THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 256, No. 18, Issue of September 25, pp. 9532-9541, 1981
Printed in U.S.A.

Enzymic Conversion of Agmatine to Putrescine in *Lathyrus sativus* seedlings

PURIFICATION AND PROPERTIES OF A MULTIFUNCTIONAL ENZYME (PUTRESCINE SYNTHASE)*

(Received for publication, November 21, 1980)

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The participation of a multifunctional enzyme (a single polypeptide with multiple catalytic activities (14)) has been demonstrated in the conversion of agmatine to putrescine in *Lathyrus sativus* seedlings. This enzyme (putrescine synthase) with inherent activities of agmatine iminohydrolase, putrescine transcarbamylase, ornithine transcarbamylase, and carbamate kinase has been purified to homogeneity and has M_r = 55,000. In the presence of inorganic phosphate, the enzyme catalyzed the stoichiometric conversion of agmatine and ornithine to putrescine and citrulline, respectively. The different activities associated with the enzyme co-purified with near constancy in their specific activity. The enzyme catalyzed phosphorolysis and arsenolysis of *N*-carbamyl putrescine. The multifunctionality of putrescine synthase was also supported by 1) activity staining, 2) intact transfer of the *ureido*-^{14}C group from labeled *N*-carbamyl putrescine to ornithine to form citrulline, and 3) the affinity of the enzyme toward structurally and functionally related affinity matrices. An agmatine cycle is proposed wherein *N*-carbamyl putrescine arising from the agmatine iminohydrolase reaction is converted to putrescine and citrulline, with the ureido group of *N*-carbamyl putrescine being transferred intact to ornithine. Preliminary results indicate that this series of reactions is also present in other plants.

Biosynthesis of putrescine, the obligatory precursor of spermidine and spermine involves different steps in different biological systems. In contrast to microorganisms and animals, in higher plants the principal source of putrescine is arginine (1, 2), although detection of ornithine decarboxylase has been reported in some plants (3–6). Earlier, Smith (7–9) and Smith and Garraway (10) proposed that in higher plants agmatine is converted to putrescine in two discrete steps, with *N*-carbamyl putrescine as the intermediate. It has been suggested that *N*-carbamyl putrescine is probably an enzyme-bound intermediate and is rapidly degraded to putrescine (2, 11).

Earlier, arginine decarboxylase from *Lathyrus sativus* was purified to homogeneity (12). During the purification of agmatine iminohydrolase, we found that, only when the assay mixture contained inorganic phosphate, significant amounts of putrescine (besides *N*-carbamyl putrescine) were produced. On the basis of the obligatory involvement of inorganic phosphate in the *N*-carbamyl putrescine → putrescine conversion, it was surmised that the *N*-carbamyl-putrescine amidohydrolase (producing putrescine, CO_2, and NH_3) might in fact represent phosphorolytic cleavage of this intermediate by a putrescine transcarbamylase-mediated reaction (functioning in the reverse direction) on lines suggested in *Streptococcus faecalis* growing on agmatine as the sole carbon source (13). Further experiments have revealed that the purified enzyme is in fact a versatile multifunctional enzyme (for reviews on this class of enzyme, see Refs. 14–17) with agmatine iminohydrolase, putrescine transcarbamylase, ornithine transcarbamylase, and carbamate kinase activities associated with a single polypeptide chain and that the metabolic conversion of agmatine to putrescine is apparently linked to citrulline production in *L. sativus*.

EXPERIMENTAL PROCEDURES[1]

RESULTS

In terms of subcellular distribution (both total and specific activity), most of putrescine synthase[2] was associated with the cytosolic fraction and hence, the postmitochondrial supernatant was routinely used for enzyme purification (for details, see Miniprint). The different reactions catalyzed by the enzyme are represented in Table I.

Co-purification of the Different Activities Associated with Putrescine Synthase

The purification of putrescine synthase was monitored by assaying the different component activities, as well as the

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[1] Portions of this paper (including "Experimental Procedures," part of "Results," Tables II, VII, and VIII, and Figs. 1, 3, and 10) are presented in miniprint at the end of this paper. Figs. 4 and 6 and Tables IV and X appear in the text in miniprint as prepared by the author. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 80M-2469, cite authors, and include a check or money order for $6.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

[2] The abbreviations and trivial names used are: putrescine synthase, the multifunctional enzyme with associated activities of agmatine iminohydrolase (agmatine deiminase, EC 3.5.3.12), putrescine transcarbamylase (carbamoyl phosphate: putrescine carbamoyltransferase, EC 2.1.3.6), ornithine transcarbamylase (carbamoyl phosphate: ornithine carbamoyltransferase, EC 2.1.3.3), and carbamate kinase (ATP-carbamate phosphotransferase, EC 2.7.2.2). The terms multifunctional, polycephalic, and chimeric enzyme used in the text represent a single polypeptide with several catalytic activities (14). CH-Sepharose, carboxyhexyl Sepharose; NBT, nitrobluetetrazolium chloride; PMS, phenazine methosulfate; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; CNBr, cyanogen bromide.

overall reactions, leading to citrulline production with agmatine or *N*-carbamyl putrescine as substrates. It is clear from Table III that the activities associated with the chimeric enzyme co-purify through the four steps, with the specific activity ratios remaining more or less constant throughout the purification steps. However, the enzyme exhibited considerable difference in terms of ornithine transcarbamylase activity in the crude extracts *vis-à-vis* the final preparation. This may conceivably be due to more than one ornithine transcarbamylase activity in the plant cell-free extracts (45, 46) and the association of only one of them with putrescine synthase. Additional activities associated with the purified enzyme are 1) that related to the synthesis of *N*-carbamyl putrescine owing to the inherent putrescine transcarbamylase activity, which had a specific activity of 33 units; 2) carbamate kinase activity, assayed in the direction of ATP synthesis, with a specific activity of 2.5 units. Generation of ATP from agmatine + ADP + P_i and *N*-carbamyl putrescine + ADP + P_i combinations (*i.e.* the overall reactions linked to carbamate kinase) could also be demonstrated. However, these component activities of putrescine synthase were not quantitated during the purification.

TABLE I

Constituent activities of putrescine synthase

Reactions

1) Agmatine + $H_2O \rightarrow$ *N*-carbamyl putrescine + NH_3

2) *N*-Carbamyl putrescine + $P_i \rightleftharpoons$ putrescine + carbamyl phosphate

3) Carbamyl phosphate + ornithine $\rightleftharpoons$ citrulline + P_i

Overall reaction

4) Agmatine + ornithine + H_2O + $P_i \rightarrow$ putrescine + citrulline + NH_3 + P_i

Additional reaction

5) Carbamyl phosphate + ADP + $H_2O \rightleftharpoons$ ATP + CO_2 + NH_3

Enzymes

1) agmatine iminohydrolase
2) putrescine transcarbamylase
3) ornithine transcarbamylase
4) putrescine synthase
5) carbamate kinase

Physicochemical Properties

Purity of the Isolated Enzyme—The final preparation obtained by both purification procedures, *i.e.* the organomercurial-Sepharose step followed by fractionation on DEAE-Sephadex (procedure I) and the putrescine-CH-Sepharose affinity step (procedure II), showed a single sharp band on polyacrylamide gel electrophoresis at pH 8.3 (Fig. 2, *a* and *b*) and at pH 4.0 (Fig. 2*c*). The fast moving band in basic gel systems was coincident with the different enzyme activities associated with the protein as evidenced by activity scanning after gel

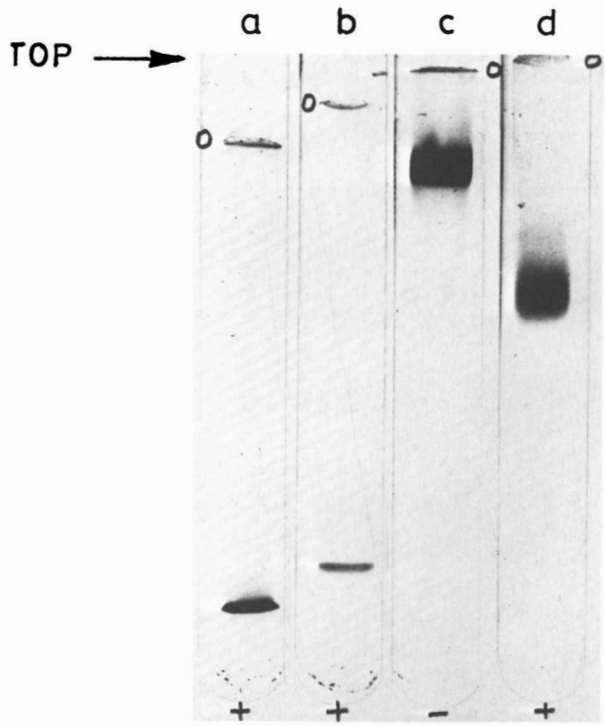

FIG. 2. **Polyacrylamide gel electrophoresis of putrescine synthase under nondenaturing conditions.** The staining pattern of the purified enzyme at pH 8.3 by procedure I (*a*); procedure II (*b*); protein from procedure II at pH 4.0 (*c*); and protein from procedure II (*d*) (purified in the presence of 1 mM phenylmethylsulfonyl fluoride) at pH 8.3. Protein used in *a* and *b*, 50 μg and in *c* and *d*, 100 μg. *O*, origin.

