BIOSYNTHESIS OF 3-NITROPROPIONIC ACID IN CREEPING INDIGO

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ABSTRACT

Candlish, Violet Elizabeth, M.Sc., The University of Manitoba, February 1968. Biosynthesis of 3-nitropropionic acid in Creeping Indigo. Major Professor: Lucien J. LaCroix.

Investigation of the biosynthesis of 3-nitropropionic acid in Indigofera spicata (Jacq.) Forsk. was undertaken in order to locate the origin of this toxic compound. Toxicity appears in animals fed this legume as a sole diet.

Gas - liquid chromatography was used to isolate and analyse quantitatively 3-nitropropionic acid in the plant extract. Considerable variation occurred in the level of this acid present in the different experiments, indicating a distinct environmental effect.

When labelled aspartate -3-^{14}C acid was fed to stem cuttings of creeping indigo there was no incorporation of label into 3-nitropropionic acid although considerable radioactivity was found in malic acid, one of the Krebs cycle acids. As deamination of aspartate leads to oxaloacetate, which is also a Krebs cycle acid, and no label appears in 3-nitropropionic acid, the conclusion was reached that the Krebs cycle acids are not directly involved in the formation of 3-nitropropionic acid in Indigofera spicata.

On feeding β-alanine-1-^{14}C to stem cuttings, there was no detectable radioactivity appearing in 3-nitropropionic acid. This suggested

that biosynthesis did not occur via oxidation of the amino group of β-alanine. Further studies using labelled malonate-2-^{14}C and labelled malonylmonohydroxamate-2-^{14}C revealed some incorporation of label into 3-nitropropionic acid, indicating that these compounds are on the biosynthetic pathway to 3-nitropropionic acid.

INTRODUCTION

Concern about the toxicity of the legume, creeping indigo, led to investigation of this plant by biologists and chemists (Bell and Everist 1951; Britten and Matsumoto 1959; Cooke 1955; Emmel and Ritchey 1941). Creeping indigo is known by the botanical name Indigofera spicata (Jacq.) Forsk. After introduction from the tropics of the Old World for experimentation by the Florida Experiment Station in 1925, the toxicity of this species was proven and it was not released for general agricultural use (Matsumoto and Unrau 1952). Before its toxicity was discovered, creeping indigo was in some use in Hawaii due to its excellent soil building characteristics and its nutritive value as a forage material.

Experimental feedings have been performed with rabbits, cattle, sheep, fowl, guinea pigs, pigs and mice (Matsumoto and Unrau 1952). All except swine and fowl found the plant palatable. Cattle developed symptoms when the diet contained 50% indigo or more and the duration of the experiment was two weeks. Sheep were more susceptible. Toxic symptoms were abortions in cattle, liver and kidney damage. Legumes completely suitable for use as forage crops and soil builders in tropical countries are difficult to find. General use of creeping indigo was curtailed until less toxic varieties could be found.

Bell and Everist (1951) reported a disease in horses which was causing concern in Australia at the time of Bell's investigations. This disease was believed to be caused by a different species of Indigofera.

A toxic substance of creeping indigo has been identified as 3-nitropropionic acid by a number of investigators including Britten, Matsumoto, et al (1959), Cooke (1955), Emmel and Ritchey (1941), Matsumoto, Nordfeldt, et al (1952), Morris, Pagan, et al (1954), Ko Sawai, Maeda, et al (1966). As the first naturally occurring aliphatic nitro compound found, 3-nitropropionic acid has interested several investigators. Dr. A. M. Unrau was one who became interested in this compound and while with the University of Manitoba initiated studies on the properties of this acid.

The function and metabolism of this nitro compound in the plant have not been studied extensively. Recently, three different groups, Birkenshaw and Dryland (1964), Gatenbeck and Forsgren (1964), Shaw and Wang (1964), Shaw (1967), have studied the metabolism of 3-nitropropionic acid in fungi. The present study was undertaken to identify precursors of 3-nitropropionic acid in Indigofera spicata. Initial experiments were designed to establish whether or not there was a relationship between the Kreb s cycle acids and 3-nitropropionic acid. Gas-liquid chromatography was used for the detection of 3-nitropropionic acid. Degradation experiments were carried out on the acid when radioactivity was detected following feeding of labelled compounds. In later experiments, the possible role of malonate and malonate derivatives as precursors was studied.

