INDOSPICINE, A HEPATOTOXIC AMINO ACID FROM *INDIGOFERA SPICATA*: ISOLATION, STRUCTURE, AND BIOLOGICAL STUDIES

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Summary

Indospicine (L-2-amino-6-amidinohexanoic acid) has been newly characterized as a component of a higher plant, being isolated from the leaves and seeds of *Indigofera spicata*. Indospicine was hydrolysed in distilled water at 120°C to 2-aminopimelamic acid and ammonia (1 mole). Mild acid hydrolysis of the latter compound yielded optically pure L-α-aminopimelic acid. The infrared, nuclear magnetic resonance, and mass spectra of indospicine and of its degradation products supported the proposed structure. When injected subcutaneously into mice (1 mg/g body weight) indospicine produced fat accumulation and cytological changes in the liver. The fat accumulation was inhibited by simultaneous injection of arginine but not by canavanine. A substantial part of the hepatotoxicity of extracts of *I. spicata* seed was accounted for in terms of indospicine. The concentration of indospicine in the seeds exceeded that in the leaves and stems of the plant.

I. INTRODUCTION

Indigofera spicata (previously called *I. endecaphylla*) would be a valuable pasture legume for the tropics if it were not for its toxic properties (see Kingsbury 1964). It has been shown to be hepatotoxic to sheep and cows and has caused abortion in pregnant animals (Norfeldt *et al.* 1952). A number of investigations to isolate and identify the toxin have been carried out using different laboratory test animals and it seems likely that more than one toxic compound is present. Britten *et al.* (1963) showed that β-nitropropionic acid, or an unknown compound closely associated with it in the leaves, was toxic to chicks which were not affected by any toxin present in the seed. Hutton, Windrum, and Kratzing (1958*a*, 1958*b*) showed that the leaves and seeds were hepatotoxic to mice and rabbits and that β-nitropropionic acid which occurs only in the leaf did not account for the liver lesions. Further work by Coleman, Windrum, and Hutton (1960) indicated that the toxic substance was water-soluble and behaved as a cation on ion-exchange resins. The substance, hepatotoxic to mammalian test animals, remained unidentified until Hegarty and Pound (1968) briefly reported the isolation from the leaves and the seeds of a new amino acid which reproduced the characteristic fat accumulation and other changes in the livers of mice.

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This substance was designated indospicine and the structure L-2-amino-6-amidinohexanoic acid was suggested for it. Indospicine is also hepatotoxic (Christie, Madsen, and Hegarty 1969) and teratogenic (Pearn 1967; Pearn and Hegarty 1970) in rats.

This paper describes in detail the isolation of indospicine from leaves and seeds, its properties, and the elucidation of its structure. Some of its biological properties are reported.

II. Experimental

(a) *Plant Material*

The principal isolation experiments were carried out with *I. spicata* seeds obtained from Ceylon through R. J. Johnson & Co., Colombo, and designated CPI 30492, 30536, and 34789 (CPI = Commonwealth Plant Introduction Number). These batches of seed contained up to 4% seeds of other species (*Mimosa invisa*, *Desmodium* sp., and *Digitaria* sp.). Tests showed that none of these seeds contained indospicine. Leaf and seed material were also obtained from plants grown in the glasshouse or in the field.

(b) *General Chemical Methods*

Melting points were determined on a Kofler hot microstage and are corrected. C, H, N, and O microanalyses were performed by the Australian Microanalytical Service, Melbourne, and by Mr. J. Kent, Chemistry Department, University of Queensland. The infrared absorption spectra were measured with a Unicam Sp 200 (for KBr disks) or a Perkin–Elmer model 237 spectrophotometer (for Nujol mulls). The n.m.r. spectra were measured in Varian A60 and HA100 spectrometers. The chemical shifts are expressed relative to the internal standard tetramethylsilane ($\delta = 0$). Mass spectra were determined with a Hitachi Perkin–Elmer RMU-6D spectrometer equipped with a direct inlet system, and optical rotations in a Perkin–Elmer Model 141 polarimeter.

Paper chromatograms were run on Whatman No. 1 and 3MM chromatographic grade filter paper. Solvents used included: A, phenol–water (4 : 1 w/v); B, a one-phase mixture of butan-1-ol – acetic acid – water (90 : 10 : 25 by vol.); C, propan-2-ol – 2N hydrochloric acid (78 : 22 by vol.). Thin-layer chromatograms were run on Eastman Chromagram cellulose sheets in the Eastman Chromagram developing apparatus. The principal solvents used were solvent B above and D, propan-2-ol – 2N hydrochloric acid (60 : 40 by vol.).

Ionophoresis was carried out on Whatman No. 1 and 3MM papers in buffer solutions of pH 2·0, 5·3, and 10·1 (Efron 1960). A horizontal method based on that of Foster (1952) was used and a potential of 25 V/cm was applied for 30–90 min while water at 2°C was circulated through the cooling plate. No decomposition of indospicine was observed at pH 10·1.