TABLE III

Co-purification of the different catalytic activities of putrescine synthase and comparison of their specific activities

The putrescine-CH-Sepharose affinity step (procedure II) was employed for purification. Enzyme activity has been expressed in total enzyme units and the numbers within parentheses refer to the specific activity (μmol of product/mg of protein/h). The specific activity of carbamate kinase in the final preparation was 2.5 μmol of ATP formed/ mg of protein/h.

Purification step	Protein	Total enzyme units: Agmatine iminohydrolase	Putrescine transcarbamylase (*N*-carbamyl putrescine arsenolysis)	Ornithine transcarbamylase	Agmatine + ornithine → citrulline	*N*-Carbamyl putrescine + ornithine → citrulline	Purification[a]	Recovery[a]
	mg						*-fold*	%
Crude extract	4680	28 (0.01)	449 (0.10)	1220 (0.26) (1:16:44:9:11)[b]	267 (0.06)	330 (0.07)	1	100
$MnCl_2$ treatment	3450	27.6 (0.01)	414 (0.12)	1000 (0.29) (1:15:36:8:9.6)	214 (0.06)	270 (0.08)	1.3	91
Ammonium sulfate fractionation	1050	21 (0.02)	409 (0.39)	950 (0.9) (1:19:5:45:8.5:11)	180 (0.17)	231 (0.22)	3.6	75
Putrescine-CH-Sepharose affinity step	5	7 (1.40)	110 (21)	140 (28) (1:15:20:8.5:10)	59.5 (11.9)	70 (14.0)	230	25

[a] Calculated for agmatine iminohydrolase.

[b] Represents the ratio of relative specific activities with respect to agmatine iminohydrolase.

electrophoresis (Fig. 4). No additional component could be detected even when 100 μg of enzyme were applied to the acrylamide gels and run under both acidic and basic conditions. Furthermore, the antiserum raised against the purified enzyme gave a single precipitin line on immunodiffusion analysis with the homogeneous preparation (not shown).

Electrophoresis on Denaturing Gels—Polyacrylamide gel electrophoresis of the enzyme in the presence of 0.1% SDS, 5% mercaptoethanol (40) reproducibly showed a single protein species (Fig. 5*a*) with an estimated M_r of 55,000 (Fig. 6).

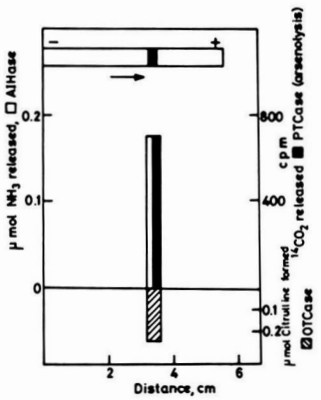

Fig. 4. Distribution of enzyme activities on polyacrylamide gel electrophoresis of putrescine synthase. After electrophoresis (pH 8.3) of the purified enzyme at 4°C, the distribution of different activities were monitored by sectioning the gels and extracting at 4°C with 50mM imidazole buffer. The enzyme activities were determined in the supernatant after removing the gel pieces by centrifugation. AIHase, PTCase and OTCase stand for agmatine iminohydrolase, putrescine transcarbamylase and ornithine transcarbamylase respectively.

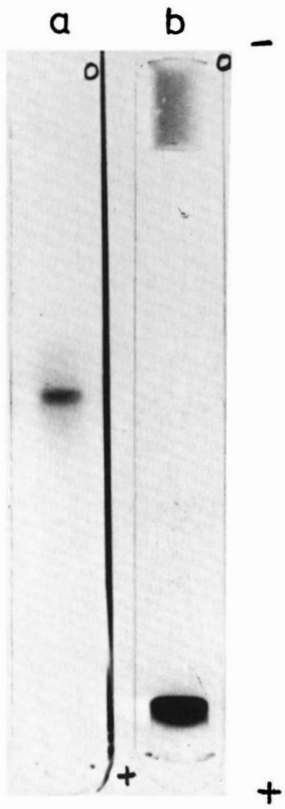

FIG. 5. **Sodium dodecyl sulfate-disc gel electrophoretic pattern of putrescine synthase.** *a*, purified enzyme (30 μg) electrophoresed for 2 h and *b*, the enzyme protein (100 μg) subjected to guanidine hydrochloride, sodium dodecyl sulfate, and urea treatment and electrophoresed for 4 h. The protein treated with 8 M guanidine hydrochloride and 2-mercaptoethanol was alkylated with iodoacetate (41). The sample was then dialyzed against 8 M urea, 0.1% sodium dodecyl sulfate in Tris-HCl buffer (pH 8.0) and subjected to gel electrophoresis. *O*, origin.

Denaturation of the protein under more severe conditions, namely employing 8 M guanidine hydrochloride followed by treatment with urea, SDS, and mercaptoethanol prior to electrophoresis (41), also revealed the presence of a single protein band on SDS gels (Fig. 5*b*). These results are consist-

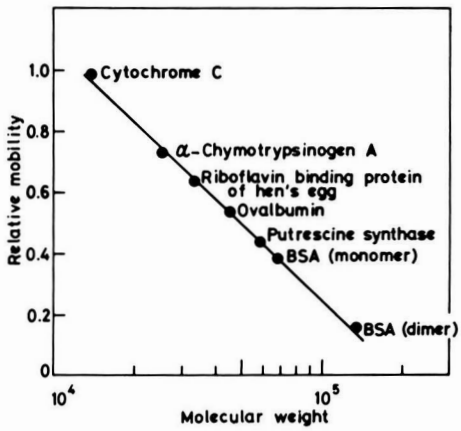

Fig. 6. Standard curve for molecular weight determination by SDS gel electrophoresis. The molecular weight of marker proteins used are: cytochrome C (13500), α-chymotrypsinogen A (23500), chicken riboflavin binding protein (34000), ovalbumin (45000), BSA monomer (68000) and dimer (136000).

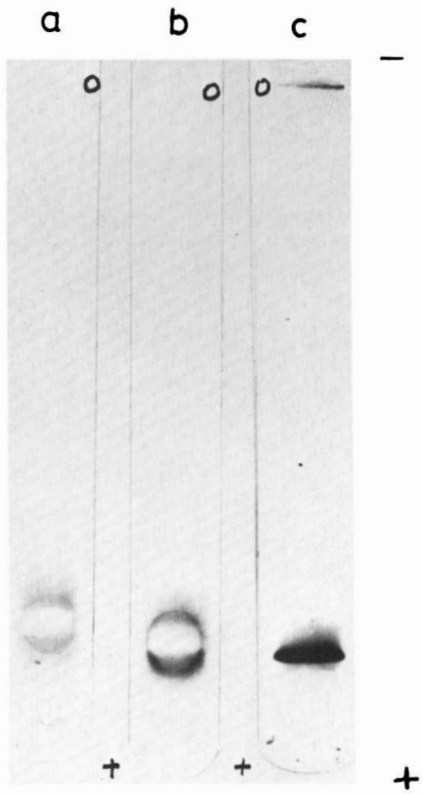

FIG. 7. **Activity staining to demonstrate ATP synthesis in the carbamate kinase-linked overall reaction catalyzed by putrescine synthase.** *a*, agmatine + ADP + P_i; *b*, *N*-carbamyl putrescine + ADP + P_i; *c*, purified putrescine synthase stained with Coomassie brilliant blue. Polyacrylamide gel electrophoresis of putrescine synthase was carried out in the cold (4 °C) at pH 8.3. Following the equilibration of the gels in 50 mM imidazole-Cl (pH 7.5) buffer, they were transferred to a solution (2.5 ml) containing 1 mM dithiothreitol, 5 mM agmatine, or *N*-carbamyl putrescine, 3 mM ADP, 5 mM Na_2HPO_4, 20 mM glucose, 5 mM $MgSO_4$, 0.5 mM $NADP^+$, 0.5 mM KCl, 150 units of hexokinase, 100 units of glucose-6-phosphate dehydrogenase, 0.5 mg of phenazine methosulfate, 1.5 mg of nitrobluetetrazolium chloride and were dissolved in 50 mM imidazole-Cl buffer and incubated at 37 °C for 1 h along with corresponding blank gels (*i.e.* minus substrate). The gels were stored in 7% acetic acid after development of the formazan band. *O*, origin.

ent with the view that the four different enzyme activities assayed reside in a single polypeptide, thereby substantiating the multifunctional nature of putrescine synthase.

Activity Staining—ATP formation *in situ* was detected by coupling with hexokinase + glucose-6-phosphate dehydrogenase + NADP and the NADPH thus generated was in turn reacted with neotetrazolium chloride and phenazine methosulfate to give rise to formazan. A blue formazan band corresponding to the protein stain was observed on gels when incubated with either agmatine + ADP + P_i or *N*-carbamyl putrescine + ADP + P_i (Fig. 7, *a*, *b*, and *c*). This finding further strengthens the polycephalic property of putrescine synthase, since it demonstrates that agmatine iminohydrolase, putrescine transcarbamylase, and carbamate kinase activities (Table I) (generating ATP as one of the products) reside in a single protein species. The formazan band was not detected in the incubation mixtures from which the substrates were omitted (not shown).