LITERATURE REVIEW

THE OCCURRENCE AND CHEMISTRY OF 3-NITROPROPIONIC ACID

The bark of the tree Hiptage mandablata Gaertn., the berries of the Karaka tree, Corynecarpus laevigata Forst., creeping indigo and the roots of the fragrant violet, Viola odorata L., were all found to contain 3-nitropropionic acid. Among the fungi it has been found in culture filtrates of strains of Aspergillus flavus Link., Aspergillus oryzae (Ahlburg) Cohn. and Penicillium atrovenetum G. Smith.

In the moulds, 3-nitropropionic acid apparently occurs in a free state whereas in higher plant species it occurs as the crystalline glycoside. Carter (1949) examined hiptagin obtained from Hiptage mandablata, which yielded hiptagenic acid when hydrolysed. This acid was found to be identical to one previously identified by Carter in the berries of Corynocarpus laevigata. In these berries, the glycoside, after acid hydrolysis, yielded carbon dioxide, ammonia and a sugar as well as hiptagenic acid. The sugar gave a glucosazone (the same osazone is given by mannose) while hiptagenic acid was found to be 3-nitropropionic acid. In Indigofera spicata several glycosides were identified and called "endecaphyllins" (Finnegan, Mueller et al 1963). These glycosides have the general formula $C_6H_{12-n}O_6(COCH_2CH_2NO_2)_n$.

In the laboratory, 3-nitropropionic acid is synthesized from silver nitrite and 3-propiolactone (Unrau 1965). After several recrystallizations,

a white needle-like crystal forms which will melt from 67-68°C. The acid can be hydrolysed and yields NO_2^- which in the past has been used for the colorimetric detection of 3-nitropropionic acid. The displacement of the nitro group is most effective at about pH 9·5 (Matsumoto, Unrau, et al 1961).

RESEARCH ON MOULDS

The production of 3-nitropropionic acid by a strain of Aspergillus flavus was recorded by Bush, Touster and Brockman in 1951. Raistrick and Stossl (1958) reported the acid as a major metabolite of Penicillium atrovenetum. The three papers by Birkenshaw and Dryland (1964), Shaw and Wang (1964), Gatenbeck and Forsgren (1964), increased available evidence for precursors of 3-nitropropionic acid in Penicillium atrovenetum.

The yield is greater in ammonium salt medium than in the nitrate medium. Birch, McLoughlin, et al (1960) showed that $NaH^{14}CO_3$ was incorporated into 3-nitropropionic acid, most of the activity being in the first carbon and approximately 1% in the second carbon. When aspartic acid-4-^{14}C was the tracer, the activity was found in the first carbon only. These results along with results obtained by Hylin and Matsumoto (1961) indicated a requirement of ammonium ion for the biosynthesis of 3-nitropropionic acid. Observations that succinic acid, fumaric acid and tartaric acid increase 3-nitropropionic acid production more than

aspartic acid, suggested that the amino group may not be directly oxidized. The possibility arises that little or no free ammonium ion is present in the medium containing aspartic acid as the sole source of nitrogen (Shaw and Wang 1964). For transamination reactions a free ammonia intermediate is not needed, rather a transaminase enzyme is utilized. Gatenbeck and Forsgren (1964) made use of uniformly labelled aspartic acid (label in both nitrogen and carbons) and showed that the resulting 3-nitropropionic acid was also uniformly labelled. This result led to the conclusion that an in situ oxidation of the amino group of aspartic acid did occur. Birkenshaw and Dryland (1964) concluded that the carbon skeleton of 3-nitropropionic acid is formed from oxaloacetic acid. The unequal distribution of radioactivity in C_2 and C_3 of 3-nitropropionic acid derived from pyruvate-2-^{14}C indicated that fumaric acid, owing to its symmetry, could not be a direct precursor.

β-alanine was shown not to be involved in the nitro acid synthesis by all the workers previously mentioned. However, Marshall (1965) suggests that aspartate is converted to β-alanine in Aspergillus flavus which then is converted to 3-nitropropionate; nitrite is then split off and converted to nitrate. If 3-nitropropionic acid is involved in nitrification by Aspergillus flavus then it is likely that the pathway of 3-nitropropionic acid synthesis in this fungus differs from that in Penicillium atrovenetum. Recent work by Shaw (1967) has shown an enzymatic reduction of 3-nitro acrylic acid to 3-nitropropionic acid.

