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Development of Cotton Fruit. III. Amino Acid Accumulation in Protein and Nonprotein Nitrogen Fractions of Cottonseed[1]

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ABSTRACT

This study was conducted to determine the metabolic events occurring during cottonseed development as revealed by N and amino acid compositions of both the whole seed and the free amino acid pool. Flowers on field grown cotton (*Gossypium hirsutum* L.) plants were tagged the day of anthesis, and bolls were sampled at intervals between 10 days after bloom and maturity. Developing seed were analyzed for both total N and nonprotein N (NPN). While total N was accumulated by the seed throughout development, NPN increased until 21 days after anthesis and then declined. At boll opening, only about 1% of the total N was in the NPN fraction.

Amino acid compositions of the seed and the NPN fraction were also determined. In juvenile seed tissue, over 50% of the NPN was accounted for by asparagine. Other amino acids prominent in this free amino acid pool of young cottonseed were, in descending order, glutamine, aspartic acid, glutamic acid, and arginine. At maturity, asparagine and glutamic acid each accounted for about 15% of the NPN. Gamma-amino butyric acid and ethanolamine were also observed in the NPN fraction. Significant shifts in total amino acid distribution were seen to occur during the fourth week of seed development. The amino acid profile of cottonseed after the fifth week of development closely approximated that of mature seed.

Additional index words: ***Gossypium hirsutum*** **L., Oilseed, Protein quality, Seed development, Free amino acids.**

PROTEIN synthesis amino acid metabolism, and transport of nitrogenous compounds to developing seed have been studied in many crops. Accumulation of N into "crude protein" has been measured both in cereals (Wiggans and Frey, 1958; Ingle, et al. 1965; Pomeranz, et al. 1966) and in oilseeds (Pickett, 1950; Schenk, 1961; Aldana, et al. 1972; Rubel et al. 1972).

Cereals assimilate nitrogen into protein, which is stored in protein bodies (Jennings and Morton, 1963). Sodek and Wilson (1970, 1971) found developing maize (*Zea mays* L.) endosperm to be capable of extensive amino acid metabolism, converting amino acids supplied into those required for protein synthesis. The amino acid composition of developing oats was found to be relatively constant from 10 days after anthesis until maturity (Brown, et al. 1970).

Atkins, et al. (1975) studied amino acid transport to the developing seed of lupine (*Lupinus alba* L.). They found that asparagine was the major phloem-mobile amino acid and the principal free amino acid in the seed. Since the phloem was the primary source of both amino and amide nitrogen (Atkins, et al., 1975), the seed must be capable of extensive amino acid metabolism to convert those amino acids supplied into those required for protein synthesis. Significantly, developing lupine seed contained asparaginase activity which provided the capacity to effect nitrogen transformations from the supplied asparagine.

Although accumulation of crude protein by oilseeds has been investigated, precedent studies of the distribution of the total and free amino acid complement during oilseed development are singularly lacking. This paper describes the composition of the free amino acid pool (NPN) in developing cottonseed (*Gossypium hirsutum* L.) and the subsequent incorporation of these amino acids into proteins. Information of this type is needed because of the continuing importance of cottonseed as a source of protein. Additionally, these data will facilitate economic and environmental investigations of nitrogen uptake and utilization in a cotton field situation, such as with the computer modeling techniques of Mutsaers (1976).

MATERIALS AND METHODS

Cotton (*Gossypium hirsutum* L. 'Stoneville 213') was planted in the field at Stoneville on 25 Apr. 1974. Flowers were tagged at anthesis (either 16 July or 27 July). Bolls developing at nodes tagged 16 July were harvested 10 days after anthesis and then weekly between 14 days and boll cracking. The second set of bolls was sampled only at maturity. Each harvest comprised either two or three bolls from each of four replications. Harvested bolls were frozen and maintained at —75 C until they were lyophilized. Developing seed were separated from the lyophilized bolls, delinted, weighed, and finely ground.

Total N in the seed was determined using a Coleman Nitrogen Analyzer[3]. Samples from two of the four replications were hydrolyzed; total amino acids were determined with a Beckman Model 121 amino acid analyzer. The standard Moore-Stein hydrolysis procedure as described in the Beckman Instruction Manual (Anonymous, 1974) was used, except that the samples were not frozen in dry ice-acetone and Pierce screw cap hydrolysis tubes were used.