Molecular Weight—The purified enzyme migrated with an apparent $M_r \cong 56{,}000$ on Sephadex G-200 (data not shown). The M_r determined by gel filtration is in close agreement with that from SDS-gel electrophoretic analysis (Fig. 6). Furthermore, it is significant that all of the component activities of putrescine synthase were recovered as a single protein peak when eluted from the molecular sieve (Fig. 8), thus providing additional evidence for the chimeric nature of the enzyme.

Stoichiometry of the Reactions

Among the various partial reactions catalyzed by putrescine synthase, the following were shown to occur stoichiometrically.

1) Agmatine Iminohydrolase Reaction—The new assay developed for agmatine iminohydrolase earlier by us (20) facilitated the quantification of *N*-carbamyl putrescine as the reaction product. Parallel estimation of ammonia was also carried out for comparison. As the purification progressed, the relative amount of NH_3 produced decreased proportionately, finally reaching approximately 1:1 stoichiometry of both the products.

2) Overall Reaction I (Agmatine + Ornithine + H_2O + P_i → Citrulline + Putrescine + NH_3 + P_i)—The stoichiometric conversion of metabolites in the above overall reaction could be easily demonstrated (Table IV). Paper chromatographic analysis of the amine fraction isolated from the above reaction mixture revealed the presence of residual agmatine and the other product, putrescine. Omission of inorganic phosphate led to accumulation of significant amounts of *N*-carbamyl putrescine as revealed by chromatographic analysis.

3) Overall Reaction II (Agmatine + ADP + P_i + H_2O → Putrescine + ATP + 2NH_3 + CO_2)—In the carbamate kinase-linked reaction, ATP synthesis was stoichiometrically coupled to the amount of agmatine degraded. Fig. 9 shows the formation of ATP in the reaction mixture containing agmatine (or *N*-carbamyl putrescine) + ADP + P_i. Since the commercial ADP sample (Sigma) was contaminated with ATP to a small extent, a small increase in absorbance at 340 nm was observed with ADP alone. The ATP accumulated in the incubation mixture resulted in a steep increase in $A_{340\ nm}$ in the coupled assay used to measure ATP (Fig. 9). These observations were confirmed with the more sensitive luciferase procedure (27).

Evidence for the Intact Transfer of the Carbamyl Group from N-Carbamyl Putrescine to Ornithine and Requirements for the Overall Reaction

Availability of synthetic [*ureido*-^{14}C]*N*-carbamyl putrescine permitted demonstration of the transfer of the carbamyl group of carbamyl phosphate to ornithine to form citrulline during the overall enzyme reaction (Table V). No liberation of $^{14}CO_2$ could be detected when the above reaction was performed in a closed Warburg flask. Furthermore, both Mg^{2+} and P_i are required for this reaction. The requirement for these components in the enzymatic production of citrulline was also evi-

TABLE IV

Stoichiometry of the overall reaction

The assay was performed in a reaction mixture containing agmatine, Na_2HPO_4, ornithine, $MgSO_4$ and purified enzyme at pH 8.8 (see Experimental Procedures) under standard conditions. Agmatine was quantitated by Sakaguchi reaction (58). Putrescine was estimated after paper chromatography and ninhydrin spray (30). Ammonia and citrulline were estimated by Nesslerization (18) and the colorimetric procedure of Prescott and Jones (28) respectively. Citrulline as one of the reaction products was conclusively identified by paper chromatography as well as by its exclusion behaviour during chromatography on Amberlite CG-50 (NH_4^+) column (32).

Agmatine utilized (μmols)	Products (μmols) in the reaction mixture		
	Putrescine	NH_3 released	Citrulline
Experiment I (0.20)	0.16	0.18	0.21
Experiment II (0.25)	0.21	0.23	0.24

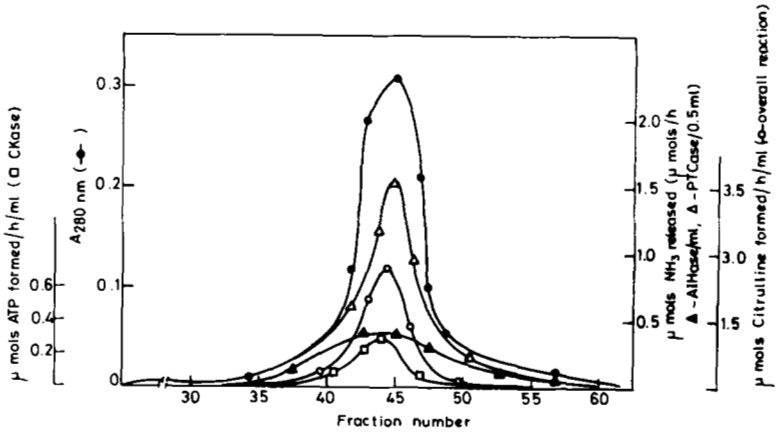

FIG. 8 (*left*). **Gel filtration of homogeneous putrescine synthase on Sephadex G-200.** Gel filtration of the purified enzyme was carried out on a Sephadex G-200 column (1.8 × 89 cm), using 50 mM Tris-Cl buffer (pH 8.0) containing 0.1 M KCl. Fractions of 3 ml were collected and the constituent activities associated with putrescine synthase were assayed as described under "Experimental Procedures." *AIHase*, agmatine iminohydrolase; *PTCase*, putrescine transcarbamylase; *CKase*, carbamate kinase.

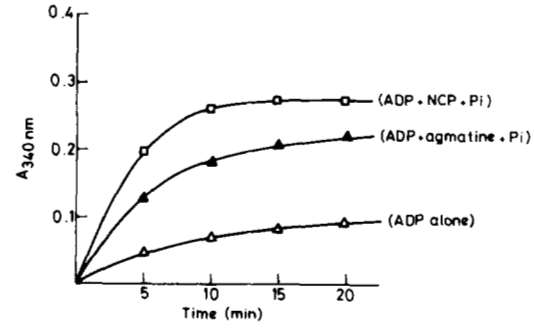

FIG. 9 (*right*). **Generation of ATP in the carbamate kinase-linked overall reaction catalyzed by putrescine synthase.** The assays were carried out under standard conditions ("Experimental Procedures") using the purified enzyme, with agmatine or *N*-carbamyl putrescine (*NCP*) as substrates. The reaction was terminated with 0.1 ml of 10% perchloric acid followed by neutralization of the solution with 3 N KOH. ATP in an aliquot of the supernatant was quantitated using the hexokinase-glucose-6-phosphate dehydrogenase coupled assay procedure (26).

TABLE V

Requirements for the overall reaction and the demonstration of transcarbamylation from N-carbamyl putrescine to ornithine catalyzed by putrescine synthase

The complete reaction mixture (1.0 ml) containing Tris-Cl (pH 8.8) 50 μmols, [*ureido*-^{14}C]*N*-carbamyl putrescine, 1 μmol (50,000 cpm), ornithine, 5 μmol, Na_2HPO_4, 1 μmol, $MgCl_2$, 5 μmol, BSA, 100 μg, and pure enzyme with appropriate blanks was incubated at 37 °C for 1 h. Citrulline was separated from labeled *N*-carbamyl putrescine in the reaction mixture by elution from NH_4OH from a Dowex 50-H^+ column as described under "Experimental Procedures." The amino acid fraction was evaporated *in vacuo* and the residue was counted for radioactivity. The identity of the labeled citrulline formed in this experiment was confirmed by paper chromatography.

Component omitted	Citrulline
	cpm
None	7800
$MgCl_2$	500
Na_2HPO_4	250
Enzyme	50

dent when the colorimetric assay procedure was used. These results indicate that phosphorolytic cleavage of *N*-carbamyl putrescine in the first step is followed by the transfer of carbamyl phosphate generated *in situ* to ornithine and are consistent with the data regarding the stoichiometry of the ornithine transcarbamylase-linked overall reaction.

Demonstration of Phosphorolysis and Arsenolysis of N-Carbamyl Putrescine by the Putrescine Transcarbamylase Component of Putrescine Synthase

Since the presumed role of the putrescine transcarbamylase component in this multifunctional enzyme is to catalyze the phosphorolysis of *N*-carbamyl putrescine, it was of interest to examine whether the enzyme can function in the reverse direction. For this purpose, the enzymic conversion of the *ureido*-^{14}C-labeled intermediate to putrescine and carbamyl phosphate was investigated (13). It is clear (Table VI) that negligible phosphorolysis was observed in the absence of P_i. Replacing P_i with arsenate resulted in the enhanced release of $^{14}CO_2$ showing that arsenolysis has occurred. The observed inhibition of arsenolysis by P_i is apparently competitive (Table VI) and agrees well with earlier observations regarding arsenolysis of citrulline (24, 47). The liberation of $^{14}CO_2$ in the presence of ADP, P_i, [*ureido*-^{14}C]*N*-carbamyl putrescine and the enzyme is indicative of the operation of the overall reaction linked to carbamate kinase.

