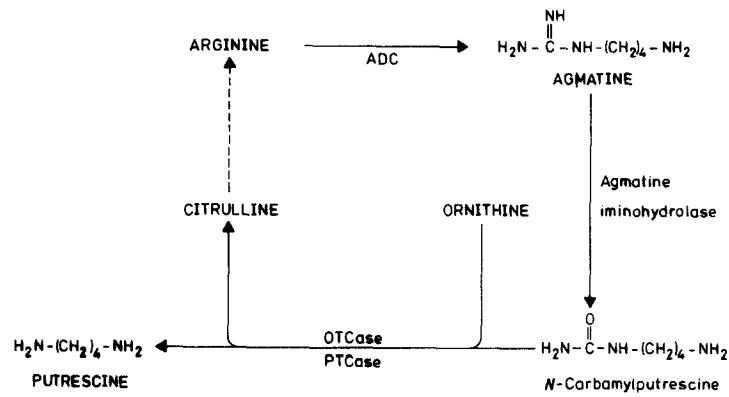

Figure 4. *Agmatine cycle.* The multifunctional enzyme, putrescine synthase of *L. sativus* plays a central role in the above scheme. The polycephalic enzyme, in addition to mediating the 3-reactions viz., AIHase, OTCase, PTCase also possesses a carbamate kinase (CKase) activity. The complete reactions catalysed by this enzyme are (i) AGM + ORN → PUT + Citrulline + NH_3 (coupled to OTCase) and (ii) AGM + ADP → PUT + ATP + NH_3 (linked to CKase). Our unpublished data (GL Prasad and PR Adiga) show that in *Cucumis sativus* also the agmatine cycle is operative and putrescine synthase exists. The cucumber enzyme harbors two more catalytic activities, viz., ADC and ODC.

Preliminary evidence has also been adduced for the operation, on similar lines, of the agmatine cycle in other higher plants. According to the proposed scheme, AGM is deiminated to NCP by the AIHase component of the enzyme, whereas NCP is cleaved to PUT by the associated PTCase activity of the multifunctional protein (not by NCP amidohydrolase). As the result of concerted actions of either of the coupling activities, viz., ornithine transcarbamylase (OTCase) or carbamate kinase (CKase) simultaneously either citrulline or ATP is produced in addition to the diamine. The essential tenets

of the agmatine cycle are the channelling of NCP generated in situ to the PTCase domain of the enzyme and coupling of PTCase activity to either OTCase or CKase in order to conserve the labile carbamyl phosphate moeity, which otherwise would be dissipated. PUT synthase from *L. sativus* is a single polypeptide chain comprising a basic unit of M_r 55 k.

Recently, we have attempted the purification of this enzyme from *Cucumis sativus* seedlings also (GL Prasad and PR Adiga, unpublished results). Furthermore all the component activities as well as the complete reactions demonstrated with *L. sativus* putrescine synthase increased progressively, yet concertedly during seedling development, reaching a peak value on day 8, and then declined. Significantly, the cucumber enzyme exhibited a more complex structural and functional organization than its counterpart in *L. sativus*. For example, the cucumber PUT synthase appears to undergo age-dependent proteolytic degradation generating fragments of polypeptides of M_r ranging from 66 k (primary translational product) through 48 k, 44 k to smaller polypeptides, as shown by resolution on SDS-PAGE. This specific progressive proteolytic modification is presumed to play an important role in altering the structural organization of the protein, as a consequence of which the directed flow of intermediates and channelling within the multifunctional protein are modified [76]. Although the exact reasons for this age-dependent proteolytic cleavage of the cucumber enzyme are unknown at present, it is conceivable that this also represents a mechanism by which the enzyme protein is catabolised. It has been postulated that after supporting the accumulation of optimal quantities of PUT and PAs for plant development, the enzyme protein is catabolized to provide energy and amino acids for re-utilization during anabolism of other proteins required for further growth. What was most unexpected was the intriguing finding that in cucumber, unlike in *L. sativus*, both ADC and ODC are also the component activities of PUT synthase i.e., in addition to the 4 enzyme activities encountered with the *L. sativus* enzyme. In other words, the PUT synthase of cucumber appears to be versatile enough to mediate PUT production from either ARG or ORN without intervention of any additional enzyme proteins. Another regulatory feature of this plant enzyme is that in crude extracts, a specific regulator of yet unknown nature seems to direct the flow of AGM to the rest of the reaction domains on the protein; however upon purification, this regulatory element is apparently lost; in other words, with ARG as the substrate, putrescine could be isolated as the sole amine end product of the complex reaction sequence. Although the selective advantage the plant derives from sequestering all the component enzyme activities of putrescine biosynthetic machinery in a single protein is unclear at present, the complex organizational features of the enzyme involved in the diamine production underscores the importance of this compound and hence PAs in plant development.