Free amino acids were extracted from samples of the remaining two replications with an acid alcohol solution as reported by Cherry (1973), except that a Dowex 2-X8 column was used as described in the Beckman Instruction Manual (Anonymous, 1974).

Acidic and neutral amino acids in this alcoholic extract were separated with the lithium citrate buffer system described by Benson et al. (1967) to determine glutamine and asparagine. Basic amino acids were determined using the Beckman standard physiological column procedure. Checks were made to determine that the acid alcohol did not hydrolyze the amide group of asparagine and glutamine during extraction. Contact with the diluting buffer (pH 2.2) for several days will hydrolyze the amide group, especially that from glutamine.

The internal standards norleucine and α-amino-β-guanidino propionic acid were added to the diluting buffer to enable correction for losses on each column. Identification and quantification of all reported amino acids were determined using the Beckman standard calibration mixture or analytical reagents from Sigma Chemical Co.

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[3] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the USDA, and does not imply its approval to the exclusion of other products that may also be suitable.

Table 1. Amino acids, ammonia, and N concentrations and weight of developing cottonseed.

Factor	Days after anthesis								
	10	14	21	28	35	42	49	56	L.S.D.†
	μmoles · gdw^{-1}								
Lysine	60	34	44	74	85	86	106	110	18**
Histidine	21	16	20	35	45	46	57	59	9**
Ammonia	640	524	567	427	371	330	396	404	109**
Arginine	63	44	58	136	179	176	214	219	38**
Aspartic Acid	500	503	557	323	236	196	252	256	84**
Threonine	47	44	52	61	71	75	96	100	15**
Serine	70	55	63	94	124	129	154	161	21**
Glutamic Acid	224	254	258	276	378	402	482	511	66**
Proline	39	25	32	62	83	88	106	108	14**
Glycine	73	52	64	103	138	148	190	202	23**
Alanine	79	78	79	98	120	125	155	163	22**
1/2 Cystine	7	5	16	7	21	13	52	34	ns
Valine	35	25	35	59	74	81	106	106	18**
Methionine	28	29	23	31	31	33	39	37	8*
Isoleucine	25	18	24	38	46	52	68	67	12**
Leucine	63	44	56	90	115	124	150	123	52**
Tyrosine	tr	tr	tr	16	32	36	46	46	3**
Phenylalanine	24	tr	10	45	66	72	89	92	17**
N, %‡	3.30	2.82	2.92	3.21	4.12	4.32	5.09	5.41	
wt, mg · seed^{-1}§	6.3	16.5	31.8	43.1	54.4	75.4	72.4	76.5	

*,** Level of significance (0.05 and 0.01, respectively) of the F test.
† L.S.D. (0.05 probability) is presented for each amino acid in which the F test from the analysis of variance was significant for differences among sample age.
‡ Data are taken from Leffler and Tubertini (1976).
§ Data are taken from Leffler (1976).

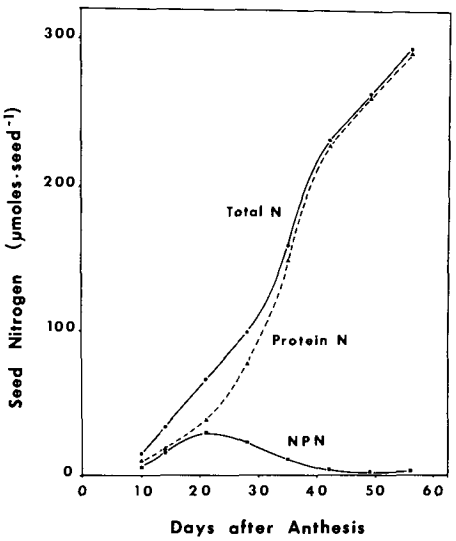

Fig. 1. Distribution of N between protein and nonprotein fractions of developing cottonseed.