Chromatography and ionophoresis papers and thin-layer sheets were dipped in ninhydrin reagent (0·5% w/v ninhydrin in absolute ethanol containing four drops of lutidine) and the colours developed at 80°C. Other detecting reagents which were applied by spraying were the pentacyanoaquoferriate (PCF) reagent of Smith and Horswell (1960) and the modified Sakaguchi's reagent of Irreverre (1965).

Indospicine was detected as a compound giving a purple colour with ninhydrin and a yellow-orange colour with the PCF reagent. This latter colour was similar to that given by acetamidine and easily distinguishable from the red colour given by arginine or the magenta colour shown by canavanine.

Quantitative amino acid analyses were carried out with a modified Paton–Simmonds (Simmonds 1958) amino acid analyser (R. D. Court, unpublished data). Neutral and acidic amino acids were determined on 140-cm columns of cation-exchange resin (Aminex Q150/S resin, Bio-Rad Laboratories, California, U.S.A.), and basic amino acids on 50-cm columns of the same resin. The buffer solutions used for analysis of neutral and acidic amino acids were those described by Simmonds (1958). Canavanine and indospicine were not separated at pH 5·26 but satisfactory separation was obtained at pH 4·60. Concentrated ethanolic extracts of leaf and dilute acid (0·1N HCl) extracts of seed were analysed without pretreatment.

(c) *Biological Assay of Hepatotoxic Effect*

Coleman, Windrum, and Hutton (1960) showed that the feeding of aqueous extracts of *I. spicata* leaves and seeds caused an accumulation of fat and patchy necrosis in the livers of mice. In the present work, similar histological changes were found to occur in the livers of mice injected subcutaneously with a crude amino acid extract and preliminary experiments were made to establish if either of these parameters could be adapted for use in a rapid biological assay of the toxin. Fat accumulation appeared to be satisfactory since it was reasonably consistent, uniform in pattern, its extent was dose-dependent, and it could be assessed by microscopic examination. Necrosis was not satisfactory since it lacked these characteristics and appeared only with the highest doses. Female mice appeared to be more susceptible than males and the maximum effect, with the crude extract on which the assay was developed, occurred about 36 hr after injection (Table 1).

TABLE 1

GRADING OF FAT ACCUMULATION IN THE LIVERS OF MICE INJECTED WITH A CRUDE EXTRACT OF *I. SPICATA* SEED AND WITH PURE INDOSPICINE

Groups of five mice were injected with a solution of the appropriate material and killed at intervals. The livers were removed, sectioned, and stained with sudan IV. The sections were graded from 0 to 4 on the basis of the intensity of the fat stain as described in the text. The mean score is the arithmetic mean of the scores of five mice. Indospicine was administered as its hydrochloride

Sex	Material Administered	Dose (ml)	Mean Score at:			
			12 Hr	24 Hr	36 Hr	48 Hr
Male	Crude extract	1·0	1·0	2·0	3·0	1·6
		0·75	0·4	1·4	1·6	1·4
		0·5	0·4	0·2	1·2	0·0
		0·25	0·0	0·0	1·0	0·8
		0·125	0·0	0·0	0·2	0·0
Female	Crude extract	1·0	1·5	2·0	3·0	2·6
		0·75	0·6	1·2	2·0	2·0
		0·5	0·0	1·8	1·4	1·2
		0·25	0·2	0·6	1·4	0·6
		0·125	0·2	0·0	0·0	0·0
Female	Indospicine	30 mg	1·2	2·6	2·5	2·2

For an assay, therefore, groups of five female mice (Royal Brisbane Hospital strain) weighing $25 \pm 1 \cdot 5$ g were injected with graded doses of extracts or toxin. The animals were killed 36 hr later and frozen histological sections of the liver were cut and stained with sudan IV or oil red O for fat. The extent of fat accumulation was graded by microscopic examination as 0, 1, 2, 3, or 4. These grades were based on the comparable histological appearances in fatty livers due to another toxin (ethionine) which contained approximately 0, 3, 7, 15, and 30 g lipid per 100 g dry weight in excess of the normal 15 g per 100 g dry weight respectively. The assessments in any assay were made in random order and were reasonably reproducible. The mean score quoted in the tables is the arithmetic mean of the scores of the individual mice. In spite of certain statistical limitations, this method of assay proved satisfactory as a means of comparing the toxic effects of different preparations.

