CHAPTER IV

DISCUSSION

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Groundnut (Arachis hypogea. L.) seeds contain large amount of protein bound arginine (Block, 1945). During germination, the storage proteins are hydrolysed to give amino acids which may either be metabolised in situ or directly transported to the growing embryo. Arginine, in addition to its role in urea cycle, may also be utilized for glutamate and proline formation (Mazelis and Fowden, 1969) or serve as a precursor for polyamine biosynthesis (Smith, 1970). Several studies have been carried out on the enzymes of arginine metabolism in the cotyledons of developing and germinating seeds (Jones and Boulter, 1968; Cheema et al, 1969; Splittstoesser, 1969b; Kollöffel and Stroband, 1973; Kollöffel and Van Dijke, 1975). No attempt, however has been made to compare these enzymes in the accompanying embryo tissue, which is associated with the cotyledon in two entirely different conditions during development and germination. Studies were, therefore carried out on arginine metabolism in groundnut seeds during development and germination under the influence of various hormones and other compounds.

It is suggested that during development there are two phases of growth (Millerd et al, 1971), an initial one of cell division and a latter one of cell expansion. The accumulation of storage material occurs mainly in the expansion phase. The

final phase of seed development (maturation) is marked by a severe water loss, a gradual reduction of metabolic activity and a very small increase in the dry weight. Rao and Rao (1978) have studied the nitrogen metabolism of developing and germinating seeds of pigeon pea (Cajanus indicus). A gradual decrease in total nitrogen occurred in the developing seed coat (testa), whereas in the embryo, total nitrogen increased. The protein content of the whole seed and the embryo increased continuously during germination but in the seed coat, protein continuously decreased. Madison et al (1976) have analysed the protein content of the seeds of soybean, pea, kidney beans and groundnut during seed development. The protein level of kidney beans was relatively constant during development, whereas that of pea, groundnut and soybean increased throughout the development. The increase in groundnut took place early and that in pea, took place later in development. The results of the present study are in agreement with the data reported by Madison et al (1976) for groundnut. The protein content of the cotyledon and the embryo increased rapidly during development of the seed but decreased with the period of germination.

The data on the level of activity of enzymes during development and germination suggest that the later stages of development which are associated with the accumulation of reserve proteins showed high OCT and low arginase and OKAT

activity. During germination, where the reserve proteins of the cotyledon are being broken down, the OCT remained unchanged but the arginase and OKAT activities increased significantly and remained high during the period of germination. The embryo, which represents a growing tissue, retained OCT activity but arginase and OKAT were considerably reduced after an initial increase, with the emergence of the radicle around 3rd day of germination. Similar observations regarding arginase have been made by Kollöffel and Van Dijke (1975) for the cotyledons of developing and germinating seeds of Vicia faba. In both dry and soaked seeds of Vicia faba arginase was higher in radicles than in the cotyledons. This was reversed in the 6 day old seedling (Genevieve and Genevieve, 1967). The OCT activity in the Vicia faba was high in the cotyledons of developing seeds, decreased sharply during maturation and further decreased during subsequent germination (Kollöffel and Stroband, 1973). In the present study however, no decrease in OCT was found during germination of seeds either in the cotyledon or the embryo. The maximum arginase and OKAT activity in the cotyledons of germinating seeds were much higher than from developing cotyledons. This is in agreement with the results of Splittstoesser (1969b) for the arginase activity in pumpkin seeds and of Kollöffel and Stroband (1973) for Vicia faba.

The pattern of changes in PCT activity of the cotyledon and embryo was different both during development and germination. This clearly indicated the difference between the cotyledon and embryo enzyme.

The enzyme arginase and OKAT of groundnut cotyledon and embryo were inhibited by auxins, kinetin and gibberellic acid at early stages of germination. The activity returned to control level with increase in the period of germination in the cotyledon but in the embryo it increased to more than the control level. OCT and PCT activity of the cotyledon and embryo remained unchanged in the presence of plant hormones.

Several investigators have reported that auxins, (Abeles, 1973), gibberellic acid (Ketring and Morgan, 1970) and kinetin (Burg and Burg, 1968; Fuchs and Lieberman, 1968) have promoting effect on ethylene production in groundnut seeds. Experiments were carried out with ethrel and chloroethanol, known to produce ethylene (Abeles, 1973). Since the pattern of changes in the activity of arginase and OKAT was similar to that of plant hormones, the effect of plant hormones appears to be mediated through ethylene.