Stability of the Enzyme

The purified enzyme was highly unstable even in the presence of glycerol, dithiothreitol, and Mg^{2+}; it lost all the component activities within 48 h after purification when stored at 4 °C. Prolonged dialysis and freeze-thawing also led to a considerable loss of activity. The carbamate kinase activity was the most labile, since dialysis of even the crude extracts for more than 10 h rendered this activity unstable. However, with the purified preparations, $(NH_4)_2SO_4$ at 1 M concentration preferentially stabilized the carbamate kinase activity. This observation is in agreement with that recorded with the purified carbamate kinase from *S. faecalis* in which case the SO_4^{2-} ion was shown to be responsible for the stabilization (50). It was consistently found that all of the component activities associated with putrescine synthase were stabilized in dilute solutions (<50 μg of protein/ml) for about 3–4 h at 37 °C by bovine serum albumin at a 250 μg/ml concentration.

TABLE VI

Phosphorolysis and arsenolysis of N-carbamyl putrescine by putrescine synthase

The reaction mixture (1.0 ml) contained imidazole-Cl buffer (pH 7.5) 100 μmol, [*ureido*-^{14}C]*N*-carbamyl putrescine (50,000 cpm) 2 μmol, BSA, 250 μg, and purified enzyme, 25 μg. The assay was carried out in Warburg flasks and the $^{14}CO_2$ liberated after an incubation period of 1 h at 37 °C was determined.

Additions	$^{14}CO_2$ released
μmol	*cpm*
None	120
Na_2HPO_4 (10)	950
ADP (1) + Na_2HPO_4 (5)	2050
Sodium arsenate (50)	7560
Sodium arsenate (50) + Na_2HPO_4 (5)	5050
Sodium arsenate (50) + Na_2HPO_4 (10)	3050
Sodium arsenate (50) + boiled enzyme	70

Interaction of Putrescine Synthase with Different Affinity Matrices: Additional Evidence for its Polycephalic Nature

Further evidence for the multifunctionality of putrescine synthase was obtained by affinity chromatography. During the preliminary studies on putrescine biosynthesis in *L. sativus*, the specific affinity of agmatine iminohydrolase toward homoarginine- and citrulline-Sepharose was repeatedly observed. Furthermore, in view of the presence of a nucleotide (ADP) binding site ascribable to the inherent carbamate kinase activity, the affinity of this chimeric enzyme to blue Sepharose was expected. These observations were exploited and the $(NH_4)_2SO_4$ fraction (step 3) was adsorbed onto homoarginine-, citrulline-, organomercurial-, and blue Sepharose affinity columns (10 × 1 cm). This was followed by extensive washing with 50 mM imidazole-Cl buffer (pH 8) to remove unadsorbed proteins. The proteins held with high affinity were eluted with their respective ligands in imidazole buffer (*i.e.* 4 mM homoarginine or citrulline in cases of the first two affinity columns, 5 mM ATP in the case of blue Sepharose and 20 mM 2-mercaptoethanol in the case of organomercurial affinity matrix). The eluates were dialyzed against 20 mM imidazole buffer thoroughly to remove the respective soluble ligands. This ligand-specific elution resulted in the recovery of all the component activities inherent in putrescine synthase, with an identical elution profile. The specific interaction of this chimeric protein toward the affinity adsorbents possessing structurally related ligands (homoarginine and citrulline) is consistent with an association of the different enzyme activities with a single protein. Binding of the enzyme to homoarginine-Sepharose could be possibly related to the structural analogy between homoarginine and agmatine, particularly with regard to guanido function and hydrocarbon backbone. The ureido group of *N*-carbamyl putrescine, a transient intermediate in the overall reaction, and ornithine transcarbamylase activity inherent in the protein are probably responsible for the affinity of putrescine synthase to citrulline-Sepharose. It is significant to note that the dialyzed eluates from all affinity matrices exhibited ratios of specific activity of the different component activities, similar to that corresponding to the purified enzyme, while still reflecting the different degrees of enzyme purity achieved by these affinity procedures (Table IX).

Polyacrylamide gel electrophoresis of the above four eluates revealed the presence among others of a fast moving protein band corresponding in position on gels to the purified putrescine synthase (not shown). Evidence for the presence of putrescine synthase in the eluates from the four affinity matrices was also obtained immunologically. The antiserum raised

TABLE IX

Specific activities of the component reactions of putrescine synthase in the eluates from different affinity Sepharoses

The enzyme activities were assayed under the standard conditions described under "Experimental Procedures," using the dialyzed enzyme eluates from different affinity Sepharoses. The values represent the specific activities of constituent activities of putrescine synthase.

Affinity Sepharose	Specific activity					
	Agmatine iminohydrolase	Putrescine transcarbamylase (*N*-carbamyl putrescine arsenolysis)	Ornithine transcarbamylase	Putrescine transcarbamylase (*N*-carbamyl putrescine synthesis)	Agmatine + ornithine → citrulline	*N*-Carbamyl putrescine + ornithine → citrulline
	μmol product formed/mg protein/h					
Citrulline-Sepharose	1.20	19	23.5	39 (1:16:19:32.5:8:9.5)[a]	9.6	11.4
Homoarginine-Sepharose	0.80	11.2	15.2	24 (1:14:19:30:8:9)	6.4	7.3
Blue Sepharose	0.42	5.46	7.8	12.6 (1:13:18.5:30:8:9)	3.4	3.8
Organomercurial Sepharose	0.54	8.0	10.8	17.3 (1:14:20:32:9.9:10)	4.7	5.4
Putrescine-CH-Sepharose (pure enzyme)[b]	1.40	21	28	47 (1:15:20:33:8.5:10)	11.9	14

[a] Represents the specific activity ratio of various activities relative to agmatine iminohydrolase.

[b] Procedure II (Table III).

TABLE X

Citrulline production coupled with putrescine synthesis in the crude extracts of some higher plants

The crude extracts (step I) from the etiolated seedlings of various plants were dialyzed against 50mM imidazole buffer and used as enzyme source for assaying the overall reaction linked to ornithine transcarbamylase (see Experimental Procedures). Citrulline was quantitated as one of the reaction products, after ion-exchange chromatography on Dowex-50(H^+) column. This ureido amino acid was also characterized by its exclusion behaviour on Amberlite CG-50 (NH_4^+) column (32) and identified conclusively by paper chromatography. Omission of either agmatine or N-carbamyl putrescine and use of boiled enzyme in the above assays did not lead to measurable citrulline formation.

Plant	nmols citrulline formed/mg protein/h with	
	Agmatine	N-carbamyl putrescine
Pisum sativum (pea)	25	38
Cucumis sativus (cucumber)	10	15
Zea mays (maize)	30	45
Lathyrus sativus (grass pea)	17	25

against putrescine synthase cross-reacted with the protein fractions eluted from the different affinity matrices, exhibiting a single precipitin line in each case (not shown).

Evidence for the Association of Putrescine Biogenesis with Citrulline Production in Other Higher Plants

The data described above show that the conversion of agmatine to putrescine in *L. sativus* is interlinked with the production of citrulline and is mediated by a multifunctional enzyme. The question then arose whether this series of reactions also occurs in other plants. For this purpose, the overall enzymatic reaction catalyzing the agmatine → putrescine conversion, namely agmatine (or *N*-carbamyl putrescine) + ornithine + P_i → citrulline + putrescine was assayed in the dialyzed crude extracts of seedlings of different plants. The results obtained clearly indicate the occurrence of similar reactions in other plants examined (Table X).

DISCUSSION

The most significant feature of the present study is the evidence for a novel multifunctional enzyme involved in agmatine to putrescine conversion in *L. sativus* and its functional significance. Two highly reproducible purification procedures, one involving an organomercurial affinity step followed by DEAE-Sephadex chromatography and the other employing affinity chromatography on putrescine-CH Sepharose, were used to purify the enzyme to a homogeneous state. In contrast to a two-step hydrolytic scheme proposed earlier for the conversion of agmatine to putrescine, involving agmatine iminohydrolase and *N*-carbamyl putrescine-amidohydrolase in plants (9, 10), the present study demonstrates that putrescine transcarbamylase rather than *N*-carbamyl putrescine-aminodohydrolase catalyzes putrescine production. The most likely explanation for this discrepancy is that a crude extract prepared in phosphate buffer was incubated for long periods in the earlier studies (9); consequently, putrescine transcarbamylase acting in the reverse direction might have degraded *N*-carbamyl putrescine into putrescine and carbamyl phosphate, the latter undergoing further hydrolysis to CO_2 and NH_3 (52). The detection of putrescine transcarbamylase activity in the pea (49) and the phosphorolytic cleavage of *N*-carbamyl putrescine in *S. faecalis* (13) are consistent with our findings.

Several lines of evidence prove the multifunctionality of putrescine synthase from the plant system: (*a*) the purified enzyme exhibits a single protein band on SDS-polyacrylamide gel electrophoresis, with $M_r = 55{,}000$; (*b*) co-purification of the different constituent activities with near constancy of specific activity ratios; (*c*) the stoichiometric conversion of the metabolites in the overall reactions catalyzed by the enzyme; (*d*) all the constituent activities band in a single region corresponding to the protein stain on polyacrylamide gel electrophoresis and co-elute as a single protein peak from Sephadex G-200; (*e*) activity staining (Fig. 7) for ATP synthesis is a result of interaction between the constituent activities; (*f*) the intact transfer of the ureido group from *N*-carbamyl putrescine to ornithine as a result of interaction between the two transcarbamylase components of the enzyme (Table V); (*g*) the affinity toward different functionally and structurally related affinity matrices and recovery of all of the activities during elution (with similar ratios of specific activities as found with the pure enzyme + immunological and electrophoretic evidence for the enzyme protein in these eluates.