It is interesting that the putrescine synthase of cucumber is a phosphoprotein

which is phosphorylated at serine residue(s). In this context, it is pertinent to recall that ODC from *Physarum polycephalum* is also a phosphoprotein and, in its phosphorylated form, the enzyme may function as a gene regulator rather than a biosynthetic enzyme [25]. Since ODC is also a component activity of putrescine synthase of this shime mould, the possibility exists that the phosphorylation status of the enzyme plays a crucial role in the modulation of its activity. Although conclusive data on these lines are still lacking, preliminary experiments seem to suggest that phosphorylation of the enzyme might act as a trigger for enzyme catabolism; purification of the [^{32}P]-labelled protein from cucumber cotyledons cultured for 12 h and 72 h and analysis of the pattern of [^{32}P] associated with degradation products of the protein resolved by SDS-PAGE seem to provide suggestive evidence of the above proposition. However, these hypotheses need confirmation by other experimental approaches.

3.4 Biosynthesis of cadaverine: Involvement of a homoarginine-lysine decarboxylase

In *L. sativus* a single enzyme responsible for decarboxylation of both homo arginine and lysine has been characterized [47] and purified 110-fold from seedlings. Results of studies on competitive kinetics, metal ion requirements and pH optima for both activities indicate that both the amino acids are utilized by a single enzyme, viz., homoarginine-lysine decarboxylase. Although affinity chromatography on homoarginine-Sepharose and lysine-Sepharose could be employed for purification of this enzyme, further attempts to obtain a homogeneous preparation of the protein and extensive characterization are severely hampered by the extremely labile nature of its catalytic activities (GL Prasad and PR Adiga, unpublished observations). It is pertinent to mention that although both ARG and homoarginine undergo similar metabolic transformations to give rise to their respective diamines, different enzymes are involved in their metabolism. ADC and homoarginine-lysine decarboxylase are two different proteins; while ADC appears to be highly specific to ARG, in *L. sativus*, homoarginine decarboxylase possesses lysine decarboxylating activity also.

3.5 S-Adenosyl-L-methionine decarboxylase (SAMDC)

S-Adenosyl-L-methionine decarboxylase (SAMDC) is the second key enzyme in SPD and SPN elaboration. In mammalian systems SAMDC is known to play a pivotal role in PA metabolism. Surprisingly, not many detailed reports on plant SAMDC are available at present. In *L. sativus* seedlings, both a PUT-sensitive (artifactual) and insensitive (biosynthetic) enzyme activities have been described [70]. The biosynthetic enzyme has been purified to near homogeneity by chromatography on an organomercurial affinity column and its activity appears to be largely localized in rapidly growing shoots. This enzyme activity is inhibited by MGBG and could be clearly separated from

the SPD synthase activity. Thus, it would appear that in plants also propylamine transferases are distinct from SAMDC. The PUT-sensitive, artifactual decarboxylation of SAM could be traced to H_2O_2 generated by the action of diamine oxidase on the diamine and as expected, this activity could be completely inhibited by added catalase. Such artifactual decarboxylation has been demonstrated in crude extracts with other amino acids used as the substrates in the presence of the diamine with varying degrees of efficiency. Unlike the biosynthetic SAMDC, this PUT-stimulated activity is independent of Mg^{2+} and sensitive to catalase. More recently, SAMDC has also been purified from etiolated corn seedlings [72]. The corn enzyme seems to attain peak activity by 5 days of growth. It has a M_r of 25 k and is also inhibited by SAM.

In plants, all the three types of SAMDC's i.e., Mg^{2+}-stimulated (*L. sativus*, [70]), PUT-stimulated (*Vinca rosea*, [7] and Mg^{2+}- and PUT-insensitive (cabbage and carrot)[11], have been encountered. PUT activation of SAMDC has often been linked to the occurrence of SPN in the organisms. It has been proposed that PUT activation of SAMDC would ensure that sufficient amounts of dSAM will be available for synthesis of SPD, thus ensuring the organism of its required supply of the triamine at appropriate time [42].