The higher activity of arginase and OKAT in the embryo in constrast to the cotyledon at later stages of germination in presence of plant hormones and ethylene producing compounds is of great significance since it suggests that the control mechanisms in the cotyledon and the embryo must be different.

Earlier studies from this laboratory (Sindhu, 1977) showed that in groundnut embryo the diamine oxidase activity is inhibited by plant hormones and ethylene producing compounds which would result in an increase in polyamine concentration. The polyamine concentration may then be regulated by an inhibitory control on enzymes involved in the synthesis of polyamines and/or by channelling arginine more for glutamate or proline pathway as a result of increase in the enzymes of this pathway. Atleast one of the enzymes involved in polyamine biosynthesis, agmatine iminohydrolase, of the groundnut embryo was found to be inhibited by polyamines (Sindhu, 1977). Thus by the inhibition of agmatine iminohydrolase and a simultaneous increase in the activity of arginase and OKAT, arginine becomes available for the growing embryo.

The question arises why such a stimulation of arginase and OKAT does not occur in the cotyledon. The presence of a natural inhibitor of diamine oxidase in the cotyledon (Sindhu, 1977), suggests that the polyamine levels in the cotyledons may not be controlled by a mechanism similar to that of embryo. Feeding experiments with polyamines showed that polyamines stimulate arginase and OKAT of embryo at later stages of germination without affecting OCT. In case of cotyledon, polyamines have no effect on these enzymes. High concentration of polyamines in cotyledons did not affect arginase and OKAT of cotyledon,

suggesting that polyamines have a different role to play in the cotyledon. The polyamines produced in the cotyledon might be transported to the growing embryo and the arginase and OKAT of the cotyledon have only degradative role.

The increased activity of arginase and OKAT in the embryo was not due to the stabilization of the existing enzyme but appeared to be due to the increase in the synthesis of these enzymes in presence of plant hormones and ethylene producing compounds. The results on the effect of cycloheximide support this suggestion. Cycloheximide has been shown to prevent protein synthesis at translational level and the action of ethylene (Abeles, 1973). In the cotyledons, in the presence of cycloheximide, the effect of hormones or ethylene producing compounds was enhanced, which suggests that the cycloheximide might interfere with the degradation of these compounds.

5-Fluorouracil, ~~an another inhibitor of protein synthesis~~ inhibits protein synthesis by suppressing the synthesis of nucleic acids. It can be incorporated into nucleic acid as an abnormal nucleotide producing a physiologically inactive product, It also prevents methylation at position 5, to produce thymidine derivatives, which are required for the synthesis of normal nucleic acids. In the present studies, 5-fluorouracil, however, had no effect on the two enzymes of embryo, either alone or in combination with auxin and ethylene producing compounds

suggesting that the increased synthesis of the two enzymes does not require the synthesis of new nucleic acids.

The arginine metabolism in the groundnut embryo thus appears to be controlled as follows : Plant hormones by affecting ethylene production would inhibit the synthesis of diamine oxidase and cause an increase in the level of polyamines in the tissue. The increased polyamines would then decrease their own synthesis by inhibiting agmatine imino-hydrolase activity. Arginine is then channelled for the formation of glutamate and proline by increasing the arginase and OKAT activity without any effect on OCT.

The regulatory control of the embryonal arginase and OKAT is not influenced by the accompanying cotyledon is demonstrated by cultivating the embryo on a synthetic medium. The pattern of changes in the enzyme activity was same as that in the embryos from whole seeds.

The studies on the differential characteristics of cotyledon and embryo arginases reported earlier indicate that the arginase of cotyledon and embryo may be two different proteins. The two enzymes were purified separately. Arginase has been isolated from animal sources and microorganisms to a marked degree of purity. However, the enzyme from higher plants has not been purified and characterised to the same extent. Arginase has been partially

purified from seeds of Canavalia ensiformis (Roosemont, 1969), Lathyrus sativus (Cheema et al, 1969), Phaseolus vulgaris (Dumitru and Dana, 1970), Glycine hispida (Dumitru, 1973), from pumpkin cotyledons (Splittstoesser, 1969b) and from 7 day old plants of bitter blue lupine (Muszynska and Reifer, 1968). In the present study the presence of mercaptoethanol in the grinding medium was essential for embryo enzyme, but it had no effect on the cotyledon enzyme suggesting that the nature of the two proteins is different. Presence of $MnCl_2$ in dialyzing medium was essential for both the enzymes during purification. Arginase from Canavalia ensiformis which was inactivated during purification could be reactivated at pH 9.0 by low concentrations of Mn^{++} and at pH 8.0 by Co^{++} and Ni^{++} (Baret and Mourgue,1960).