RESEARCH ON INDIGOFERA

The toxicity of Indigofera spicata for rabbits was reported by Emmel and Ritchey (1941). Experiments were conducted in two different countries. Plants grown in the Philippines showed no evidence of toxicity, but grazing plots in Gainesville, Florida produced a definite toxicity in rabbits. Morris, Pagan, et al (1954) associated this toxicity with 3-nitropropionic acid. Subsequent experiments by Hutton, Windrum, et al (1958); Britten, Matsumoto, et al (1959); Britten, Palafox, et al (1963) and Murray, Moore, et al (1965) have not solved the problem of toxicity of indigofera. As these experiments verify, 3-nitropropionic acid is certainly a toxic component, but whether or not there are any other toxic components in creeping indigo is open to question. Hutton, Windrum, et al (1958) produced evidence of toxicity when seeds were fed to rabbits. As the seeds do not contain 3-nitropropionic acid this group concluded that toxicity was not related to the content of the nitro acid. The other experimenters mentioned did relate toxicity to 3-nitropropionic acid level by feeding the synthesized acid at levels found in indigo meal.

In unpublished work carried out by Unrau, experimental evidence was obtained which showed that aspartic acid, succinic acid and fumaric acid when fed in solution to cuttings of creeping indigo resulted in highly significant increases in the concentration of the nitro acid. Nitrate and ammonium ion increased the concentration of the nitro acid with ammonium ion being slightly more effective. This work led to the conclusion that the Krebs cycle acids were involved in 3-nitropropionic acid synthesis.

NITROGEN METABOLISM

Legumes are plants which fix nitrogen due to a symbiotic relationship with bacteria. As a legume, Indigofera will have the peculiar nitrogen metabolism associated with nitrogen fixation. Nodules are produced by bacteria in the root and these nodules have vascular connections to the plant proper. Rhizobium is the genus of bacteria responsible for nodule formation (Bonner and Varner 1965).

Nitrogenous compounds are absorbed from the soil by roots of green plants. Most of the nitrogen is absorbed in the form of nitrates which are rapidly reduced on entering the plant. Since conversion to nitrite is the first step in nitrate reduction, nitrites can also serve as a nitrogen source. However nitrites are not an important source of nitrogen. Ammonium salts and organic nitrogen compounds can be important nitrogen sources where certain soil conditions prevail. These four sources of nitrogen must all be taken into consideration when examining the biosynthesis of 3-nitropropionic acid.

Reduction of nitrate does not appear to be reversible in plants and is accompanied by a decrease in the carbohydrate stores and a marked increase in the rate of respiration to supply energy for the endergonic reactions involved. Various workers (Bonner and Varner 1965; Webster 1959; McKee 1962) have suggested that throughout nitrate reduction the nitrogen atom at various stages of reduction forms a part of a series of organic molecules. This could infer a role for 3-nitropropionic acid as

one of these organic molecules in conversion of nitrate to ammonia. The molecular species definitely involved in nitrate reduction are N_2, NO_3^-, NO_2^-, NH_2OH and NH_3. These are not enough for a series of biochemically plausible steps. Nitrite reduction is affected by photochemical processes which indicate a photochemical influence on 3-nitropropionic acid level if this compound is involved in nitrate reduction. Losada, Ramirez, et al (1965) and Wessels (1965) have investigated spinach chloroplasts. A nonenzymatic photoreduction of nitro- and nitroso-aryl compounds by reduced ferredoxin was found.

Nitrogen compounds in plants arise through four metabolic processes (Steward and Pollard; Holden, Editor 1962). Protein synthesis requires amino acids, which make up many of the nitrogen compounds found in plants. Accumulation of a nitrogenous compound may result due to a metabolic block brought about by genetic, nutritional, or environmental means. Some nitrogen compounds act as inhibitors or antimetabolites which arrest metabolic sequences that would otherwise occur. Storage organs may accumulate nitrogen compounds where growth and protein synthesis are arrested. Nitrogenous end products of plant metabolism are rarely lost as volatile compounds, nor are they excreted. These must all be considered as ways in which 3-nitropropionic acid might arise in the nitrogen metabolism of the creeping indigo.

