Seeds of *Indigofera* Species: Their Content of Amino Acids That May Be Deleterious

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To provide information about their suitability as field crops, several *Indigofera* species have been examined for possible deleterious constituents. The occurrence of indospicine (2-amino-6-amidinohexanoic acid) and other free amino acids in extracts of seeds from 17 different species of *Indigofera* was determined by thin-layer and ion-exchange chromatography. The hepatotoxic and teratogenic amino acid, indospicine, was detected only in *I. endecaphylla*, the species from which it had been isolated originally. Complete amino acid composition (excluding tryptophan) of defatted seed meal of three species (*I. endecaphylla, I. pilosa,* and *I. schimperi*) was determined. A basic amino acid of unknown structure and unknown physiological properties was detected in two species (*I. schimperi* and *I. oblongifolia*).

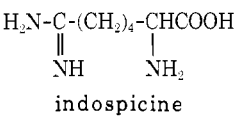
Few legumes are suitable forage crops for tropical regions of the earth. Trailing (creeping) indigo (*Indigofera endecaphylla*, also known as *I. spicata*) is one that showed great promise. It was introduced to the U. S. by the Florida Agricultural Experiment Station in 1925 (Emmel and Ritchey, 1941) and recommended for Puerto Rico in 1940 (Davis and Villalobos, 1940). It also has been studied as a forage in: Hawaii (Takahashi and Ripperton, 1949); Ceylon (Jeganathan, 1953), where it had been used as a cover crop since 1925 (Joachim, 1932); Australia (Hutton *et al.*, 1958a); and Fiji (Yelf, 1959). Hairy indigo (*I. hirsuta*) was introduced into subtropical U. S. in 1902 (Ritchey, 1950), grown in Cuba (Beliuchenko, 1964), and promoted in North Carolina as a green manure crop (Lovvorn *et al.*, 1963). Other species of *Indigofera* have been studied in Australia (Hassell, 1945) and in the Virgin Islands (Oakes, 1970).

Some species of *Indigofera* have been used in folk medicine (Hartwell, 1970; Nagata, 1971). Although several have been examined for alkaloids, only a few have given a positive test for their presence (Aplin and Cannon, 1971; Fong *et al.*, 1972; Smolenski *et al.*, 1972).

The favorable agronomic aspects of *I. endecaphylla* were marred by the discovery that it is toxic to rabbits (Emmel and Ritchey, 1941), ruminants (Jeganathan, 1953; Nordfeldt and Younge, 1949; Yelf, 1959), and chickens (Rosenberg and Palafox, 1950). Other species of indigo also noted as toxic were *I. patens* (Van der Walt and Steyn, 1939), *I. enneaphylla* (Bell and Everist, 1951), and *I. subulata* (Cooke, 1955).

After chickens proved to be sensitive to the toxic substance(s) in *I. endecaphylla*, they served to test species of *Indigofera* and other legumes for toxicity (Rosenberg and Zoebisch, 1952; Zoebisch *et al.*, 1952). Hiptagenic acid, identified in 1949 by Carter and McChesney as β-nitropropionic acid (β-NPA), was identified as the substance in *I. endecaphylla* foliage toxic to chicks (Morris and Pagán, 1953; Morris *et al.*, 1954). A colorimetric procedure was developed to test plant foliage for β-NPA, and of six species of *Indigofera* examined, only two (*I. endecaphylla* and *I. subulata* (Cooke, 1955)) showed it. Ten years later, β-NPA was found in another species, *I. enneaphylla* by Murray *et al.* (1965). Other workers isolated a series of β-NPA-containing glucosides, called endecaphyllins, from the leaves and stems of *I. endecaphylla* (Finnegan *et al.*, 1963). No β-NPA was detected in seeds of any *Indigofera* species.