(d) *Isolation and Characterization of Indospicine*

(i) *Preparation of Crude Amino Acid Extract of Seed*

Coleman, Windrum, and Hutton (1960) suggested that the hepatotoxin was water-soluble and behaved as a cation on ion-exchange resins. This was confirmed and biological assays indicated that the major part of the toxicity was associated with the strongly basic amino acid fraction. This

fraction was isolated on a preparative scale by a modification of the adsorption-dialysis technique (Hunter, Houston, and Kester 1955; Beyermann, Maat, and Hegarty 1964; Hegarty, Schinckel, and Court 1964). Zeo-Karb 226 resin (14–52 mesh) was used inside the dialysis bags, the quantities of seed (2 kg) and resin used being the same as described by Beyermann, Maat, and Hegarty (1964). The resin from the dialysis bags was mixed and transferred to a large glass column and washed with water (1 bed volume), 80% aqueous ethanol (v/v) (1·5 bed volumes), and finally with water (2 bed volumes). Cations were eluted from the resin with 2N acetic acid (10 litres). The eluate was concentrated to a thick syrup in a rotary evaporator and finally dried over KOH under vacuum. The solid (90 g) was dissolved in distilled water and adjusted to a final volume of 200 ml (1 ml = 10 g seed). Analysis of a typical crude extract showed the following composition (mg/ml): potassium 25, sodium 1, magnesium 12, calcium 5, indospicine 105, canavanine 48, arginine 10. Traces of other ninhydrin- and Sakaguchi-positive compounds and a substance chromatographically identical with choline were present. Biological assay showed that no detectable toxicity remained with the residual aqueous extract of the seed, but that it was recovered in the crude amino acid extract (see Table 3).

(ii) *Isolation of Indospicine from Crude Extract*

Attempts to obtain crystalline picrates or flavianates from the crude extract were unsuccessful because of its high salt content. Several methods of desalting crude extracts were therefore developed. One of these involved chromatographing the extract on sheets of Whatman 3MM paper (0·25 ml/sheet) in solvent C, in which indospicine and canavanine were separated, locating the indospicine band with PCF reagent, cutting out the band, and eluting the amino acid. Biological assay showed that the major part of the toxicity was present in this band. No other band showed any evidence of toxicity when tested at comparable concentrations. This method has already been briefly described (Hegarty and Pound 1968) and no further details will therefore be presented.

Crude extracts were desalted on a preparative scale with only slight decomposition of indospicine by the following low temperature ion-exchange method. Extract (50 ml, adjusted to pH 4 with 6N HCl) was applied to a column (length 12 cm, diam. 4 cm) of Dowex 50-X4 resin (50–100 mesh), which was surrounded by a cooling jacket through which water at 1°C was circulated. The column was washed with water until the effluent was free of chloride. The bases were then eluted from the resin with the 2N triethylamine solvent of Harris, Tigane, and Hanes (1961) and collected in a flask cooled in solid CO_2. The effluent (2·5 litres) was evaporated in a rotary evaporator under high vacuum at 5°C (20 ml), adjusted to pH 4·5 with 6N HCl, and heated to boiling. Flavianic acid (15 g) was added and, on cooling, indospicine monoflavianate separated out as brilliant orange plates together with a small amount of arginine monoflavianate of similar crystalline form; canavanine diflavianate remained in solution. After several crystallizations from boiling water the flavianate (8 g) was converted to the hydrochloride by stirring the hot solution with excess Deacidite FF anion-exchange resin (acetate form). The resin was filtered off, washed, and the almost colourless filtrate concentrated to a small volume (5 ml). When adjusted to pH 4·5 with 2N HCl it yielded on evaporation crude indospicine monohydrochloride. This material contained 3–10% arginine.

Final purification was effected by an ion-exchange method based on that of Blau (1961) but using volatile buffer solutions. Crude hydrochloride (600 mg) was applied to a column (120 cm long, 2·0 cm diam.) of Zeo-Karb 226 (< 200 mesh) which had been equilibrated with ammonium acetate–acetic acid buffer solution pH 5·9 (Hirs, Moore, and Stein 1952). Elution of the amino acids was carried out with the same buffer at a flow rate of 1·2 ml/min. After 1250 ml of eluant had passed through the column, fraction collection (50 ml) was commenced. Indospicine was present in fraction Nos. 25–40 and arginine in fraction Nos. 44–50. Those fractions containing indospicine from two such experiments were combined and the ammonium acetate removed by vacuum sublimation at 40°C as described by Hirs, Moore, and Stein (1952). The residue was dissolved in 10 ml of boiling water and treated with flavianic acid (3 g) to yield indospicine monoflavianate (2·1 g). This was recrystallized from boiling water and then converted to the monohydrochloride as previously described. After two recrystallizations from aqueous ethanol 800 mg of pure substance was obtained.

(iii) *Physical Properties of Indospicine*

The amino acid was isolated as the monohydrochloride which crystallized from aqueous ethanol as white needle-like crystals of the monohydrate, m.p. 131–134°C. It had $[\alpha]_D^{22}+18\pm1°$ (*c*, 1 in 5N HCl) (Found: C, 37·4; H, 8·1; N, 18·5; Cl, 16·0. $C_7H_{15}N_3O_2.HCl.H_2O$ requires C, 37·0; H, 8·0; N, 18·5; Cl, 15·6%). The water of crystallization was not completely removed on drying for 72 hr at 100°C under vacuum over P_2O_5. When the temperature was raised to 110°C some decomposition occurred. The main absorption bands in the i.r. spectrum (Nujol) were at 3420, 3290, 3100, 2060, 1690, 1630, 1510, and 1420 cm^{-1}. N.m.r. spectrum: (δ p.p.m.) 1·8 (six-proton broad multiplet), 2·5 (two-proton triplet), 3·8 (one-proton triplet). The mass spectrum is discussed below.