Only the young leaves really participate very actively in the metabolism of the whole plant (Holden 1962). In Indigofera spicata higher levels of the compound, 3-nitropropionic acid, were found in the younger leaves (Unrau 1965). McKee (1962) indicated a possible relationship between hydrogen cyanide and 3-nitropropionic acid. HCN combined in glucoside form is known to occur in several hundred species. Formation of some cyanogenetic glucosides is associated with amino acid metabolism. Gentle chemical treatment can convert 3-nitropropionic acid to HCN.

In a recent paper by Doxtader (1966) results of his experiments indicated that malonylmonohydroxamic acid and 3-nitropropionic acid were both excellent substrates for nitrate production. The possible role of malonylmonohydroxamic acid as a precursor of 3-nitropropionic acid was suggested in the conclusions.

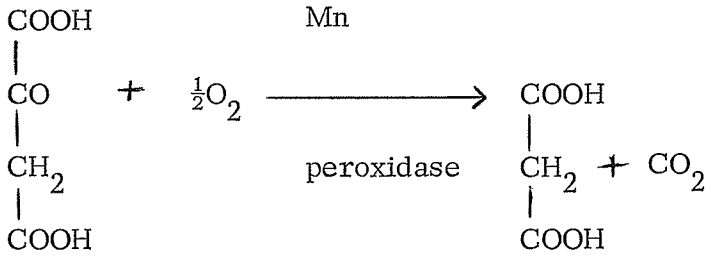
CARBOHYDRATE METABOLISM

The Kreb s tricarboxylic acid cycle provides energy and a source of carbon skeletons in aerobic organisms. The acids involved in this cycle are malic, oxaloacetic, citric, cis-aconitic, isocitric, oxalosuccinic, ∝-ketoglutaric, succinic and fumaric (Bonner and Varner 1965). As a result of experiments carried out with indigo cuttings, Unrau (1965) suggested a metabolic relationship between aspartic, fumaric, succinic acids and 3-nitropropionic acid. The

Kreb's cycle acids were thought to be a likely source of the carbon skeleton for the nitro acid.

Compartmentation of organic acids in plant tissues presents a conceptual picture which may be helpful in considering the origin of carbon skeletons for various natural compounds (MacKennan, Beevers, et al 1963; Lips and Beevers 1966). Malate produced by dark fixation carboxylation reactions is in a pool physically separated from malate produced by reactions of the tricarboxylic acid cycle.

There is indication that enzymes are widely distributed among plant species which are capable of converting oxaloacetate to malonate (Pattee 1965).



Malonic acid is a common constituent of mature leaves of certain species of Leguminosae (Bonner and Varner 1965). There are many ways malonate may be synthesized in cells of one sort or another. In higher plants the two main ways are

(1) carboxylation of acetyl CoA to yield malonyl CoA, a precursor of malonic acid and

(2) by -decarboxylation of oxaloacetic acid.

Malonyl CoA is utilized in a number of reactions such as biotin formation, fatty acid synthesis and flavonoid synthesis.

MATERIALS AND METHODS

STEM CUTTING EXPERIMENTS

Creeping indigo plants were grown in pots in the greenhouse at a temperature greater than 75° F. Twelve to fourteen inch cuttings were taken from the runners of the plants. The nutrient solution used was 0.01 M in $CaCl_2$, $MgSO_4$, NH_4Cl, NaCl and 0.0015 M in KH_2PO_4. This solution was adjusted to pH 6.5 with NaOH when necessary. Fifty-ml plastic tubes were used to hold the solution and cuttings. At the conclusion of the experiment, the cuttings were removed from the tubes, washed with water and dried at 65 °C. After drying, the cuttings were ground in a food chopper.

INTACT PLANT FEEDING EXPERIMENTS

Stem cuttings were rooted in moist sand. After two months, roots were established and these plants were moved to boxes containing a nutrient medium made up as follows: 0.005 M $Ca(NO_3)_2 \cdot 4H_2O$; 0.005 M KNO_3; 0.002 M $MgSO_4 \cdot 7H_2O$; 0.001 M KH_2PO_4; 1 ml of micronutrients and 5 mg of sequestrene were added per liter of nutrient medium. The micronutrients were made up as follows: 0.046 M H_3BO_3; 0.0092 M $MnCl_2 \cdot 4H_2O$; 0.0008 M $ZnCl_2$; 0.0003 M $CuCl_2 \cdot 2H_2O$; 0.0001 M $NaMoO_4 \cdot 2H_2O$. The sequestrene used was technical sodium ferric diethylenetriamine pentaacetate, containing 14.2% iron as Fe_2O_3; and is an iron chelate. For feeding experiments the plants were transferred

to bottles containing the same nutrient medium as was used for the stem cutting experiments. Labelled precursors were added to the nutrient medium and air was bubbled through the solution for the duration of the experiment. The plants were dried on completion of the treatment and chopped, the roots being treated separately from the stems and leaves.