Australian investigators studied the toxicity of *I. endecaphylla* and came to the conclusions that liver damage observed in test animals was not caused by β-NPA (Coleman *et al.*, 1960; Hutton *et al.*, 1958a, 1958b). This conclusion was reached because β-NPA could not be found in seeds of this plant, which were as toxic as the leaves or stems or both, and because ingestion of pure β-NPA did not produce the liver lesions noted with the natural toxicant. A basic fraction from extracts of both seeds and leaves yielded a highly toxic amino acid, which was identified as 2-amino-6-amidinohexanoic acid (indospicine)



indospicine

(Hegarty and Pound, 1968). Hegarty and Pound (1970) found 0.04–0.15% indospicine in mature leaves and 0.5–2% indospicine in harvested seeds. This amino acid produced liver damage in mice and rats (Christie *et al.*, 1969). Indospicine has been synthesized (Culvenor *et al.*, 1969). It inhibits the incorporation of arginine into protein in a rat liver cell-free system (Hegarty and Pound, 1970; Madsen *et al.*, 1970), and it is antagonistic to arginine in bacteria (Leisinger *et al.*, 1972).

In addition to interest in *Indigofera* species as forage crops, one has also been cited as a possible pulp crop (White *et al.*, 1971) or seed crop (White, 1969). Accordingly, we were prompted to survey the *Indigofera* species in our seed collection for the presence of indospicine so that we could provide information about their suitability as field crops.

EXPERIMENTAL SECTION

Established procedures were used to grind the seeds and defat them. The micro-Kjeldahl method was followed to determine nitrogen content of the samples. The meals (500-mg samples) were hydrolyzed by refluxing each with 250 ml of constant boiling HCl for 24 hr. Liquid samples were hydrolyzed for 24 hr by taking 5 ml of the extracts to dryness and refluxing the residue with 50 ml of constant boiling HCl.

Extraction. The "nonprotein nitrogen" (NPN) was extracted with one of the following solvent mixtures: A, 95% ethanol–water (1:1, v/v); B, 95% ethanol–0.1 *N* HCl (1:1, v/v); C, 70% ethanol; D, 0.1 *N* HCl in 70% ethanol, prepared by mixing 95% ethanol and 1:3 *N* HCl (7:3); and E, 2 *N* NH_4OH in 50% ethanol, prepared by mixing 526 ml of 95% ethanol, 343 ml of water, and 131 ml of concentrated NH_4OH.

Chromatography. Thin-layer plates were spread with a slurry prepared from 20 g of silica gel G and 45 ml of 1% aqueous starch solution. The developing solvent was chloroform–methanol–concentrated NH_4OH–water (40:40:15:5). Spots were visualized with a solution prepared by dissolving 500 mg of ninhydrin in 15–20 ml of acetone,

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Table I. Amino Acid Composition of Meals and Extracts of *Indigofera* Seeds

Amino acid	Mg of amino acid/g of nitrogen in the samples analyzed[a]							
	I. endecaphylla				*I. hirsuta*		*I. pilosa*	*I. schimperi*
	Hydrolyzed defatted seed meal	NPN[b] extract	Hydrolyzed NPN extract[b]	Hydrolyzed residue[c] meal	Hydrolyzed NPN extract[b]	Hydrolyzed residue[c] meal	hydrolyzed defatted seed meal	hydrolyzed defatted seed meal
Lysine	356	5	210	366	195	308	323	367
Histidine	166	Trace	107	176	83	152	166	155
Canavanine	197	503	492	Trace	39	0	0	24
Indospicine	92	466	186	0	0	0	0	0
Arginine	579	34	443	584	812	736	662	619
Aspartic acid	731	30	457	655	495	640	808	799
Threonine	260	4	127	247	119	210	248	250
Serine	340	27[d]	186	338	182	327	387	345
Glutamic acid	1304	43	778	1226	794	1318	1487	1443
Proline	280	5	164	267	157	262	304	285
Glycine	391	4	170	367	172	352	424	388
Alanine	209	12	120	293	220	266	247	245
Cysteine	51	76	274	35	176	32	76	63
Valine	302	8	156	344	169	316	292	281
α-Aminopimelic acid	206	10	341	5	0	0	0	0
Methionine	40	e	41	100	e	33	45	36
Isoleucine	279	1	150	269	154	262	278	278
Leucine	411	1	238	466	224	454	442	458
Tyrosine	239	3	170	204	128	186	236	230
Phenylalanine	367	11	225	358	163	301	349	367
Unidentified[f]								
R_{asp} 0.88	Trace	Trace	Trace	25	Trace	Trace	Trace	Trace
R_{glu} 1.19	0	47	0	0	0	0	0	0
R_{leu} 0.94	0	0	0	7	Trace	Trace	0	0
R_{leu} 0.95	0	1	Trace	0	Trace	0	0	0
R_{leu} 0.96	Trace	2	0	0	0	0	Trace	Trace
R_{leu} 1.02	0	0	51	0	0	0	0	0
R_{lys} 0.63	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace
R_{arg} 0.84	0	0	0	0	0	0	0	86