One of the basic features of the reaction catalyzed by putrescine synthase is the intact transfer of the carbamyl moiety of *N*-carbamyl putrescine to ornithine, due to coupled activities of the two transcarbamylase components of the enzyme. In the absence of ornithine, the putrescine transcarbamylase component acting in the reverse direction can conceivably catalyze the phosphorolytic cleavage of *N*-carbamyl putrescine to putrescine and carbamyl phosphate, provided carbamyl phosphate is depleted from the catalytic site to drive

the reaction in favor of putrescine synthesis. That the higher plant efficiently uses ornithine transcarbamylase activity for this purpose to channel carbamyl phosphate generated *in situ* to citrulline production is clearly evident from the data presented. Of relevance in this context is that, in *S. faecalis*, ornithine transcarbamylase activity is intrinsic to the purified putrescine transcarbamylase (48). Thus, it would appear that an "agmatine cycle" (Fig. 11) functions in the higher plants; one of the essential tenets of this cycle is the intact transfer of the carbamyl group from *N*-carbamyl putrescine to ornithine, thus sparing two ATP molecules otherwise needed for the *de novo* synthesis of carbamyl phosphate. The coupling of these novel, highly organized enzymatic reactions would readily explain the nonaccumulation of *N*-carbamyl putrescine (1, 2, 11) as well as the choice of such a carbamyl intermediate by the higher plants. Preliminary data indicate that these reactions comprising the agmatine cycle are functional in other plants also. The above series of reactions are similar to those of the arginine dihydrolase pathway in microorganisms although three separate enzymes catalyze the latter reactions (53).

The higher catalytic efficiency (approximately 10 times) of putrescine synthase in the overall reaction compared to agmatine iminohydrolase assayed in isolation (Table III) is in line with the cooperativity in multienzyme systems (15). The requirement of Mg^{2+} in the ornithine transcarbamylase-linked overall reaction is also consistent with its known stabilizing influence on carbamyl phosphate (54). It is intriguing that the putrescine synthase with M_r = 55,000 harbors four functionally discrete reaction domains on its single chain polypeptide backbone. This is not suprising since several such multifunctional proteins with similar molecular size were reported earlier (14). While a subunit structure for the purified putrescine synthase can be ruled out, the possibility of an oligomeric form functioning *in vivo* is to be considered. To test whether proteolytic cleavage during purification accounted for the relatively smaller size of the enzyme (55), purification of the synthase was attempted in the presence of 1 mM phenylmethylsulfonyl fluoride. Under these conditions, the enzyme showed a considerably slower mobility during gel electrophoresis at pH 8.3 (Fig. 2 *d*) *vis-à-vis* the protein purified in the absence of phenylmethylsulfonyl fluoride (Fig. 2, *a*, *b*, and *c*). The relatively larger size of the enzyme prepared in the presence of the proteolytic inhibitor was also evident during gel filtration on Sephadex G-200 since the activity was eluted in the void volume itself.

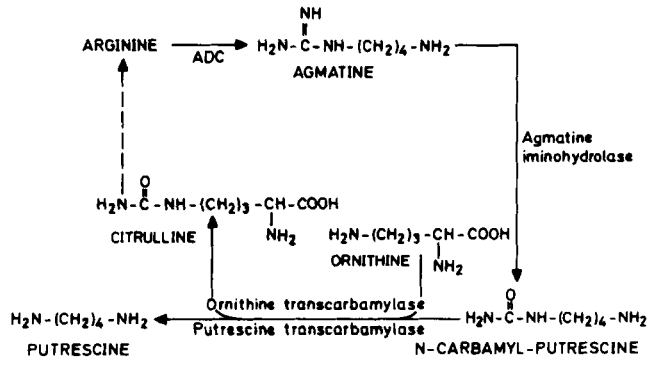

FIG. 11. **Agmatine cycle and its relation to arginine synthesis in higher plants.** The scheme proposed is based on the sequence of reactions investigated in the present study. *N*-Carbamyl putrescine arising from agmatine iminohydrolase reaction is converted to putrescine and citrulline due to the interaction between putrescine transcarbamylase and ornithine transcarbamylase components of putrescine synthase.

Thus, it is clear that the putrescine synthase of *L. sativus* is another example of a highly organized chimeric protein, catalyzing a set of sequential reactions (14). The coexistence of both the coupling activities (carbamate kinase and ornithine transcarbamylase) in putrescine synthase is not easily understood at present. Both of these activities catalyze reversible reactions, with their K_{eq} favoring useful anabolic reactions and thus are ideally suited for coupling mechanisms, which in turn enhance the catalytic efficiency in a multistep reaction. The transfer of the carbamyl group in the series of reactions leading to citrulline synthesis (Table V) is one of the few examples of its kind; the other two instances are (*a*) generation of citrulline from carbamyl oxamate and ornithine in *Streptococcus allantoicus* cell-free extracts (56) and (*b*) synthesis of arginine from carbamyl aspartate and ornithine in the crude extracts of wheat seedlings (57).

REFERENCES

1. Smith, T. A. (1971) *Biol. Rev. Camb. Philos. Soc.* **46,** 201-241
2. Smith, T. A. (1975) *Phytochemistry* **14,** 865-890
3. Hasse, K., Ratych, O. T., and Salnikow, J. (1967) *Hoppe-Seyler's Z. Physiol. Chem.* **348,** 843-851
4. Yoshida, D. (1969) *Plant Cell Physiol.* **10,** 393-397
5. Bagni, N., and Spernaza, A. (1977) in *Plant Growth Regulators* (Kudrev, T., Ivanova, I., and Karmov, E., eds) pp. 75-78, Bulgarian Academy of Sciences, Sofia
6. Heimer, Y. M., Mizrahi, Y., and Bachrach, U. (1979) *FEBS Lett.* **104,** 146-148
7. Smith, T. A. (1970) *Ann. N. Y. Acad. Sci.* **171,** 988-1001
8. Smith, T. A. (1969) *Phytochemistry* **8,** 2111-2117
9. Smith, T. A. (1965) *Phytochemistry* **4,** 599-607
10. Smith, T. A., and Garraway, J. L. (1964) *Phytochemistry* **3,** 23-26
11. Smith, T. A. (1977) *Prog. Phytochem.* **4,** 27-81
12. Ramakrishna, S., and Adiga, P. R. (1975) *Eur. J. Biochem.* **59,** 377-386
13. Roon, R. J., and Barker, H. A. (1972) *J. Bacteriol.* **109,** 44-50
14. Kirschner, K., and Bisswanger, H. (1976) *Annu. Rev. Biochem.* **45,** 143-166
15. Gaertner, F. H. (1978) *Trends Biochem. Sci.* **3,** 63-65
16. Welch, G. R. (1977) *Prog. Biophys. Mol. Biol.* **32,** 103-191
17. Stark, G. R. (1976) *Trends Biochem. Sci.* **2,** 64-66
18. Suresh, M. R., Ramakrishna, S., and Adiga, P. R. (1976) *Phytochemistry* **15,** 483-485
19. Gerhart, J. C., and Pardee, A. B. (1962) *J. Biol. Chem.* **237,** 891-896
20. Srivenugopal, K. S., and Adiga, P. R. (1980) *Anal. Biochem.* **104,** 440-444
21. Smith, L. H. (1955) *J. Am. Chem.* **77,** 6691-6692
22. Williams, D. L., and Ronzio, A. R. (1952) *J. Am. Chem. Soc.* **74,** 2407
23. Savage, C. R., Schumer, J. A., and Weinfeld, H. (1973) *Anal. Biochem.* **53,** 431-440
24. Krebs, H. A., Eggleston, L. V., and Knivett, V. A. (1955) *Biochem. J.* **59,** 185-193
25. Raijman, L., and Jones, M. E. (1973) in *The Enzymes* (Boyer, P. D., ed) Vol. 9, pp. 97-119, Academic Press, New York
26. Lamprecht, W., and Trautschold, I. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H-U., ed) Vol. 4, pp. 2101-2110, Academic Press, New York
27. Kimmich, G. A., Randles, J., and Brand, J. S. (1975) *Anal. Biochem.* **69,** 187-206
28. Prescott, L. M., and Jones, M. E. (1969) *Anal. Biochem.* **32,** 408-419
29. Inoue, H., and Mizutani, A. (1973) *Anal. Biochem.* **56,** 408-416
30. Ramakrishna, S., and Adiga, P. R. (1973) *J. Chromatogr.* **86,** 214-218
31. Suresh, M. R., and Adiga, P. R. (1977) *Eur. J. Biochem.* **79,** 511-518
32. Schimke, R. T., and Barile, M. F. (1963) *J. Bacteriol.* **86,** 195-206
33. Hartman, W. W., and Dreger, E. E. (1931) *Organic Synthesis,* Vol. 2, pp. 150-151, John Wiley & Sons, New York
34. March, S. C., Parikh, I., and Cuatrecasas, P. (1974) *Anal. Biochem.* **60,** 149-152
35. Cuatrecasas, P. (1970) *J. Biol. Chem.* **245,** 3059-3065
36. Sluyterman, L. A. A. E., and Wijdenes, J. (1974) *Methods En-*