RESULTS AND DISCUSSION

Most amino acids increased (gdw^{-1} basis) during cottonseed development (Table 1). At least part of the increase in concentration resulted from the accumulation of protein in the seed. However, the aspartic acid concentration decreased significantly, from over 500 μmoles · gdw^{-1} in the first three samples to half that in mature seed. Aspartic acid accounted for nearly 40% of the total amino acid complement 21 days after anthesis, but only for about 10% of the total at maturity. The concentration of ammonia also fell with maturity, probably reflecting a decrease in amide nitrogen in the seed. Only cysteine did not change significantly with seed age; this probably is due to a low precision of estimation for this amino acid.

In general, other investigators have not found drastic shifts in the concentration of any amino acid during seed development. This is true at least for developing seed of oats, lupine, and wheat (Jennings and Morton, 1963; Pomeranz et al., 1966; Brown et al., 1970; Atkins, et al., 1975). However, in developing pea (*Pisum arvense* L.) seed, Flinn and Pate (1968) found that homoserine accounted for over 40% of the amino acids early in development, but it diminished rapidly with maturity. While homoserine is not a common protein amino acid, it is an intermediate in the conversion of aspartic acid to other amino acids. Upon the decline of homoserine, arginine and aspartic acid accumulated in the free amino acid pool.

In developing cottonseed, we observed a similar pattern. Aspartic acid was predominant early in seed development. Upon its decline, aspartic acid was replaced by those amino acids most needed for protein synthesis: arginine and glutamic acid.

Soluble-pool N increased from 10 to 21 days, and then declined until maturity (Fig. 1). Total N in the seed increased throughout development. Similar patterns of soluble and total distribution were reported by Rubel et al. (1972) for N accumulation by developing soybean (*Glycine max* L. Merr.). Asparagine was quite high in the NPN fraction (primarily free amino acids) early in development (Table 2). The concentrations of all amino acids in the soluble pool, including asparagine, declined as the seeds matured.

We believe that the composition of the free amino acid pool during early seed development reflects the accumulation of the "supply" amino and amide N. This pool then becomes the substrate for amino acid synthesis and metabolism during subsequent development.

The metabolic conversion of "supply" amino acids into "demand" amino acids probably occurs in all developing seed. Clearly, significant shifts occurred in the composition of the free amino acid pools of both cottonseed and field pea (Flinn and Pate, 1968). In

Table 2. Concentration of soluble amino acids, ammonia and nonprotein N in developing cottonseed.

Factor†	Days after anthesis								
	10	14	21	28	35	42	49	56	L.S.D.‡
	μmoles · gdw^{-1}								
Lysine	8.6	7.7	11.3	4.9	0.6	0.1	0.1	0.2	6.6*
Histidine	4.3	5.3	8.1	6.2	3.4	0.6	0.2	0.2	2.1**
Ammonia	95.6	54.8	44.7	30.4	14.3	10.9	10.7	9.0	51.9*
Arginine	15.1	19.5	23.7	28.8	15.2	4.5	2.4	3.2	12.6*
Aspartic Acid	27.8	18.0	12.3	6.4	3.1	1.1	2.4	2.6	ns
Asparagine	238.8	272.3	242.6	126.7	36.4	1.3	2.3	2.8	25.2**
Threonine	4.5	8.6	9.0	5.2	2.6	0.9	0.2	0.2	1.4**
Serine	6.9	9.8	7.2	4.4	3.3	1.9	0.6	0.4	1.0**
Glutamic Acid	22.2	16.3	15.9	9.0	5.2	2.2	2.0	3.2	11.3*
Glutamine	44.7	67.8	84.8	25.5	11.6	2.8	0.2	tr	17.2**
Glycine	0.7	1.7	1.2	0.6	0.6	0.4	0.2	0.4	ns
Alanine	6.7	19.6	10.1	3.5	2.6	1.4	0.5	0.5	3.2**
1/2 Cystine	2.4	3.2	1.6	1.1	0.4	tr	0.2	0.7	1.4*
Valine	tr	tr	0.3	0.4	0.2	0.1	tr	tr	ns
Methionine	tr	tr	0.3	0.2	0.1	tr	tr	tr	ns
Isoleucine	tr	0.8	0.8	0.6	0.1	tr	tr	tr	ns
Leucine	tr	1.2	1.1	0.8	0.5	0.2	tr	tr	ns
γ amino butyric acid	1.6	3.5	2.4	6.5	4.0	2.6	1.4	1.0	2.5*
Ethanolamine	3.2	2.8	2.1	2.0	1.1	0.9	0.5	0.3	ns
Non-protein N	829.2	929.6	905.5	518.7	206.6	51.3	34.7	38.5	141.3**