[a] To convert to g of amino acid/16 g of N, multiply by 0.016 (or divide by 62.5). [b] Nonprotein nitrogen (NPN) extract obtained with solvent D. About 39% of the N of *I. endecaphylla* defatted seed meal was extracted. About 24% of the N of *I. hirsuta* defatted seed meal was extracted. [c] After two extractions with solvent D. [d] This serine value is questionable, as the shape of the curve suggested that other components were present. [e] Value for methionine was not calculated, as doubt existed as to its identity. [f] Elution position given in relation to identified amino acid; *i.e.*, $R_{\text{amino acid}}$ = milliliter of effluent to elution peak of unidentified/milliliter of effluent to elution peak of identified amino acid. Values for first seven unidentified compounds are calculated as if each unidentified compound has color yield and molecular weight of leucine; value of unknown at R_{arg} 0.84 is calculated as if it has color yield and molecular weight of arginine.

adding 3 ml of acetic acid, and then diluting to 100 ml with 1-butanol.

A glass column (3.5 × 58 cm) was filled to a height of 26 cm with 50–100 mesh Dowex 50X2 cation exchange resin in H^+ form. The neutralized (pH 7) extract was applied to the resin, which then was washed thoroughly with water. Basic amino acids were eluted from the column with about 250 ml of 2 *N* NH_4OH as one fraction which was concentrated and spotted on paper.

Preparative separations were achieved on Whatman 3MM paper using 1-propanol–concentrated NH_4OH (70:30) as developing solvent. The unknown amino acid and arginine were extracted from the appropriate areas of the paper with water.

The Beckman Spinco model 120 amino acid analyzer was operated according to the procedure of Benson and Patterson (1965). Their procedure was modified by using the long (50-cm) column (containing Beckman UR30 resin) for the separation of the basic amino acids with the pH 5.28 buffer rather than the more usual short (5-cm) column (containing PA-35 resin). The acidic and neutral amino acids were determined as described in the published procedure.

Tryptophan content was not determined on any of the samples.

Materials Analyzed. Preliminary examination utilized the extraction procedure of Bell and Tirimanna (1965), which employed 200 mg of seed and 2 ml of solvent A or B, to obtain the NPN extracts from separate samples of the defatted seed meals of the following 17 species of *Indigofera: I. arrecta* A. Rich., *I. brachystachya* E. Mey., *I. densiflora* Mart. and Gal., *I. endecaphylla* Jacq., *I. frutescens* L. f., *I. hirsuta* L., *I. jaliscensis* Rose, *I. kirilowii* Maxim., *I. linifolia* (L. f.) Retz., *I. oblongifolia* Forsk., *I. pilosa* Poir., *I. schimperi* Jaub. and Spach., *I. sphaerocarpa* A. Gray, *I. suffruticosa* Mill., *I. sulcata* DC, *I. tinctoria* L., and *I. zoilingeriana* Miq. The 34 NPN extracts were examined qualitatively by thin-layer chromatography (tlc) for their amino acid content. One NPN extract (obtained either by solvent A or B) of each species was examined for its basic amino acid content by the automated ion-exchange procedure. The results were only qualitative because nitrogen could not be measured in the small amount of extract available.