The optimum pH for cotyledon and embryo enzyme in present case was 9.5 with carbonate bicarbonate buffer and 10.5 with glycine - NaOH buffer. Arginase from etiolated cotyledons of pumpkin seeds had a pH optimum of 9.5 in pyrophosphate buffer (Splittstoesser, 1969b). The crude and purified preparations of enzyme from Phaseolus vulgaris seeds showed pH optimum at 7.3 and 8.7 respectively at $38^{o}C$ (Dumitru and Dana, 1970), whereas the enzyme from Limonium vulgare hadpH optimum of 9.8 (Francois and Goas, 1967). Pierrette (1962) has reported an optimum pH of 10.3 for arginase from leaves of Aspidium aculeatum. In case of jack bean arginase the optimum pH varied from 7.5 to 9.0

depending on the concentration of Co^{++}. The optimum pH with Mn^{++} was about 8.8 and was independent of the Mn curve (Anderson,1945).

The Km value for arginine for the cotyledon and embryo arginase in present case was found to be 5.8 mM and 5.6 mM respectively at pH 9.5. The reported Km values for arginase from plant sources has been shown to vary widely. A Km value as high as 50 mM for arginase from Glycine hispida (Dumitru,1973) to as low as 0.026 mM for pumpkin cotyledon arginase (Splittstoesser, 1969b) has been reported. However, the values obtained in present case are in agreement with the Km value of 5.5 mM for arginase from Lathyrus sativus (Cheema et al, 1969) (A comparative data on arginase from various sources is given in Table 80).

The cotyledon and embryo arginase in present study also differ in their energy of activation and heat stability. The cotyledon enzyme was comparatively more stable than the embryo enzyme when heated at $80^{o}C$ for 10 minutes. Arginase from pumpkin cotyledons was quite resistant to heat and could be heated for 5 minutes at $60^{o}C$ with little loss of activity (Splittstoesser, 1969b).

The differences observed in certain kinetic characteristics of the cotyledon and embryo arginase may result from the differences in the procedures used for the purification of the two enzymes such as heat treatment and use of DEAE Sephadex for cotyledon enzyme. The environment of the two purified preparation was however the same during the studies.

Table 80 : Some collected data on arginase from various sources.

Source	Optimum pH	Km for arginine (mM)	Reference
Rabbit liver	9.8	1.4	Francoise et al (1971)
Rat liver	9.5	3.8	Herzfeld and Raper (1976)
Rat small intestine	10.0	4.9	Herzfeld and Raper (1976)
Rat brain	9.8	16.0	Bunyatyan et al (1970)
Human leukemic lymphocytes and granulocytes	8.5	2.7-3.1	Cristina and Friedrich (1975)
Human full term placenta	9.1	27.0	Porta et al (1976)
Ureotelic vertebrate liver		∠50	Mora et al (1965)
Fish liver		68	Patnik et al (1976)
Frog liver		87	Patnik et al (1976)
Bat liver		24	Patnik et al (1976)
Chick kidney			
(a) High arginine requiring strain		14 (at pH 9.5) 37 (at pH 7.5)	Kadowaki and Nesheim (1979)
(b) Low arginine requiring strain		12 (at pH 9.5) 29 (at pH 7.5)	Kadowaki and Nesheim (1979)

contd...

Table 80 : contd.

Source	Optimum pH	Km for arginine (mM)	Reference
Saccharomyces cerevisiae	9.2	12.8	Chan and Cossins (1973)
Pumpkin cotyledon	9.5	0.026	Splittstoesser (1969b)
Phaseolus vulgaris	8.7		Dumitru and Dana (1970)
Lathyrus sativus	9.5-10.0	5.5	Cheema *et al* (1969)
Glycine hispida		50	Dumitru (1973)
Groundnut			
Cotyledon	9.5	5.8	Present studies
Embryo	9.5	5.6	Present studies

The enzyme OCT has been isolated from animal tissues (Burnett and Cohen, 1957; Reichard, 1957; Grillo and Bedino, 1968; Grillo and Pinna, 1972; Mataix and Ruiz - Amil, 1975) microorganisms (Rogers and Novelli, 1962; Schimke et al, 1966; Bishop and Grisolia, 1967; Dundas, 1970; Marshall and Cohen, 1972; Penninckx et al, 1974; Boggess and Naylor, 1975; Dolgikh and Blagoveshchenskii, 1975; Abdelal et al, 1977) and from higher plants (Kleczkowski and Cohen, 1964; Tam and Patil, 1972; Waly and Abdelal, 1974; Glenn and Maretzki, 1977).