*,** Level of significance (0.05 and 0.01, respectively) of the F test. † Proline, tyrosine, phenylalanine and tryptophan were also observed as traces in most analyses, but were not consistently present in sufficient amounts to be quantitated. The NPN value is obtained by summing the total nitrogen content of the amino acids actually observed. ‡ L.S.D. (0.05 probability) is presented for each amino acid in which the F test from the analysis of variance was significant.

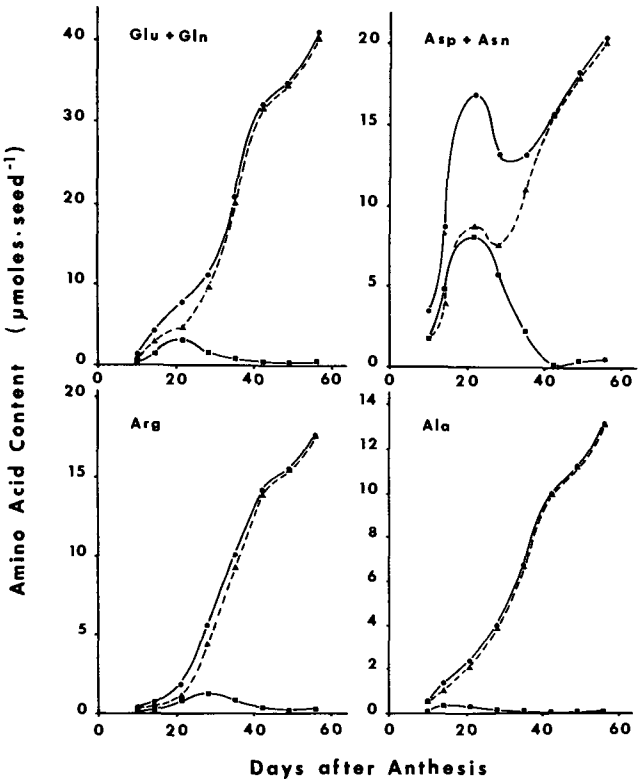

Fig. 2. Distribution of glutamyl, aspartyl, arginyl, and alanyl residues between protein and nonprotein fractions of developing cottonseed. ●, total; ▲, protein; ■ nonprotein.

those seeds where shifts in free amino acid distribution were not observed, the metabolic pool may either be small or be in balance with the amino acid profile of the storage proteins.

Two non-protein amino acids, gamma-amino butyric acid (GABA) and ethanolamine, were always found in the NPN fraction. These may be two of the "unknown" amino acids described by Carter et al. (1966) in an alcoholic extract of mature cottonseed. A third peak they referred to may be tryptophan, since we occasionally observed it in the chromatograms (our technique does not protect tryptophan during extraction). Burks and Earle (1965) reported similar unidentified peaks in extracts of cotton fruit and suggested that GABA might be one of them. We found no other unidentified peaks in chromatograms of the basic amino acids.

Content, as contrasted with concentration, of four amino acids is illustrated in Fig. 2. For most amino acids — of which glutamic acid-glutamine, arginine, and alanine are representative — the free portion declined after a low, early peak. Total content increased throughout development, indicating that most of each amino acid was present in combined form as protein. Aspartic acid-asparagine, however, exhibited a different pattern. Content of these amino acids reached an early peak at 21 days, and was approximately evenly divided between soluble and protein fractions. Between 21 and 28 days, however, the content of these amino acids in both fractions declined. Beyond 28 days, the soluble asparatic acid-asparagine continued to decline while the total aspartic acid in the hydrolysate increased again. This indicates that the increase was the result of protein synthesis.