zymol. **34,** 544–547
37. Suresh, M. R., and Adiga, P. R. (1979) *J. Biosci. (India)* **1,** 109–124
38. Davis, B. J. (1964) *Ann. N. Y. Acad. Sci.* **121,** 404–427
39. Reisfeld, R. A., Lewis, V. J., and Williams, D. E. (1962) *Nature (Lond.)* **195,** 281–283
40. Laemmli, U. K. (1970) *Nature (Lond.)* **227,** 680–685
41. Weber, K., Pringle, J. R., and Osborne, M. (1972) *Methods Enzymol.* **26,** 3–27
42. Andrews, P. (1964) *Biochem. J.* **91,** 222–233
43. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
44. Ouchterlony, O. (1967) in *Experimental Immunochemistry* (Weir, D. M., ed) pp. 655–656, Blackwell Scientific Publications, Oxford
45. Eid, S., Waly, Y., and Abdelal, A. T. (1974) *Phytochemistry* **13,** 99–102
46. Glenn, E., and Maretzki, A. (1977) *Plant Physiol.* **60,** 122–126
47. Knivett, V. A. (1954) *Biochem. J.* **58,** 480–486
48. Wargnies, B., Lauwers, N., and Stalon, V. (1979) *Eur. J. Biochem.* **101,** 143–152
49. Kleckzkowski, K., and Wielgat, B. (1968) *Bull. Acad. Pol. Sci. Ser. Sci. Biol.* **16,** 521–526
50. Ravel, J. M., Sund, R. F., and Shive, W. (1959) *Biochem. Biophys. Res. Commun.* **1,** 186–190
51. Reichard, P. (1957) *Acta Chem. Scand.* **11,** 523–536
52. Tabor, H., and Tabor, C. W. (1972) *Adv. Enzymol. Relat. Areas Mol. Biol.* **36,** 203–268
53. Abdelal, A. T. (1979) *Annu. Rev. Microbiol.* **33,** 130–168
54. Allen, C. M., Jr., Richelson, E., and Jones, M. E. (1966) in *Current Aspects of Biochemical Energetics* (Kaplan, N. O., and Kennedy, E. P., eds) pp. 401–412, Academic Press, New York
55. Pringle, J. R. (1975) *Methods Cell Biol.* **12,** 149–184
56. Valentine, R. C., Bojanowski, R., Gaudy, E., and Wolfe, R. S. (1962) *J. Biol. Chem.* **237,** 2271–2277
57. Kleckzkowski, K., and Grabarek-Bralczyk, J. (1968) *Acta Biochim. Pol.* **15,** 283–291
58. Akamatsu, S., and Watanabe, T. (1961) *J. Biochem.* **49,** 566–569

MINIPRINT SUPPLEMENT TO

ENZYMIC CONVERSION OF AGMATINE TO PUTRESCINE IN LATHYRUS SATIVUS SEEDLINGS: PURIFICATION AND PROPERTIES OF A MULTIFUNCTIONAL ENZYME (PUTRESCINE SYNTHASE)

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EXPERIMENTAL PROCEDURE

Materials - Lathyrus sativus seeds were procured from the plant breeding section of Indian Agricultural Research Institute, New Delhi. The germination of seeds and growth conditions for obtaining the 5 day old etiolated seedlings have been described elsewhere (18). The following chemicals were puchased from Sigma Chemical Company: amino acids, aliphatic amine hydrochlorides, 1,3(dimethylaminopropyl)carbodiimide, NADP, ADP, ATP, dithiothreitol, p-aminophenylmercuric acetate, NBT, PMS, diacetyl monoxime, antipyrene, p-diphenylamine sulfonic acid (Na salt), ion exchange media, Sephadex G-200, Sepharose CL-4B, Blue Sepharose, beef liver catalase, yeast hexokinase, glucose-6-phosphate dehydrogenase and firefly luciferase. The ion exchange resins - Dowex-50 and Dowex-1 and Amberlite CG-50 were procured from Biorad Laboratories, Richmond, CA, whereas acrylamide and bisacrylamide were the products of Eastman Organic Chemicals, Rochester, N.Y. [1-4,^{14}C]-*putrescine* dihydrochloride (54 mCi/mmol) was purchased from the Radio Chemical Center, Amersham, U.K. [^{14}C]**Urea** (38 mCi/mmol) was from Bhabha Atomic Research Centre, Bombay. Agmatine SO_4 (Sigma) was purified on a Dowex-50(H^+) column using a HCl gradient (0->4M) to remove putrescine contamination. Carbamyl phosphate was purified as described (19). Synthesis and purification of N-carbamyl putrescine was accomplished by the new proce-**dure** *detailed elsewhere* (22).

Synthesis of [ureido-^{14}C]N-carbamyl putrescine - [^{14}C]KCNO prepared by reacting [^{14}C]urea (2 mmols) and K_2CO_3 (1.2 mmols) (21,22) was mixed with putrescine.HCl (100 µmoles). Further procedure for synthesis and separation of N-carbamyl putrescine by paper chromatography were the same as outlined in the ref.13. The purified product had a sp.act. of 50 µCi/mmol.

Enzyme assays - The different catalytic activities associated with putrescine synthase, namely agmatine iminohydrolase, putrescine transcarbamylase, ornithine transcarbamylase and carbamate kinase were monitored by assaying the individual reactions as well as the overall reactions linked to either ornithine transcarbamylase or carbamate kinase. All the assays were conducted at 37°C unless otherwise stated, with an incubation period of 1h using the enzyme protein in the range of 0.02-2mg in a final volume of 1.0ml. In case of assays using the purified enzyme, 100µg of BSA was routinely included in the reaction mixtures, for stabilization purpose. Brief descriptions of the different assays employed are given below.

(i) Agmatine iminohydrolase - The activity was *measured by quantitating* either of the two products, namely, NH_3 in case of enzyme preparations devoid of diamine oxidase (for which agmatine is also a good substrate (18)) and N-carbamyl putrescine in other purification steps. NH_3 liberation was followed in microdiffusion vials (18) containing 100mM Tris-Cl buffer (pH 8.8), 3mM dithiothreitol, 0.5mM $MnCl_2$, 5mM agmatine and the enzyme. The NH_3 released was estimated by Nesslerization (18). The procedure for N-carbamyl putrescine estimation was the same as detailed earlier (20), except that N-ethyl maleimide was used to obviate the thiol interferences in the color reaction (23).

(ii) Putrescine transcarbamylase in the direction of N-carbamyl putrescine synthesis - The product was quantitated by the radiometric assay procedure (20) or colorimetrically by the method of Gerhart and Pardee (19) after removing the thiol interference (23). The reaction mixture contained 0.5ml imidazole-borax-glycyl glycine (10:10:50mM) buffer (pH 7.5), 2mM dithiothreitol, 5mM putrescine and the enzyme and was incubated at 30°C. The reaction was initiated by the addition of 10mM carbamyl phosphate and terminated with 0.1ml of 20% perchloric acid. Substrate and enzyme blanks were routinely included and the amount of N-carbamyl putrescine formed was quantitated by reference to a standard curve.

(iii) Putrescine transcarbamylase in the reverse direction (arsenolysis) - The enzymic cleavage of N-carbamyl putrescine in the presence of arsenate does not reach an equilibrium, as the product carbamyl *arsenate spontaneously* degrades into CO_2 and NH_3 (13,24). The arsenolysis of N-carbamyl putrescine was measured by quantitating the NH_3 produced in a reaction mixture containing 50mM imidazole-Cl buffer (pH 7.5); N-carbamyl putrescine, 5mM; sodium arsenate (pH 7.5), 50mM and the enzyme. The reaction mixture was incubated in the microdiffusion vials and NH_3 estimated (18). For calculating the equilibrium of putrescine transcarbamylase reaction, the assay was conducted using [ureido-^{14}C]N-carbamyl putrescine in Warburg flasks and $^{14}CO_2$ released under acidified conditions was determined (13).

(iv) Carbamate kinase - This activity was assayed in the forward direction by measuring ATP formed (25). The components of the reaction mixture were: imidazole-Cl buffer (pH 7.5), 50mM; ADP, 2mM; *carbamyl phos*-phate, 5mM; $MgSO_4$, 5mM; KCl, 0.5mM; and the enzyme protein. Carbamyl phosphate was replaced by either agmatine (5mM) plus Na_2HPO_4 (2mM) or N-carbamyl putrescine (5mM) plus Na_2HPO_4 (2mM) in the overall reactions catalyzed by putrescine synthase to demonstrate ATP synthesis with concomitant conversion of the substrates to putrescine. ATP generated in these reactions was determined by the hexokinase-glucose-6-phosphate dehydrogenase coupled assay (26) or by the sensitive luciferase procedure using the Beckman LS-100 scintillation spectrometer (27).