We reexamined several species by extracting with various solvents. One-gram samples of defatted seed meals of *I. arrecta, I. endecaphylla, I. hirsuta,* and *I. suffruticosa* were extracted twice with 20-ml portions of solvent D. Five grams of defatted seed meal of *I. oblongifolia* was extracted four times with 50-ml portions of solvent C. Thirty grams of defatted seed meal of *I. schimperi* was extracted five times with 180-ml portions of solvent E; the combined extracts of this particular sample were separated by ion-exchange and paper chromatography.

Table II. Canavanine, Indospicine, and Unknown Content of *Indigofera* Species

Indigofera species	Nitrogen in whole meal, % dry basis	Mg of amino acid/g of nitrogen in the whole meal[a]			
		Canavanine	Indospicine	Unknown[b]	APM[c]
I. arrecta	4.90				
Solvent D extract		587	0	0	n.d.[d]
Hydrolyzed solvent D extract		564	0	0	n.d.
Hydrolyzed residue meal		15	0	0	n.d.
I. endecaphylla	4.62				
Hydrolyzed defatted seed meal		176	82	0	184
Solvent D extract		192	178	0	4
Hydrolyzed solvent D extract		188	71	0	130
Hydrolyzed residue meal		Trace	0	0	3
I. hirsuta	4.34				
Solvent D extract		11	0	0	n.d.
Hydrolyzed solvent D extract		10	0	0	0
Hydrolyzed residue meal		0	0	0	0
I. oblongifolia	3.71				
Hydrolyzed defatted seed meal		41	0	18	n.d.
Hydrolyzed residue meal (after extraction with solvent C)		16	0	13	n.d.
I. pilosa	4.62				
Hydrolyzed defatted seed meal		0	0	0	0
I. schimperi	4.46				
Hydrolyzed defatted seed meal		22	0	79	0
I. suffruticosa	4.45				
Solvent D extract		532	0	0	n.d.
Hydrolyzed solvent D extract		504	0	0	n.d.
Hydrolyzed residue meal		13	0	0	n.d.

[a] To convert to g of amino acid/16 g of N, multiply by 0.016 (or divide by 62.5). [b] R_{arg} 0.84. Calculated as if color yield and molecular weight are same as those of arginine. [c] APM = α-aminopimelic acid, probably derived from indospicine. [d] n.d. = not determined.

In the case of *I. arrecta, I. endecaphylla, I. hirsuta,* and *I. suffruticosa,* the basic amino acid content of the NPN extracts obtained with solvent D, the hydrolyzed NPN extracts, and the hydrolyzed residue seed meal after extraction were determined by the modified Benson and Patterson procedure. The acidic and neutral amino acids of the hydrolyzed NPN extracts and residue meals of *I. endecaphylla* and *I. hirsuta* were also measured by the automated ion-exchange procedure. A complete amino acid analysis was carried out on the unhydrolyzed NPN extract of *I. endecaphylla*; however, since only 30% of its nitrogen could be accounted for, complete analyses were not carried out on corresponding extracts from other species. The basic amino acid contents of the hydrolyzed defatted seed meal and the hydrolyzed residue meal of *I. oblongifolia* were also determined by the modified Benson and Patterson procedure. Hydrolyzed defatted seed meals of *I. endecaphylla, I. pilosa,* and *I. schimperi* were examined for complete amino acid content *via* the automated ion-exchange procedure.

RESULTS AND DISCUSSION

Qualitative examination of NPN extracts by tlc and by the automated ion-exchange procedure provided the basis for selecting 7 of the 17 *Indigofera* species for further investigation. The amino acid composition (excluding tryptophan) of four species is given in Table I. Table II lists the amounts of three basic amino acids found in seed meals from seven species (including the four species of Table I).