The enzyme from several tissues has been shown to catalyse the carbamylation of lysine (Jasiorowska and Kleczkowski,1970). Plant and bovine liver OCT also catalyse the carbamylation of putrescine with much higher efficiency than that of lysine (Kleczkowski and Wielgat, 1968; Wielgat and Kleczkowski,1970, 1971). Kleczkowski and Wielgat (1968) studied the carbamylation of ornithine and putrescine in pea seedlings and found that the ratio of OCT/PCT remained constant even after extensive purification of the enzyme, suggesting that the same enzyme protein is responsible for the carbamylation of both ornithine and putrescine though some differences in optimal reaction conditions for ornithine and putrescine carbamylating activities were reported. Optimum pH for OCT activity was found to be 8.45 and higher pH caused a drop of activity (Kleczkowski and Cohen, 1964). In case of putrescine optimum pH was found to be

10.5 and at pH 8.45 this activity is only about 1/10th of that found at optimum pH (Kleczkowski and Wielgat, 1968).

In the present studies although it was not possible to separate the two activities during purification, the ratio of OCT/PCT was significantly altered. The optimum reaction conditions for OCT and PCT showed some differences. With glycine NaOH buffer, the optimum pH for OCT was found to be 10 and for PCT, the activity steadily increased with the increase in pH. The energy of activation for OCT and PCT was also found to be different. Table 81 shows the comparision of pH optimum, Km values and energy of activation of OCT from various sources. Pea seedling PCT showed Km value for putrescine and carbamyl phosphate to be 8.1 mM and 0.5 mM respectively. The Km values for PCT in present case are 4.03 mM and 0.19 mM for putrescine and carbamyl phosphate respectively.

Wielgat and Kleczkowski (1973) while comparing moleeular weights of OCT from various sources, suggested that OCT may be composed of several subunits. Plant and bovine liver OCTs which can carbamylate putrescine, have a molecular weight of 110,000 whereas E. coli OCT which can not carbamylate putrescine, has a molecular weight of 60,000. If this is true, and assuming that plant and mammalian OCTs are not able to dissociate into subunits, then the results of the present study may be explained by assuming the groundnut OCT as an enzyme with subunits and that the specificity of the enzyme depends on its subunit structure.

Table 81 : Some collected data on OCT from various sources.

Source	Optimum pH	Km for ornithine (mM)	Km for carbamyl phosphate (mM)	Energy of activation Kcal/mole	Reference
Mycoplasma hominis 07	8.4	3.6	2.3		Schimke et al (1966)
Streptococcus lactis	8.5	2.0	3.7	20.5	Ravel et al (1959)
Streptococcus faecalis	8.5	2.0	3.7		Jones (1962)
E. coli	8.5-8.9	1.5	0.2		Rogers and Novelli (1962)
Rat liver	8-9	1.4	0.4		Reichard (1957)
Beef liver	8.0	3.0	1.2	13.1	Burnett and Cohen (1957) Joseph et al (1963)
Frog liver	8.0			13.1	Brown and Cohen (1959)
Guinea pig liver	7.8	4.4	5.0		Mataix and Ruiz-Amil (1975)
Neurospora crassa	9.0	1.9	0.3		Davis (1962)
Phaseolus vulgaris	8.5	5.0	1.7		Tam and Patil (1972)

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Table 81 : contd.

Source	Optimum pH	Km for ornithine (mM)	Km for carbamyl phosphate (mM)	Energy of activation Kcal/mole	Reference
Pea seedling	0.45	2.7	0.25		Wielgat and Kleczkowski (1973) Kleczkowski and Cohen (1964)
Nostoc Muscorum	9.5	2.5	0.7	12.3	Boggess and Naylor (1975)
Vitis vinifera L.	8.4 - 8.8	5.5	3.5		Roubelakis and Kliewer (1978a)
Groundnut cotyledon	10	1.2	0.2	17.2	Present studies