(v) Ornithine transcarbamylase - This catalytic activity was determined in a reaction mixture containing 50mM triethanolamine-Cl buffer (pH 8.0), 2mM dithiothreitol, 5mM ornithine and the enzyme. *The reaction was* initiated by the addition of 10mM carbamyl phosphate. Appropriate blanks lacking either the enzyme or substrates were always included. After terminating the reaction and adding N-ethyl maleimide to remove interference by sulfhydryl compounds (23), citrulline was estimated by the colorimetric procedure of Prescott and Jones (28).

(vi) Assay for overall reaction linked to ornithine transcarbamylase - The overall reaction catalyzed by putrescine synthase (namely the combined activities of agmatine iminohydrolase, putrescine transcarbamylase and ornithine transcarbamylase) was assayed by measuring citrulline production with either agmatine or N-carbamyl putrescine as the substrate (reaction 4, Table I). The assay components were: 50mM Tris-Cl (pH 8.8), 3mM dithiothreitol, 10mM $MgSO_4$, 5mM ornithine, 2.5mM Na_2HPO_4, agmatine or N-carbamyl putrescine (5mM) and the enzyme. During each assay, appropriate blanks containing the boiled enzyme were regularly included. Following the termination of the reaction with 20% perchloric acid (0.1ml) and removal of denatured proteins, the sample was applied on to a Dowex-50x12(H^+) column (4x1cm). After washing the column thoroughly with distilled water, the amino acid fraction containing citrulline was selectively eluted with 3ml of 2M NH_4OH. The amine substrates which interfere in the color reaction by producing excess of chromogen, are preferentially retained on the ion exchange resin during the above step. An aliquot of NH_4OH eluate was subjected to color reaction (28) and the difference between the reaction due to active enzyme and boiled enzyme was taken to represent the citrulline formed.

Enzyme unit - One unit of activity is defined as the amount of enzyme required to produce 1µmol product (NH_3 or N-carbamyl putrescine or citrulline) per h under the standard assay conditions described above. Specific activity is represented by units/mg protein.

Identification of the reaction products by paper chromatography - The amino acid and/or amine fractions of the reaction mixtures were purified on Dowex-50(H^+) columns (29) and resolved by circular paper chromatography using phenol:0.066M HCl-KCl buffer (pH<2) (4:1 v/v) as described (30). The radioactivity associated with amines was measured as detailed earlier (31) after making quenching *corrections. Amberlite CG-50 (NH_4^+)* resin was used to characterize citrulline fraction with concomitant retention of ornithine (32) in the overall reaction.

Preparation of affinity Sepharoses - CNBr was synthesized according to Hartman and Dreger's procedure (33). The method followed to prepare putrescine-*CH Sepharose is as follows:* The activation and washing procedures described by March et al. (34) were carried out to obtain CH-Sepharose by reacting 6-amino hexanoic acid with CNBr-activated Sepharose. The free carboxyl groups were coupled to putrescine by carbodiimide condensation (35) at pH 4.8 with two additions of 1,3(dimethylaminopropyl)carbodiimide. The coupling of the diamine was confirmed by picrylsulfonate test (35).

Organomercurial Sepharose - The coupling of p-aminophenylmercuric acetate to CNBr-activated Sepharose and the determination of its capacity were carried out by the established procedure (36). The affinity matrix had a capacity of 3.5µmol/ml gel.

Other affinity Sepharoses - Citrulline and homoarginine were linked through their α-amino groups to CNBr-activated Sepharose (34). The immunosorbent (IgG fraction specific to L. sativus diamine oxidase coupled to Sepharose) used to remove the diamine oxidase in earlier steps of purification was prepared as described earlier (37). Blue Sepharose was regenerated by washing with 2M NaCl solution prior to protein purification.

Disc gel electrophoresis - Polyacrylamide gel electrophoresis at pH 8.3 and 4.0 were carried out according to Davis (38) and Reisfeld et al. (39) respectively. The procedure of Laemli (40) was utilized for SDS-gel electrophoresis. To ensure complete denaturation, the protein was treated with guanidine hydrochloride followed by urea and SDS before electrophoresis on denaturing gels, as described previously (41).

Determination of M_r by gel filtration - Gel filtration of putrescine synthase was conducted on a Sephadex G-200 (coarse; 40-100µ) column (1.8x89cm) pre-equilibrated with 50mM Tris-Cl buffer (pH 8.0) containing 2mM 2-mercaptoethanol and 0.1M KCl. The mixture of standard proteins and enzymes (ovalbumin,45000; BSA monomer, 68000; dimer, 136000; alcohol dehydrogenase, 150000 and catalase 240000) was applied and the column eluted with the above buffer at a flow rate of 20ml/h. Fractions of 2ml were collected. The M_r was determined by the method of Andrews (42).

Other methods - Protein was estimated by the procedure of Lowry et al. (43) with BSA as standard. Antibodies to putrescine synthase were raised in rabbits by administering 0.5-1mg of pure enzyme protein in phosphate-buffered saline emulsified with an equal volume of Freund's complete adjuvant (Difco) (by injecting subcutaneously at 10 day intervals). Following three injections, a booster shot of 1mg protein in saline was given and after 6 days, the animal was bled and serum prepared. Ouchterlony double diffusion analysis was carried out on 1.25% agar gels (44).

RESULTS

Purification of putrescine synthase

Procedure I - All the steps were carried out at 4°C and centrifugation at 25000xg for 30min, unless otherwise indicated.

Step 1: Preparation of crude extract - Fresh 5-day old L. sativus seedlings were washed and homogenized in a chilled Waring blendor with one volume of 50mM imidazole-Cl buffer (pH 8.0) containing 5mM 2-mercaptoethanol. The homogenate was passed through four layers of cheese cloth to remove the fibrous material and centrifuged.

Step 2: Precipitation of nucleoproteins - The crude extract from the first step was adjusted to 7.5mM $MnCl_2$ concentration. After stirring for 30min, the precipitated nucleoproteins were removed by centrifugation.

Step 3: Ammonium sulfate fractionation - The step 2 supernatant fraction was brought to pH 7.0 with addition of 2M NH_4OH. Precooled solid $(NH_4)_2SO_4$ was added in small amounts with stirring to achieve 45% saturation. After 30min of stirring in the cold, the precipitate was centrifuged and discarded. The supernatant was adjusted to 85% saturation with $(NH_4)_2SO_4$ and stirred for 1h. The precipitate obtained after centrifugation was dissolved in 5mM imidazole buffer (pH 7.5, containing 2mM 2-mercaptoethanol) and dialyzed against two changes of 2L of the same buffer.

Step 4: Organomercurial Sepharose affinity step - Taking advantage of the sulfhydryl nature of agmatine iminohydrolase (8) and the total absence of cysteine residues in the diamine oxidase of L. sativus (37), a group specific affinity step for the purification of sulfhydryl proteins was employed. The regenerated p-aminophenylmercuric acetate column (3.5x25cm) was equilibrated with 50mM *imidazole buffer (without mercaptoethanol)* and the dialyzed $(NH_4)_2SO_4$ fraction (step 3) was applied at a slow flow rate (0.2ml/min). After washing off all the unadsorbed proteins, until the effluent had a A_{280} of <0.05, the proteins held on the column were eluted with 50mM imidazole buffer (pH 7.5) containing 20mM 2-mercaptoethanol and fractions of 2ml were collected. This fraction was dialyzed against 20mM imidazole buffer (2L), overnight to remove mercaptoethanol.

Step 5: Chromatography on DEAE-Sephadex - The concentrated protein fraction from the organomercurial-Sepharose step was fractionated on a column of DEAE-Sephadex. The elution and activity profiles clearly show that the different catalytic activities associated with putrescine synthase emerged with the single protein peak *eluted with* 0.5M KCl (Fig.1). At this stage of purification, polyacrylamide gel electrophoresis of the protein showed a single fast moving component (Fig.2a). The protocol employed has been summarized in Table II which shows a 157-fold purification and 15% recovery with respect to agmatine iminohydrolase activity.

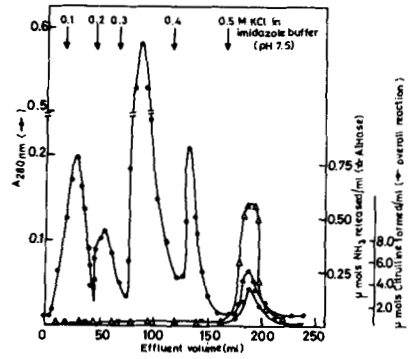

Fig.1. DEAE-Sephadex chromatography of putrescine synthase. The protein fraction eluted from *organomercurial Sepharose with 2-mercapto*-ethanol was applied on a column (2.2x30cm) of DEAE-Sephadex (A50-medium) preequilibrated with 50mM imidazole buffer (pH 7.5). The column was washed with the same buffer and the proteins eluted step-wise with 0.1, 0.2, 0.3, 0.4 and 0.5M KCl in the above buffer. The activity of agmatine iminohydrolase in the eluent was followed by estimating ammonia liberated while the overall *reaction was assayed* by quantifying citrulline formed. AIHase represents agmatine iminohydrolase.