Of the 17 species examined, only *I. endecaphylla* contained any detectable indospicine. This was the species from which the Australian workers isolated indospicine (Hegarty and Pound, 1968). Presumably, the α-aminopimelic acid (Tables I and II) is an artifact derived from indospicine. Hegarty and Pound (1968, 1970) demonstrated that this compound is degraded to α-aminopimelic acid under acidic conditions. Virtually all the indospicine of *I. endecaphylla* is removed during NPN extraction since the hydrolyzate from the residual meal contains no indospicine and only a trace of α-aminopimelic acid.

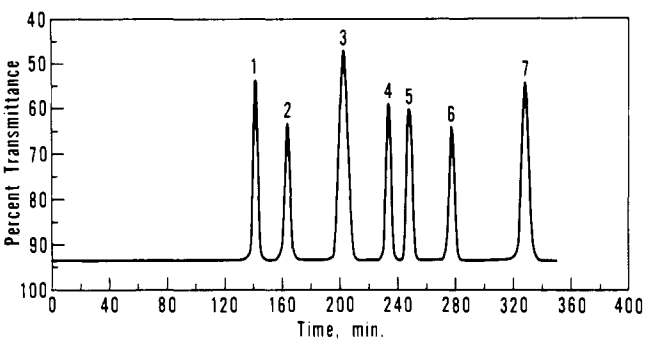

Figure 1. Elution sequence of basic amino acids and ammonia. Conditions: 50-cm column, Beckman UR-30 resin, 55°, pH 5.28 (0.35 *N*) sodium citrate buffer, and a flow rate of 80 ml/hr. Identification: 1, lysine; 2, histidine; 3, ammonia; 4, canavanine; 5, indospicine; 6, unknown; and 7, arginine.

Another "unusual" basic amino acid, canavanine, was detected in widely varying amounts in the NPN extracts of all 17 species examined, except for that of *I. pilosa.* Canavanine is an antimetabolite of arginine and is also claimed to be toxic (Tschiersch, 1962).

In addition, an unidentified basic amino acid was found in NPN extracts from *I. schimperi* and *I. oblongifolia.* This unidentified compound is eluted from the ion-exchange column before arginine (*i.e.*, at R_{arg} 0.84) but behind canavanine and indospicine. Figure 1 shows that these four basic amino acids were clearly resolved under the operating conditions used in this work. The structure of the unknown has not been determined, nor do we know the color yield in its reaction with ninhydrin. The values listed for this unknown (R_{arg} 0.84) in Tables I and II were calculated by assuming that its color yield and molecular weight are the same as those of arginine. This arbitrary assumption was made because the molecular weights and

color yields for canavanine, indospicine, and arginine are quite similar. Examination of Table II reveals that apparent content of this unknown is greater in *I. schimperi* than in *I. oblongifolia* and not much of the unknown was removed from *I. oblongifolia* seed meal, even after four extractions. The quantity of the unknown amino acid isolated from *I. schimperi* (see Experimental Section) was not sufficient for structural characterization. However, using this crude isolate, we were able to show that an unfamiliar spot appearing at R_f 0.10 in thin-layer chromatograms of *I. schimperi* extracts probably is due to the same component that elutes from the resin column at R_{arg} 0.84. Whenever more seed becomes available, we hope to complete the isolation and identification of this unknown amino acid.

Our analyses were confined to seeds of *Indigofera* species, since the foliage and other parts of them were not generally available to us. However, since indospicine occurs throughout the plant in *I. endecaphylla,* we have no reason to suppose that deleterious amino acids in other *Indigofera* species would be restricted to the seed.

Some other minor unknown ninhydrin-positive components were encountered in the analysis of four *Indigofera* species. Some of these unidentified compounds are shown in Table I by their elution positions compared to those of known amino acids. Apparently, one (or more) unknown coincides with the serine peak in the NPN extract of *I. endecaphylla,* causing the value for serine to be erroneously high.

The amount of the nutritionally essential amino acids present in defatted seed meals of three *Indigofera* species is similar to the amounts present in legumes generally (VanEtten *et al.,* 1967). The quantity of glycine, aspartic acid, and serine in these *Indigofera* species is more than usually found in legumes.

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