COLORIMETRIC DETERMINATION OF NITRITE

Nitrite Reagent

Solution A: sulfanilic acid solution

to 70 ml water and 30 ml concentrated HCl was added 0.5 g sulfanilic acid.

Solution B: Naphthylamine reagent

0.5 g Naphthylamine hydrochloride was dissolved in 25 ml ethanol (95%). 75 ml of water was added.

To 1 ml aliquots of sample, cooled in ice water, was added 3 ml of solution A, contents mixed and after several minutes 3 ml of solution B was added, and 5 ml of 95% ethanol. The contents were mixed and read at 520 mμ within 15 - 20 minutes in a Bausch and Lomb colorimeter. Silver nitrite was used to construct the standard curve. Solutions were made up to contain in the region of 50 μg NO_2^- per ml.

SAMPLE PREPARATION

Samples of two grams or more were extracted with 50 ml distilled water by heating in a pressure cooker. Pressure was maintained at the five pound level for one hour, after which, the heat was turned off and the samples removed from the cooker to stand overnight before proceeding with the sample separation.

The water extract, obtained by filtering the hydrolysed mixture, was separated into basic, acidic and neutral fractions by ion exchange chromatography. The cation exchange column material used was Dowex 5OW-X8 and the anion exchange resin was Dowex 1-XIO (converted to the formate form). The organic acid fraction, containing the 3-nitropropionic acid, was methylated using diazomethane and analysed in the gas Chromatograph (Canvin 1965).

For the gas chromatography, two different columns were used.

(1) Chromosorb W (60-80 mesh) coated with 10% reoplex 400 in a copper tube 15 inches x $\frac{1}{4}$ inch.

(2) 5% versamid 900 on silanized Chromosorb W (60-80 mesh) in a copper tube 4 feet x $\frac{1}{4}$ inch.

The helium gas flow rate through the column was adjusted to 100 ml per minute. Some of the peaks on the chromatogram were identified by comparison with standard mixtures. Various peaks were collected and counted in a Nuclear - Chicago (720 series) Liquid Scintillation counter using dioxane scintillator.

DEGRADATION OF LABELLED 3-NITROPROPIONIC ACID

The methylated ester of 3-nitropropionic acid was collected from the gas chromatograph in water. The sample was deesterified by refluxing for at least an hour with 3 ml of 2N NaOH. To the amount obtained was added 400 mg of unlabelled 3-nitropropionic acid and the degradation procedure continued.

The Nef reaction (Birkenshaw and Dryland 1964) was used to degrade the nitro acid to acetaldehyde dinitrophenylhydrazone, liberating the first carbon as CO_2. This carbon dioxide was trapped in 3 ml of a 1:2 mixture of ethanolamine: ethylene glycol monoethyl ether (ethyl cellosolve). The trapped $^{14}CO_2$ was combined with 15 ml of toluene scintillator (1:2 mixture of ethyl cellosolve in toluene containing 6 g/liter of 2.5 diphenyloxazole) and counted in the Liquid Scintillation counter.

Acetaldehyde dinitrophenyl hydrazone was degraded to sodium acetate by Kuhn-Roth oxidation (Stickings and Townsend 1961). The Schmidt reaction (Colowick and Kaplan 1962) was used to degrade the sodium acetate. The CO_2 was trapped and counted as described above. The amounts of reagents used were multiplied by five in order to be able to degrade the complete sample from the Nef degradation at one time.