Some plants which elaborate significant quantities of SPN (e.g. *L. sativus*) contain a PUT-insensitive rather than PUT-stimulated SAMDC. Such plants apparently make use of an alternate pathway starting from aspartic acid (Figure 3) for preferential synthesis of SPD as demonstrated in the case of *L. sativus* [65]. However, the nature of biochemical and physiological in vivo signals which dictate the relative contribution of the two coexisting pathways of SPD synthesis in this plant need further study. Since leguminous plants in general have limiting concentrations of methionine [29] it is possible that the alternate mechanism of SPD synthesis via carboxyspermidine employing the relatively abundant aspartic acid might bestow on them adequate capacity for SPD production. Carboxyspermidine synthase has been partially purified by affinity chromatography and shown to be absolutely specific for NADPH as the cofactor. Interestingly, carboxyspermidine decarboxylase is marginally affected by MGBG but profoundly curtailed by SPD. How widely this novel alternate pathway of SPD synthesis is distributed in the plant kingdom is a matter for future investigation.

3.6 Enzymic synthesis of sym. *homospermidine from PUT in plants*

Sym. Homospermidine (1,9-diamino-5-azanonane) was first shown to occur in the free state in significant quantities in *Santalum album* (sandal) leaves [26] and is now known to have a fairly widespread distribution among living organisms. Kuttan and Radhakrishnan [26] have suggested that the amine might derive all its carbon and nitrogen atoms from PUT and have proposed a reaction sequence according to which the Schiff base formed between PUT and its oxidation product, viz., 4-aminobutyraldehyde, is subsequently

reduced to *sym*-homospermidine (Figure 5). The enzyme involved, viz., homospermidine synthase, has been partially purified (100-fold) by Blue-Sepharose affinity chromatography [67]. This enzyme activity could also be demonstrated in sandal leaf extracts. Interestingly, attempts to demonstrate the natural occurrence in *L. sativus* of this unusual amine have not been successful. Although many possible explanations can be proposed for the absence of this amine in *L. sativus*, it is speculated that *L. sativus*, being a hardy plant capable of withstanding nutritional and water stress under drought-like conditions for a considerable time, might utilize with advantage the existing enzyme machinery to elaborate *sym*-homospermidine under these adverse climatic conditions as an adaptive mechanism. Of significance in this connection is the postulate that the occurrence and functional significance of the unusual PA in biological systems may be related to the extreme environmental habitats to which such organisms are exposed.

4. Miscellaneous aspects

It is conceivable that in addition to the primary biosynthetic pathways of PUT and PA in higher plants described above there are other, albeit minor, routes of amine production under certain physiological circumstances. For example the elaboration of PUT arising from decarboxylation of citrulline via NCP in *Sesamum* and sugarcane has already been mentioned [12, 30]. Additionally, pyridoxal-dependent aminotransferases which catalyse the

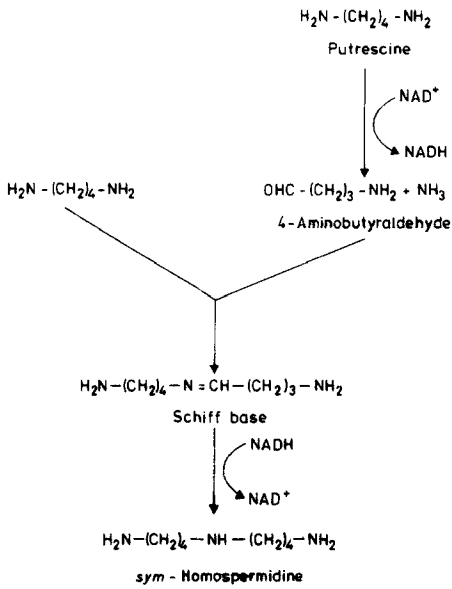

Figure 5. Biosynthesis of sym-Homospermidine.

reaction of various α-ω-diamines with 2-oxoglutarate yielding glutamate and ω-amino aldehyde (diamine amino transferase) have been detected in plants and microbial extracts [57] and it is possible that this reversible reaction could mediate PUT production using 4-aminobutyraldehyde derived from glutamate under certain conditions. More plausibly, PUT could also arise in plants from SPD and SPN through the action of polyamine oxidase on lines suggested for the mammalian systems [52] since such enzymes capable of oxidation at the secondary amino groups of PAs have been detected in Gramineae [58, 59]. Recent observations that in *L. sativus* seedlings a trans-amidinase with a broad substrate specificity can efficiently mediate amine interconversions assumes significance since it could reversibly catalyse AGM → PUT conversion and thus may have significance in terms of intracellular concentration of the diamine [66].