This 21 to 28-day interval was a period during which there was a noticeable shift in the amino acid composition. While aspartic acid declined, arginine, theonine, and lysine — which can be derived from aspartic acid (Mahler and Cordes, 1971) — increased. This interval may, therefore, reflect a transition from predominance of one class of protein to the predominance of another class. The presence of a specific quantity of an amino acid (i.e., asparagine) does not appear to result in its incorporation into protein in like amounts.

The maximum soluble arginine level was not reached until 28 days, a week later than the time at which the other soluble amino acids were maximal. This may indicate that arginine was derived from other carbon skeletons and that this week reflects a

Table 3. Amino acid and N composition and weight of cottonseed from bolls produced from early and late flowers.

Factor	Flowering date	
	16 July	27 July
Lysine	4.82†	5.00
Histidine	2.74	2.73
Ammonia	2.06	2.09
Arginine	11.44	11.31
Aspartic Acid	10.20	10.79
Threonine	3.58	3.75
Serine	5.08	5.40
Glutamic Acid	22.58	22.94
Proline	3.74	3.95
Glycine	4.54	4.73
Alanine	4.34	3.59
1/2 Cystine	1.30	1.02
Valine	3.72	3.71
Methionine	1.68	1.91
Isoleucine	2.64	2.59
Leucine	6.04	6.26
Tyrosine	2.52	2.59
Phenylalanine	4.54	4.63
N, %	5.41 ± 0.13‡	4.26 ± 0.29
Seed wt/boll, g	2.44 ± 0.07‡	1.83 ± 0.02

† Amino acids are presented as g · 16 g N^{-1} recovered.
‡ Data are presented as means ± S.E. of the means.

lag time of synthesis. It may also indicate that proteins high in arginine were beginning to be synthesized during this period. Resolution of the relative contributions of transport and of synthesis within the seed to the soluble arginine pool will require additional investigation.

The data presented in Fig 1, Table 1 and Table 2 show that cottonseed continued to accumulate protein N throughout development. This continual accumulation can be accounted for only by continued input of N into the developing seed. Continued input of N would require that the funiculus remain intact throughout cottonseed development, in agreement with the data of Benedict et al. (1976) and Leffler and Tubertini (1976).

It is evident that translocation of N continues until boll opening. The form of translocated N is not readily apparent, but it is likely to be already reduced to amino or amide form. This interpretation is derived from the data of Radin and Sell (1975), who found that NO_3^- transported to the seed could account for only about 5% of the reduced N accumulated by cottonseed.

Although translocation of amino acids was not studied in these experiments, it appears to us that asparagine would be a likely candidate for the transport form of amino N in *Gossypium*. This is suggested by the extremely high concentration of asparagine in the NPN fraction of cottonseed less than 21 days old.

The amino acid compositions of cottonseed from early and mid-season (16 July and 27 July) flowers are presented (in the conventional manner: g · 16 g N^{-1}) in Table 3. Seed in the earlier formed bolls were both larger and accumulated a higher concentration of N. The amino acid distributions of the seed proteins were the same, however, regardless of flowering-developmental period. Clearly, seasonal influences were minimal on amino acid composition although they were significant on protein quantity.

The nutritional quality of cottonseed protein, as determined by the amino acid profile, is relatively desirable. According to the FAO (1970), isoleucine is the first limiting amino acid and the sulfur-containing amino acids are secondarily limiting. Although there is appreciable lysine present, it is generally regarded as low or even limiting in cottonseed meal (Bressani and Elias, 1974). This may be because gossypol complexes with lysine during processing (Damaty and Hudson, 1975). Lysine availability may be less of a problem if either glandless cultivars (McMichael, 1959) or liquid cyclone processing (Ridlehuber and Gardner, 1974) were used.

Even in its present unimproved condition, cottonseed flour is being used as a protein supplement in developing countries. Examples are the commercial products marketed in Columbia by the Institute of Nutrition of Central America and Panama (Dimino, 1969). The effects of agronomic and cultural practices on cottonseed proteins must therefore be investigated. Relatively little information is currently available about the effects of environment and genotype on protein quantity, quality and amino acid composition. These data must be obtained if cotton is to be fully utilized as a source of protein for food and animal feed.

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