CHEMICALS

Chemical s used as precursors were as follows:

D-L aspartic-3-^{14}C acid (2.4 mc /mM) Baird-Atomic Inc.;

β-alanine-I-^{14}C (0.05 mc / 1.3 mg) Radiochemical Centre;

sodium acetate -2-^{14}C (56.8 mc /mM) New England Nuclear Corp.;

sodium carbonate - ^{14}C (2.00 mc /mM) Radiochemical Centre;

diethyl malonate-2-^{14}C (2.00 mc /mM) Radiochemical Centre;

malonic acid-2-^{14}C (0.1 mc/1.4 mg or 7.27 mc/mM) New England Nuclear Corp.;

Malonyl monohydroxamic acid was prepared in the laboratory by using malonic acid-2-^{14}C. Malonic acid monochloride was prepared according to Colowick and Kaplan (1962). The monohydroxamate was prepared by adding neutralized hydroxylamine to malonic acid monochloride (Lipmann and Tuttle 1945). Neutralized hydroxylamine was prepared by adding one volume of 4N hydroxylamine hydrochloride to one volume of 3.5 M NaOH.

RESULTS

STANDARD SAMPLES EXPERIMENT

1. Standard samples of 3-nitropropionic acid were prepared and analysed by the colorimetric procedure used in earlier indigo experiments by Unrau (1965). These were compared with standard samples put through the ion exchange columns, eluted and determined colorimetrically. Recovery in 4 determinations was between 95 and 100% of the sample put onto the column.

2. Two methods for treating the methyl esters were compared and the results analysed statistically to determine which method would be used in future analysis.

Method A: Methyl esters were made up to 5 ml with methanol and 50 µl taken for injection into the gas chromatograph.

Method B: Methyl esters from method A were blown down, in a stream of air, to a volume of 0.5 ml and 20 µl taken for injection into gas chromatograph.

Table I shows the results of this experiment. Blowing the methylated organic acid fraction to dryness loses much of the 3-nitropropionic acid portion.

IDENTIFICATION OF SOME PEAKS

The acids included in a standard mixture were oxalic, malonic, succinic, tartaric and citric. This mixture was compared with a leaf sample by combining the two and injecting a portion. Malonic, succinic, malic and citric acid peaks coincided with peaks in the leaf extract. Table II shows the results of these experiments. A sample chromatogram from the reoplex column is illustrated in Figure 1.

The main peak of oxaloacetic acid was found to emerge coincidental with 3-nitropropionic acid on the reoplex column. On switching to the versamid column, separation was effected, with 3-nitropropionic acid emerging first. When standard methylated 3-nitropropionic acid was injected an average value of 0.83 I.U./μg was obtained. Figure 2 shows an example of a versamid column chromatogram.

TABLE I - Comparison of two methods of analysis of 3-nitropropionic acid (methyl ester).

Injected sample g	I.U. (1)	I.U. (2)	I.U./μg (1)	I.U./μg (2)	Average I.U./μg
A					
100	83.2	80.0	0.8320	0.8000	0.8160
150	126.4	116.0	0.8426	0.7733	0.8080
200*	142.0	142.0	0.7100	0.7100	0.7100
250	206.0	206.4	0.8240	0.8256	0.8248
300	240.0	260.0	0.8000	0.8670	0.8330
350	301.2	296.0	0.8605	0.8457	0.8530
B					
400	200.0	187.2	0.5000	0.4680	0.4840
600	366.0	338.4	0.6100	0.5640	0.5870
800*	358.0	368.0	0.4475	0.4600	0.4537
1000	588.0	551.2	0.5880	0.5512	0.5676
1200	856.0	832.0	0.7133	0.6933	0.7033
1400	1092.0	1020.0	0.7800	0.7285	0.7542

* omitted from "method" calculations due to loss of sample.

I.U. means integration units.

Average method A: 0.82696 ± 0.01732 % error 2.1%

Average method B: 0.61925 ± 0.1086 % error 17.5%

TABLE II - Identification of plant organic acids by gas chromatography of their methyl esters.

Sample * injected	Peak areas (integration units)				
	peak 1	peak 3	peak 4	peak 7	peak 11
leaf sample	2464	220	196	250	308
leaf sample + malonic acid	4675	210	176	---	340
leaf sample + succinic acid	----	>2000	---	---	---
leaf sample + 3 NPA	----	---	582	---	---
leaf sample + malic acid	----	---	---	>2000	---
leaf sample + citric acid	2500	---	182	---	480

* For identification purposes, known methylated organic acids were added to the methylated leaf sample.

Peak numbers referred to are as in Figure 2, p. 21.