The monoflavianate formed sparingly soluble orange plates from boiling water which had no true melting point but darkened above 250°C. (Found: C, 42·0; H, 4·5; N, 14·2; O, 32·2. $C_{17}H_{21}N_5O_{10}S$ requires C, 41·9; H, 4·3; N, 14·2; O, 32·8%). The monopicrate crystallized from aqueous ethanol as yellowish brown needles arranged in sheaves, m.p. 200–201°C (decomp.) (Found: C, 38·8; H, 4·8; N 20·8. $C_{13}H_{18}N_6O_9$ requires C, 38·8; H, 4·6; N, 20·9%). The molecular weight of the free amino acid was calculated from the picrate-ion absorption at 380 nm, a value of 175 being obtained ($C_7H_{15}N_3O_2$ requires 173). Indospicine had the following R_{Ala} values on paper chromatography: solvent A, 1·23; solvent B, 0·41; and on thin-layer chromatography (t.l.c.): solvent B, 0·33; solvent D, 0·65. It was separated from arginine and canavanine in solvent A but the amino acids streaked badly. Good separation of these three amino acids was obtained on t.l.c. in solvent D even when crude extracts were chromatographed directly. On paper ionophoresis it moved as a positively charged ion at pH 2, 5·3, and 10·1. At pH 10·1 it moved slightly faster than arginine but the separation was only marginal. Complete separation was achieved at 100 V/cm. Indospicine was readily estimated in the amino acid analyser (leucine colour equivalent 1·02) by the use of pH 4·60 buffer. The peak effluent volumes of the relevant amino acids were: histidine, 126 ml; canavanine, 164 ml; indospicine, 186 ml; arginine, 238 ml.

(iv) *Aqueous Hydrolysis of Indospicine*

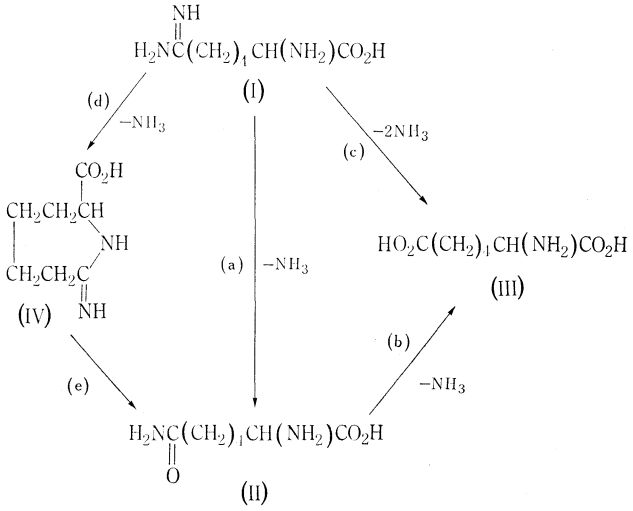
A column (length 15 cm, diam. 1·2 cm) of AG2-X8 anion-exchange resin (100–200 mesh) was prepared in the free base form and washed exhaustively with distilled water to remove the last traces of alkali. Indospicine monohydrochloride (500 mg) in water (2 ml) was applied to the column and then washed with water at a flow rate of 3·4 ml/min (see Blau 1961) until the effluent gave a negative ninhydrin reaction. The eluate was freeze-dried and indospicine (290 mg) obtained as a white hygroscopic powder. This was dissolved in water (5 ml), sealed in a glass tube, and held at 120°C for 20 hr. The hydrolysate on evaporation gave 120 mg 2-aminopimelamic acid (II, scheme I). White crystals were obtained from aqueous ethanol, m.p. 232–235°C (decomp.), $[\alpha]_D^{22}+3°$ (*c*, 0·5 in water) (Found: C, 48·4; H, 8·1; N, 16·0. $C_7H_{14}N_2O_3$ requires C, 48·2; H, 8·1; N, 16·0%). I.r.: ν_{max} 3400, 3190, 2870, 1650, 1630, 1580, 1530, 1440 cm^{-1}. N.m.r.: (δ, p.p.m.) 1·7, 2·3, 3·7. On paper chromatography it gave a purple colour with ninhydrin but did not react with the PCF reagent [R_{Ala} (paper): solvent A, 1·23; solvent B, 0·68. R_{Ala} (t.l.c.): solvent B, 0·85; solvent D, 0·88]. On ionophoresis at pH 2 it behaved as a neutral amino acid and on the amino acid analyser emerged with glycine.