TABLE II

Purification of putrescine synthase (Procedure I)

The purification was monitored by assaying agmatine iminohydrolase component of the multifunctional enzyme.

Purification step	Protein mg	Total enzyme units	Sp.act.	Fold purification	Recovery %
1. Crude extract	9360	56.4	0.007	1.0	100
2. $MnCl_2$ treatment	6898	68.9	0.01	1.4	120
3. $(NH_4)_2SO_4$ fractionation	1944	58.3	0.03	4.2	102
4. Organomercurial Sepharose affinity	72	25.2	0.35	50.0	45
5. DEAE-Sephadex chromatography	7.7	8.5	1.10	157.0	15

Purification of putrescine synthase by affinity chromatography using putrescine-CH Sepharose (Procedure II)

A specific putrescine-CH Sepharose affinity step was later developed for the rapid purification of the chimeric enzyme with good recovery of all associated activities, in lieu of the more laborious organomercurial affinity step plus DEAE-Sephadex chromatography (Procedure I, steps 1-5).

The dialyzed protein fraction (step 3) was subjected to affinity chromatography on the putrescine-CH Sepharose column. It is clear from Fig.3 that 2mM putrescine effectively eluted the protein as a single peak which had different component activities associated with putrescine synthase. The pooled, dialyzed and concentrated protein fraction of this step showed on polyacrylamide gel electrophoresis, a single protein species (Fig.2b) coincident with and inseparable from the one obtained by the procedure I. Therefore, in further studies, this affinity procedure was routinely utilized for enzyme purification.

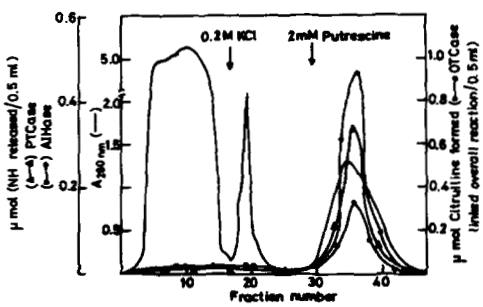

Fig. 3. Affinity chromatography of putrescine synthase on putrescine-CH Sepharose. The dialysed ammonium sulfate fraction (step 3) was adsorbed on the affinity column (10 ml bed vol.), preequilibrated with 50 mM imidazole buffer. The unadsorbed proteins were removed by washing with the same buffer, and then with 0.2M KCl in this buffer, which however failed to dislodge the enzyme activity. The enzyme was eluted specifically by the addition of 2 mM putrescine in the imidazole buffer. The enzyme activities in different fractions were determined after dialysis to remove the diamine. AIHase, PTCase and OTCase stand for agmatine iminohydrolase, putrescine transcarbamylase and ornithine transcarbamylase respectively.

General properties

The pH optima for the different activities of putrescine synthase are listed in Table VII along with the buffers used. Phosphate buffers (Na or K) were found to be inhibitory to all the component activities, to different extents. In general, an incubation temperature of 37°C was found optimal for the assay of different reactions; however, putrescine transcarbamylase assay in the direction of N-carbamyl putrescine synthesis was performed at 30°C to minimizethe nonenzymatic destruction of carbamyl phosphate.

TABLE VII

Optimal pH conditions for the different catalytic activities of putrescine synthase

Component activity	pH optimum	Buffer used
Agmatine iminohydrolase	8.8*	Tris-Cl
Putrescine transcarbamylase (N-carbamyl putrescine synthesis)	7.5	Imidazole-borax-glycyl glycine (10:10:50 mM)
Putrescine transcarbamylase (arsenolysis)	7.5	Imidazole-Cl
Carbamate kinase and the overall reactions linked therewith	7.5	Imidazole-Cl
Ornithine transcarbamylase	8.0	Triethanolamine-Cl
Overall reaction linked to ornithine transcarbamylase	8.8*	Tris-Cl

* pH values have not been corrected for temperature effect.

Substrate specificity - Arginine, arcain and creatinine failed to serve as substrates for agmatine iminohydrolase activity. Apart from putrescine, the putrescine transcarbamylase component also carbamylated cadaverine and spermidine with an efficiency as low as 4% and 0.5% respectively, compared to putrescine. The broad substrate specificity observed for putrescine transcarbamylase is consistent with the findings with other systems (48,49). However, putrescine transcarbamylase did not attack citrulline and N,N'-dicarbamyl putrescine in the arsenolysis assay. Aspartic acid could not replace ornithine in the ornithine transcarbamylase-linked overall reaction.

Effect of metal ions, sulfhydryl reagents and other compounds - Among the monovalent cations K^+ was found to be essential for carbamate kinase activity, while metal ions like Mg^{++} did not affect either arsenolysis or carbamylation of putrescine. It is also clear from the data presented in Table VIII, that among divalent cations tested, Mn^{++} enhanced the agmatine iminohydrolase activity to a considerable extent. Mn^{++} also replaced Mg^{++} to some extent in the overall reactions I and II. The thiol nature of the agmatine iminohydrolase activity is exemplified by its profound inhibition by all sulfhydryl blockers used, and also by its affinity to organomercurial Sepharose referred to earlier. However, influence of sulfhydryl blockers on other individual activities were not tested. Arcain, the diguanido analogue of agmatine exerted a 30% inhibition of agmatine iminohydrolase at 0.5mM concentration, which is in agreement with the previous observation (8).

TABLE VIII

Effect of different compounds on agmatine iminohydrolase component of putrescine synthase

Agmatine iminohydrolase activity was assayed under standard assay conditions by quantitating the NH_3 released. The enzyme activity without the addition of any test compound was taken as 100% and the results have been expressed in terms of % relative activity.

Compounds tested (mM)	Relative activity %	Compounds tested (mM)	Relative activity %
Divalent cations		Sulfhydryl blockers	
Mn^{2+} (0.5)	400	p-Hydroxymercuri-benzoate (0.2)	65
Mn^{2+} (2.0)	350	Iodoacetate (1.0)	50
Mg^{2+} (1.0)	110	N-Ethyl maleimide (0.5)	30
Fe^{2+} (1.0)	105	Other compounds	
Hg^{2+} (0.5)	70	Arcain (0.5)	70
Thiol compounds		Pyridoxal phosphate (0.2)	100
Dithiothreitol (2.0)	150	Semicarbazide (0.1)	100
2-Mercaptoethanol (2.0)	120		
GSH (2.0)	125		

Initial velocity studies on agmatine iminohydrolase - The enzyme exhibited a typical Michaelis-Menten kinetics, when the velocity of the reaction (NH_3 liberation) was plotted against the concentration of agmatine. The highest activity was observed in the range of 3-5mM of the guanidoamine and significant substrate inhibition was observed. When agmatine concentration was >5mM. From the Lineweaver-Burk plot, a K_m value of 1 mM for agmatine was obtained.

Equilibrium of the putrescine transcarbamylase reaction - It has been earlier noted that the bacterial putrescine transcarbamylase exhibits a reaction equilibrium highly favourable in the direction of N-carbamyl putrescine synthesis and is thus analogous to ornithine transcarbamylase in this respect (13). To examine whether this observation is applicable to the putrescine transcarbamylase activity associated with the plant putrescine synthase, the putrescine transcarbamylase reaction in backward direction was followed by measuring the formation of carbamyl phosphate from [ureido-^{14}C]N-carbamyl putrescine and Pi at short intervals. From the Fig. 10, it can be seen that there was a progressive increase in the $^{14}CO_2$ released during the first 15 min, which was followed by a relatively slower increase thereafter. The end of initial rapid phase presumably represents the attainment of the equilibrium. The carbamyl phosphate formed could be expected to undergo a slow non-enzymic decomposition during incubation at 37°C, which in turn would drive the reaction forward, resulting in a decreased production of carbamyl phosphate during second slower phase (Fig. 10). A K_{eq} of 10^5 was obtained for the putrescine transcarbamylase reaction, which is similar in magnitude to those of the bacterial putrescine transcarbamylase (13) and ornithine transcarbamylase of rat liver (51).

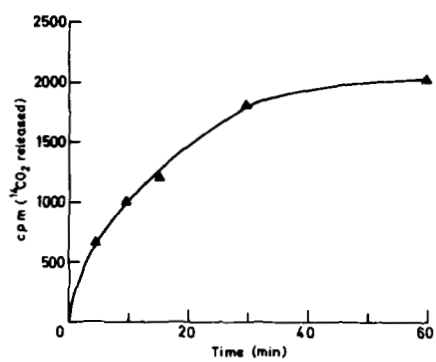

Fig.10. Equilibrium of putrescine transcarbamylase reaction. Formation of ^{14}C carbamyl phosphate from [ureido-^{14}C]N-carbamyl putrescine and Pi was determined. The assay was carried out in closed Warburg flasks, in a reaction mixture (1.0ml) containing potassium phosphate buffer (pH 6.2), 200 μmols; [ureido-^{14}C]N-carbamyl putrescine ($2x10^5$cpm), 4μmols; BSA, 500 μg and 100 μg of homogeneous putrescine synthase. From the above data, an equilibrium constant

$$K_{eq} = \frac{(\text{N-carbamyl putrescine})(\text{Pi})}{(\text{putrescine})(\text{carbamyl phosphate})} = 10^5$$

was calculated.