3 NPA - 3-nitropropionic acid.

Sample chromatograms of organic acids (methylated) of

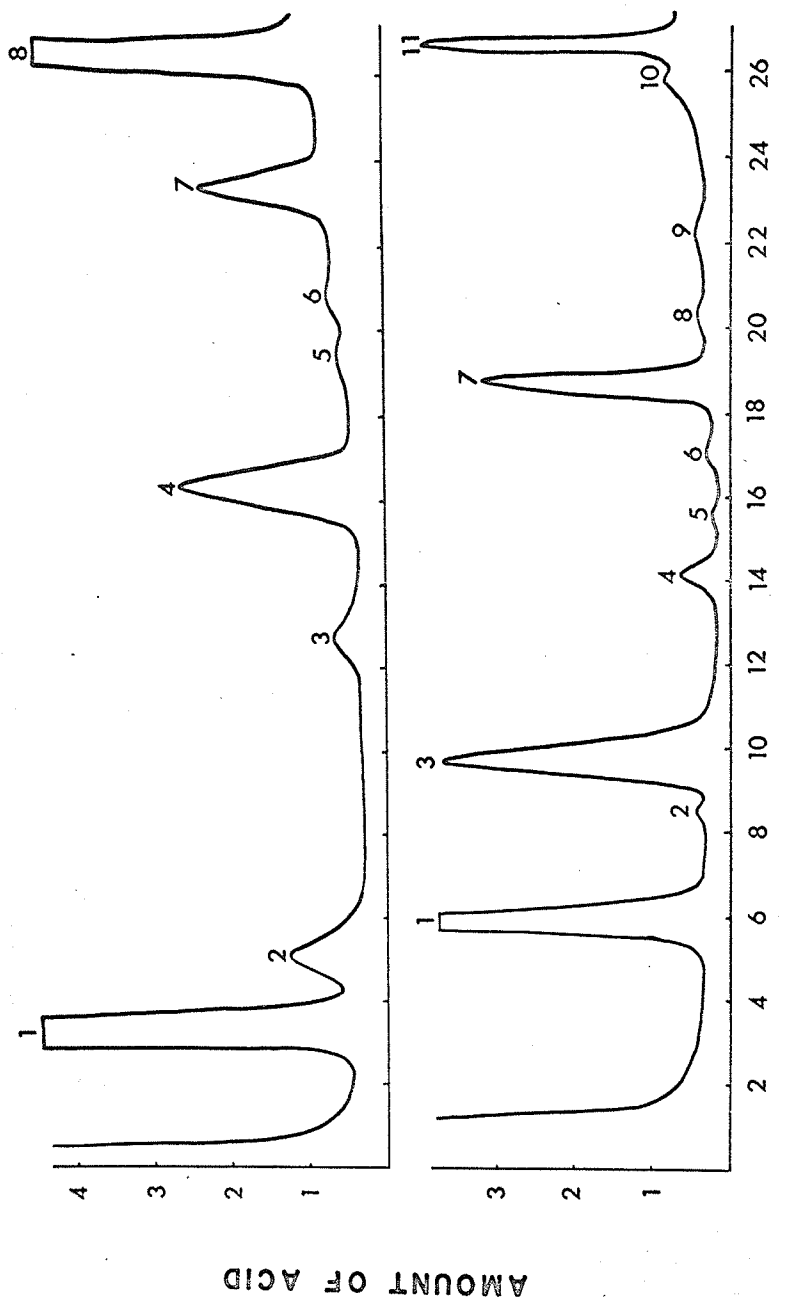
Indigofera spicata water extract.

Figure 1: (top) using reoplex treated column

Figure 2: (bottom) using versamid treated column

Legend:

acid	peak number	
	reoplex column	versamid column
malonic acid	1	1
succinic acid	2	3
3-nitropropionic acid	3	4
oxaloacetic acid	3	5 and 6
malic acid	4	7
citric acid	8	11


AMOUNT OF ACID
TIME IN MINUTES
1
2
3
4
5
6
7
8
9
10
11

PRELIMINARY PRECURSOR EXPERIMENTS

Stem Feeding

Aspartic acid, β-alanine, propionic acid and oxaloacetic acid were fed at a concentration of 0.1 M in the nutrient medium previously indicated. The malonic acid was found to be toxic at this concentration and therefore was fed at 0.02 M in nutrient medium. The aspartic acid and β-alanine were labelled with ^{14}C. The uptake of the labelled compounds by the cuttings is shown in Table III.