In another experiment, indospicine (9·6 mg, 55 μmoles) was hydrolysed under the same conditions used above. Analysis of the solution showed the presence of ammonia (46 μmoles) and 2 aminopimelamic acid (53 μmoles). The reaction took place at a lower temperature in the presence of 0·1M Na_2CO_3. Indospicine (192 mg, 1·11 m-mole) was hydrolysed with 0·1M Na_2CO_3 (10 ml) at 50°C for 15 hr in a modified Conway apparatus (Smith and Horswell 1960). Ammonia (18 mg, 1·05 m-mole) was detected in one compartment. The hydrolysate in the other compartment was neutralized and desalted by passing it through several columns of AG 11A8 ion-retardation resin (Rollins, Jensen, and Schwartz 1962) to yield 2-aminopimelamic acid (90 mg) (m.p. and mixed m.p., identical i.r.).

(v) *Hydrolysis of 2-Aminopimelamic Acid*

Amide (95 mg) was heated with 1N HCl (3 ml) in a sealed tube at 120°C for 2 hr. The hydrolysate was evaporated to dryness and the free amino acid isolated with a short column of

AG 50W-X4 resin. Evaporation of the 2N ammonia eluate gave L-α-aminopimelic acid (III, scheme I) (80 mg), crystals from aqueous ethanol, m.p. 217–218°C, $[\alpha]_D^{22}+20\cdot4\pm0\cdot5°$ (*c*, 1 in 5N HCl) (Found: C, 47·6; H, 7·3; N, 7·8. Calc. for $C_7H_{13}NO_4$: C, 48·0; H, 7·4; N, 7·8%). Lit. m.p. 217–218°, $[\alpha]_D^{16}+21°$ (*c*, 1 in 5N HCl) (Wade *et al.* 1957), undepressed mixed m.p. with authentic sample [Koch–Light Laboratories, Colnbrook, Bucks. $[\alpha]_D^{22}+21\pm0\cdot5°$ (*c*, 1 in 5N HCl)].



Scheme 1.—Summary of reactions of indospicine described in the text. (*a*) Water, 120°C, 20 hr or 0·1M Na_2CO_3, 50°C, 15 hr; (*b*) 1N HCl, 120°C, 2 hr; (*c*) 6N HCl, 120°C, 20 hr; (*d*) vacuum pyrolysis; (*e*) water, 120°C, 44 hr.

The substance was chromatographically identical with L-α-aminopimelic acid [R_{Ala} (paper) solvents A and B, 0·88 and 1·53; R_{Ala} (t.l.c.) solvents B and D, 1·57 and 1·13] and on paper ionophoresis at pH 2·0. It emerged at the same peak effluent volume on the amino acid analyser. Its n.m.r. (peaks centred at $\delta = 1\cdot7$, 2·5, and 3·8 p.p.m.) and mass spectra were identical with those of the authentic material and the i.r. spectra of the two substances were identical in the wavelengths of all absorption bands.

In another experiment, amide (13·66 mg, 78 μmoles) gave on hydrolysis L-α-aminopimelic acid (13 mg, 75 μmoles) and ammonia (1·30 mg, 79 μmoles).

(vi) *Acid Hydrolysis of Indospicine*

Indospicine monohydrochloride (48 mg, 211 μmoles) was hydrolysed with 6N HCl (5 ml) in a sealed tube at 120°C for 20 hr. Analysis of the hydrolysate showed the presence of ammonia (6·52 mg, 383 μmoles) and L-α-aminopimelic acid (36 mg, 204 μmoles) (m.p. and mixed m.p., elemental analysis, optical rotation not measured).

(vii) *Vacuum Pyrolysis of Indospicine*

Indospicine (50 mg) was pyrolysed at 10^{-2} mm. At 220°C a white ring of sublimate formed on the cooler parts of the tube. The tube was held at about 230°C until no more sublimate formed; towards the end of the experiment a second band of sublimate began to form in the still cooler part of the tube but because of the small amount of this material available it has not been investigated in detail. The main band of white sublimate (about 5 mg) had no true melting point but turned black above 300°C. It moved as a single spot on t.l.c. in the solvent D which gave a strong magenta colour with the PCF reagent similar to that given by canavanine but more stable. It was less basic than indospicine as indicated by its behaviour on paper ionophoresis at pH 10·1 and did

not react with ninhydrin. Because there was insufficient material for elemental analysis, structural information on this new compound was obtained in the following ways. The i.r. spectrum (KBr disk) showed peaks at 3200, 2900, 1640, 1620, 1510, 1470, 1390, and 1360 cm^{-1} while the n.m.r. spectrum gave peaks centred about $\delta = 1 \cdot 9$, $2 \cdot 7$, and $4 \cdot 1$ p.p.m. The mass spectrum was identical with that of indospicine measured in the same instrument on the same day and showed characteristic peaks at *m/e* 156, 111, 97, 94, 84. Preliminary experiments indicated that the compound was slowly hydrolysed to 2-aminopimelamic acid in distilled water at 120°C. The compound, tentatively named desaminoindospicine (IV), (0·95 mg, 6·1 μmoles) in distilled water (1 ml) was held in a sealed tube at 120°C for 44 hr. Analysis of the solution showed that 2-aminopimelamic acid (5·7 μmoles) and α-aminopimelic acid (0·3 μmole) had been formed. No ammonia was detected in the hydrolysate before or after the addition of magnesium oxide. Structure IV, scheme I, has been tentatively assigned to desaminoindospicine on the basis of the evidence presented here but further work is necessary to confirm this structure.