5. Conclusions and future prospects

From the above discussion, it is clear that the most important achievements hitherto in the field of PA metabolism in higher plants have been the mapping of the major biosynthetic pathways and the recognition of the intermediary enzyme reactions that are amenable to modulatory influence by growth and environmental signals. Admittedly, several aspects of PA metabolic machinery require clarification before the molecular aspects of PA biogenesis and regulation can be fully understood. With the recognition that PAs play important roles in several facets of plant development and physiology, the major thrust of future research on PAs will undoubtedly continue to deal with gross alterations in activity of enzymes involved in PA elaboration in response to various natural and artificial manipulations of plant growth and development. In this connection, a cautionary note is in order and concerns the interpretation drawn by the quantification of ADC/ODC activity in plant crude extracts solely by measurement of [$^{14}CO_2$] liberated from the respective labelled substrates, unsupported by correlation with parallel changes in the levels of the other products of the reaction, viz., the diamines. Relevant in this context is our clearcut demonstration that a number of amino acids can undergo non-enzymatic decarboxylation due to liberation of H_2O_2 during amine oxidase-mediated catabolism of diamines (like AGM and PUT) and thus giving spurious results. It is surprising that despite the fact that ADC is the most studied enzyme of plant PA metabolism, its regulation at the molecular level by a variety of small and large molecular effectors, (particularly by K^+ and osmolarity of the medium) has not yet received the attention it deserves, unlike its counterpart viz., ODC in microbial and animal systems. With the availability of specific antibodies to the purified enzyme and well-defined in vivo model systems responding rapidly to modulatory influence of effectors such as amine intermediates, substrate analogues and inhibitors etc., further studies on these lines should be rewarding. Development of plant

cell culture systems that are drug-resistant over-producers of the biosynthetic enzymes (i.e., similar to DFMO-resistant mammalian cell lines [31, 32] should prove highly valuable in this respect since such desirable features should solve many problems related to yields and stability of the enzymes. Unlike microbial and animal systems, where the participation of an antizyme as a dominant factor in ODC regulation is a proven fact, unequivocal evidence for the existence and functionality of such a regulator of either ADC or ODC in plants is lacking despite the lone claim [27] for an ODC-antizyme in enzyme regulation in barley. Similarly available information regarding the various other possible modes of ADC regulation such as enhanced rate of biosynthesis, decreased rate of catabolism, interconversion among active and inactive forms of the enzymes or a combination thereof to account for rapid fluctuation in biosynthetic activity is still fragmentary. Likely involvement of post-translational protein modifications, such as phosphorylation, in enzyme stability and function as factors governing in vivo regulation of ADC and ODC clearly deserves further study. As already pointed out, a close examination of the enzymes of PA metabolism in plants reveals a high degree of organizational complexity. Although evidence for the operation of the proposed AGM cycle in the two plant systems referred to is conclusive, the general validity of its significance needs to recruit many more respresentative species for further study. Detailed kinetic and functional characteristics of the various enzyme domains of the main component of the cycle viz., the multiheaded PUT synthase may reveal the nature of the various regulatory elements which monitor the directed flow of the intermediates to various catalytic sites on the protein. Further investigations at the level of genomic and structural organization of this versatile enzyme may unravel many exciting facets of its functions.

Another promising approach to plant PA metabolism is the study of molecular and regulatory properties of SAMDC on which available information hitherto is at best fragmentary compared to its animal or microbial counterpart. Similarly, the aminopropyl transferases in plants have received little attention and development of rapid and simple radiometric assays should facilitate studies in this direction. Finally, the question of the relative contribution of ADC and ODC to provide PUT in plants as a physiological trigger as well as the intracellular biochemical milieu that dictate the recruitment of either or both the enzyme mechanisms remains highly enigmatic. Our own findings that at least in one plant system, both the activities are manifested by a single protein on purification should be viewed as a clue that there may be many regulatory elements which govern the expression of the two activities in vivo; further exploration of the nature and functional characteristics of such factors should be highly rewarding in terms of understanding the complexities of plant PA metabolism.

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