(viii) *Mass Spectra of Indospicine and Its Hydrolysis Products*

The mass spectrum of the free base showed no reproducible peaks of higher mass number than *m/e* 111 ($M-NH_3-CO_2H$); a peak of *m/e* 156 ($M-NH_3$) was observed on one occasion only. Other major peaks were at *m/e* 97, 94, 84. 2-Aminopimelamic acid (II, scheme I) did not give a parent ion but showed characteristic peaks at *m/e* 129, ($M-CO_2H$) 112, 84, 72, 69, 67, and 56. α-Aminopimelic acid (III, scheme I) gave peaks at *m/e* 157 ($M-H_2O$), 130, 112, 84, 69, and 67. The fragmentation patterns of II and III were readily interpreted in terms of those already established for known amino acids (Biemann 1962). Desaminoindospicine (IV) gave a spectrum identical with that of I and it is likely that the reaction I → IV can take place in the ion source.

(ix) *Isolation of Indospicine from Leaves*

Fresh leaves (850 g, CPI 16069) were collected in the field and a 75% (v/v) ethanolic extract prepared immediately from them. The extract was concentrated in a rotary evaporator at 40°C and the volume was adjusted so that 1 ml = 5 g of leaf. Extract (60 ml, containing about 300 mg of indospicine) was placed on a column (17 cm by 4 cm diam.) of Zeo-Karb 226 (ammonium form; 100–200 mesh) which had been equilibrated with ammonium acetate–acetic acid buffer pH 5·9. Water was passed through the column until no more ninhydrin-positive compounds emerged and ammonium acetate buffer pH 5·9 was then passed through the column at a flow rate of 1·2 ml/min. Fractions (25 ml) were collected and tested for indospicine by t.l.c. in solvent D. Indospicine was detected in fraction Nos. 44–112 and these were combined and the ammonium acetate was removed as described previously. The crude indospicine was purified by conversion to the flavianate which was recrystallized several times from boiling water (350 mg). This contained a small amount of arginine which was removed as described for the corresponding isolate from seed. Indospicine monohydrochloride monohydrate was crystallized from aqueous ethanol. It was identical (m.p. and mixed m.p., i.r. spectrum, and behaviour on paper chromatography and ionophoresis) with the material isolated from seed.

(x) *Distribution of Indospicine in* I. spicata *Plants*

To gain some idea of the concentrations of indospicine in the various plant parts, mature single plants (about 2·5 kg fresh weight) of two accessions of *I. spicata* (CPI 18557 and 16110) growing in the field were harvested 2 in. above the ground and separated into the following parts; tips (the growing point and the last leaf to be fully expanded), the remainder of the leaves including petioles, stems, and (from CPI 16110) green seeds and seed pods. The various plant parts were cut up into small pieces which were thoroughly mixed and then divided into three portions. One portion was used to determine the dry weight and a second was extracted with 75% (v/v) ethanol as described previously. The extracts were concentrated and the volume adjusted so that 1 ml = 5 g fresh weight of plant and analysed for indospicine. As Table 2 shows, indospicine occurred in appreciable concentrations in the growing tips, the leaves, the stem, and the green seeds. In other experiments, it has been shown that the green seed pods contain only traces of indospicine so that the concentration in the seeds is actually higher than in the other plant parts.

The range of concentrations of indospicine in the leaves of mature plants analysed in this investigation has been 40–150 mg/100 g fresh weight, while values of 8–12 mg/100 g fresh weight have been obtained for seedlings 1 month old. Harvested seed has been found to contain 0·5–2% indospicine on a dry weight basis.

TABLE 2

DISTRIBUTION OF INDOSPICINE WITHIN FIELD-GROWN PLANTS OF TWO ACCESSIONS OF *I. SPICATA*

Plant Part	Accession CPI 18557		Accession CPI 16110	
	Fresh Wt. (g)	Indospicine Content (mg/100 g fresh wt.)	Fresh Wt. (g)	Indospicine Content (mg/100 g fresh wt.)
Tips	221	84	208	121
Leaves	1040	92	996	92
Stem	1035	52	1054	99
Seeds	—	—	661	124

III. BIOLOGICAL PROPERTIES

Subcutaneous injection of the crude extract of *I. spicata* produced extensive local oedema, often haemorrhagic, which undoubtedly led to gross disturbances in fluid balance of the animals and was a limiting factor in the amounts of extract which could be injected. The oedema increased for some hours and persisted for at least 24 hr. It was less with the various fractions of the extract and minimal with the pure toxin.

Histologically, fat commenced to accumulate in the livers about 6 hr after injection. It reached a maximum level at about 36 hr in the case of the crude extract and considerable amounts persisted at 48 hr. With the pure toxin the maximum infiltration was seen at about 24 hr, but fat still persisted at 48 hr (Table 1). The later maximum in the former instance is possibly due to the gross oedema produced by the crude extract with consequent delay in absorption of the toxin. The fat occurred predominantly in the peripheral zones of the lobules, extending inwards to the central veins as the accumulation proceeded and as the amount of toxin was increased. Female mice accumulated somewhat more fat than males (Table 1 and unpublished data) with the crude extract; it seems likely that this would be the case with the pure indospicine which appears to be the only toxin present, although this point has not been tested in this investigation.

The amounts of fat in the liver were reduced by simultaneous injection of arginine in amounts about equal to the amount of indospicine but were not altered by a much smaller amount of arginine, nor was the amount of fat affected by simultaneous injection of canavanine (Table 3). Canavanine itself produced no accumulation of fat.

It is therefore clear that the amounts of arginine and canavanine in the seed extracts did not affect the assay.

In haematoxylin–eosin-stained sections of the livers of mice injected with 40 mg of indospicine the cells throughout the lobules were enlarged, had lost the cytoplasmic basophilic granulation, and appeared pale and finely vacuolated or foamy. The vacuolation was coarser at the peripheral zones of the lobules presumably in

TABLE 3

TYPICAL ASSAY OF AN EXTRACT OF *I. SPICATA* SEEDS, COMPARISON WITH INDOSPICINE, AND INFLUENCE OF ARGININE AND CANAVANINE ON THE HEPATOTOXICITY OF INDOSPICINE

Groups of five female mice were injected with solutions of the various materials and the accumulation of fat in the livers assayed as described in the text and in Table 1. Extracts were prepared and described in the text and their indospicine content determined with an amino acid analyser. Indospicine, canavanine, and arginine were administered as their hydrochlorides

Material Administered	No. of Mice with Score of:					Mean Score
	0	1	2	3	4	
Extract 29A containing:						
Indospicine (30 mg)	—	—	1	2	2	3·2
Indospicine (20 mg)	—	—	1	2	2	3·2
Indospicine (10 mg)	1	1	2	1	—	1·6
Indospicine						
75 mg	—	—	1	2	2	3·2
40 mg	—	—	2	2	1	2·8
20 mg	1	2	—	2	—	1·6
10 mg	2	1	1	1	—	1·2
Indospicine (20 mg):						
+Arginine (5 mg)	1	1	2	—	1	1·8
+Arginine (25 mg)	4	—	1	—	—	0·4
Extract 91 containing:						
Indospicine (20 mg)	—	2	1	2	—	2·0
Indospicine (20 mg)						
+arginine (25 mg)	2	2	—	1	—	1·0
Indospicine (40 mg)	—	—	—	3	2	3·4
Indospicine (40 mg)						
+canavanine (40 mg)	—	—	1	2	2	3·0
Canavanine (40 mg)	2	3	—	—	—	0·6
Control mice						
(no treatment)	5	5	—	—	—	0·5

association with the accumulation of fat. However, stainable fat did not account for the finer vacuolation more centrally, which therefore is most likely due to water. Scattered isolated necrotic cells were present in small numbers in the mid zones and central zones of the lobules; many cells in these zones contained prominent phagosomes. The nuclei appeared to be slightly enlarged and depleted in chromatin whilst nucleoli were enlarged and prominent.

These changes were also seen to a lesser degree in animals injected with 20 mg and were grossly accentuated in those injected with 75 mg when groups of necrotic cells were found but massive necrosis was not observed. The livers of mice injected

with 40 mg of the toxin and 40 mg of canavanine showed similar changes while animals injected with canavanine alone showed no significant changes. Simultaneous injection of mice with arginine 25 mg and indospicine 20 mg resulted in mitigation of the above changes. Cytoplasmic basophilia was retained and the nuclei were less affected. Some hydropic change however persisted in the cytoplasm. An LD_{50} was not established as the material was in short supply. The highest dose given (75 mg) was well tolerated by the animals. In other experiments, mice fed the crude amino acid extract developed severe fatty change in the livers. The histological features were much exaggerated.

IV. Discussion

The isolation of indospicine adds a new type to the already wide spectrum of plant amino acids. Structure (I) was indicated by the hydrolysis experiments summarized in scheme I. Only a compound possessing structure (I) could give rise to these identifiable hydrolysis products. The conversion of the amidino group to the corresponding amide (II) on mild alkaline hydrolysis and to the carboxylic acid (III) on acid hydrolysis was in agreement with the results obtained by Arcamone *et al.* (1964) with the amidine-containing antibiotic distamycin A. The isolation of L-α-aminopimelic acid, optically pure within the limits of experimental error, under conditions unlikely to have caused any alteration in configuration showed that indospicine has the L-configuration. Structure I has been confirmed by the synthesis of DL-indospicine (Culvenor, Foster, and Hegarty 1969), which, except for those physical properties involving optical activity, was identical with the natural compound. The synthetic material inhibited the incorporation of [^{14}C]arginine into protein in a cell free system from rat liver with about half the activity of the L-isomer.

Although several antibiotics, e.g. distamycin A from a *Streptomyces* sp. (Arcamone *et al.* 1964) contains the simple amidino group, compounds containing this group do not appear to have been previously isolated from higher plants. With one possible exception we have not detected any other compounds giving the characteristic yellow-orange colour with the PCF reagent in numerous extracts of leaves and seeds of a range of *Indigofera* species. No evidence for the existence of indospicine in combined form was obtained by hydrolysis of several extracts and residues of *I. spicata* leaves. The pathway of biosynthesis of indospicine remains to be determined.

Indosipicine appears to be the only substance isolated from *I. spicata* which is hepatotoxic to mice. Its toxic properties account for the major part of the toxicity of the seed and of its crude extracts for mice. No other toxic compound was isolated and no solid evidence for the existence of another toxic for mice was found. The other unusual component of the seed, canavanine, did not lead to fat accumulation or necrosis of the liver in the dosage used, nor did it enhance the toxic properties of indospicine. No canavanine has been detected in the large number of ethanolic extracts of leaves of *I. spicata* which we have analysed so that this compound is not involved in the demonstrated toxicity of the leaves to rabbits and mice (Hutton, Windrum, and Kratzing 1958*a*, 1958*b*) and to grazing animals (Norfeldt *et al.* 1952).

Simultaneous injection of arginine mitigated the effects of indospicine—the amount of fat accumulation was less and the other cytological changes not so apparent.

Gullino *et al.* (1955) showed that L-arginine markedly reduced the toxicity of an $LD_{99.9}$ mixture of the other nine essential amino acids in rats and also acted as a protective agent against ammonia toxicity. The protective effect of L-arginine was considered (Greenstein *et al.* 1956) to be due at least in part to a mobilization and acceleration of the classic Krebs–Henseleit urea synthesis in the liver. However, it has now been shown that competitive antagonism occurs between arginine and indospicine in various pathways of arginine metabolism in rat liver and this may explain the protective action of L-arginine in our experiments. Thus, Madsen, Christie, and Hegarty (1970) showed that incorporation of L-[^{14}C]arginine into protein was depressed in cell-free fractions from the livers of female rats treated with indospicine and that a similar depression could be produced *in vitro*. The formation of [^{14}C]arginyl-*t*RNA was inhibited by indospicine *in vitro* while the formation of [^{14}C]leucyl-*t*RNA was not impaired. Madsen and Hegarty (1970) have shown that L-indospicine is a competitive inhibitor of rat liver homogenate arginase and that the affinity of the enzyme for indospicine is about the same order as that for arginine.

Britten *et al.* (1963) found that chicks receiving meal of *I. spicata* seed showed no symptoms of toxicity macroscopically through failure to gain weight or on histological examination. Birds given leaf meal from the plant failed to gain weight and showed hepatic lipidosis. They concluded that β-nitropropionic acid or an unknown compound closely associated with it in the plant was responsible for its toxicity to chicks. However, no direct experimental evidence is available to show that β-nitropropionic acid causes hepatic lipidosis in chicks.

The effect of feeding indospicine to chicks has not been investigated. However, in view of the indications that indospicine may interfere with arginine metabolism in mammalian test animals, a possible explanation of the different results obtained by feeding leaf meal and seed to chicks and rats and mice may lie in the different metabolic pathways in these animals. Chicks are uricotelic animals while rats and mice are ureotelic animals. It is of interest that another naturally occurring amino acid, L-2,4-diaminobutyric acid has been shown to interfere with urea synthesis in rat liver (O'Neal *et al.* 1968) and the results indicated a different mechanism of toxicity of the substance in uricotelic and ureotelic laboratory animals. Further work is necessary to determine if a similar difference occurs with indospicine.

Britten *et al.* (1963) concluded that the critical experiment on the substance in *I. spicata* which is toxic to cattle must be done directly on cattle despite the difficulties and expense involved. However desirable such an experiment might be, the isolation of sufficient indispicine for administration to cattle would be an extremely expensive and time-consuming task.

Indospicine has now been shown to be the principal free amino acid in the leaves of *I. spicata* and to be hepatotoxic to mice and rats (Christie, Madsen, and Hegarty 1969) as well as causing abortion and teratogenic effects in rats (Pearn and Hegarty 1970). There is a close similarity between these toxic effects and those reported in sheep and cattle fed *I. spicata* leaves (Norfeldt *et al.* 1952) which strongly suggests that strains of *I. spicata* likely to be non-toxic to cattle should have a very low indospicine content. A plant-breeding program aimed at developing such strains is now in progress at the Cunningham Laboratory, Division of Tropical Pastures, CSIRO, Brisbane.

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VI. References

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