Of the labelled compounds fed, between 9-13% of the radioactivity was detected in the sample applied to the ion exchange column. The distribution of radioactivity after gas chromatography is shown in Table IV. The amount of 3-nitropropionic acid present in samples was calculated from the chromatograms and the results tabulated in Table V. This is the only experiment reported in this thesis in which the reoplex column was used for the gas chromatography and the conversion factor for this table only was 0.75 I.U./μg 3-nitropropionic acid.

Intact Plant Feeding

Sodium acetate, diethyl malonate and malonic acid (all labelled in position 2) and labelled sodium carbonate were fed in nutrient medium. Information obtained from this experiment is tabulated in Tables VI, VII, VIII and IX. The total amount of radioactive material added to the

bottles at the beginning of the 9 day run, expressed in disintegrations per minute, were as follows:

sodium acetate	9.61×10^7
sodium carbonate	8.35×10^7
diethyl malonate	11.73×10^7
malonic acid	0.983×10^7

TABLE III - The uptake of ^{14}C labelled aspartate and β-alanine by cuttings of creeping indigo.

No. of samples	Precursor	Mean radioactivity (dpm X 10^{-6})		
		fed	uptake	% uptake
6	aspartate-3-^{14}C	4.8	4.013	83.6
range			3.325-4.566	70.0-95.1
7	aspartate-3-^{14}C	5.61	4.100	73.0
range			3.290-4.788	59.0-86.0
3	aspartate-3-^{14}C	6.42	5.483	85.5
range			4.390-6.183	69.0-96.9
6	β-alanine-1-^{14}C	0.808	0.721	89.2
range			0.618-0.777	76.5-96.1

TABLE IV - Distribution of radioactivity in the aqueous extract of plant material after gas chromatography.

component	Aspartate -3-^{14}C feeding: mean radioactivity of 5 samples (dpm x 10^{-6})	Aspartate -3-^{14}C feeding: % of fed	β-alanine -1-^{14}C feeding: mean radioactivity of 2 samples (dpm x 10^{-6})	β-alanine -1-^{14}C feeding: % of fed
total sample put on ion exchange column	1.88	10.9	0.28	12.8
organic acid fraction	0.51	2.92	0.013	0.5
3-nitropropionic acid	none	----	none	----
malic acid	0.08	0.46	not determined	

TABLE V - Amount of 3-nitropropionic acid (3 NPA) in water extract samples from preliminary stem feeding experiments.

precursor	No. of samples	mean and range I.U. *	mean μg 3 NPA x 10^{-2}	mg 3 NPA / g dry wt.
aspartate	10	40.9 26.4-58.0	54.6	1.24
β-alanine	3	32.9 13.6-54.0	43.8	1.02
propionate	4	19.6 14.4-25.6	26.2	0.57
malonate	4	37.9 34.0-42.0	50.6	1.51
oxaloacetate	4	36.4 29.6-44.0	48.6	1.06
control	4	42.2 22.0-60.8	56.2	0.78

* I.U. = integration units

TABLE VI - Comparison of 3-nitropropionic acid level (mg /g dry wt.) in leaf, root and stem portions of the preliminary intact plant feeding experiments.

precursor fed	leaf	root	stem
sodium acetate	0.130	1.250	0.559
sodium carbonate	1.030	1.325	1.100
diethyl malonate	1.086	1.448	0.334
malonate	0.830	4.490	0.705

TABLE VII - Distribution of radioactivity in leaf, root and stem of indigo plants fed various substrates.

substrate fed	leaf	root	stem
Radioactivity in sample prior to ion exchange chromatography (dpm)			
sodium acetate	12.6×10^5	24.5×10^5	13.2×10^5
sodium carbonate	0.712×10^5	0.48×10^5	1.4×10^5
diethyl malonate	22.0×10^5	53.7×10^5	16.9×10^5
malonate	1.62×10^5	3.22×10^5	2.15×10^5
Radioactivity in total organic acid sample (dpm)			
sodium acetate	1.5×10^5	3.3×10^5	1.4×10^5
sodium carbonate	0.116×10^5	00.143×10^5	0.177×10^5
diethyl malonate	7.7×10^5	20.0×10^5	5.4×10^5
malonate	0.42×10^5	00.4×10^5	0.2×10^5
Radioactivity in 3-nitropropionic acid (dpm)			
sodium acetate	22×10^2	19×10^2	31×10^2
sodium carbonate	7×10^2	9×10^2	9×10^2
diethyl malonate	19×10^2	31×10^2	20×10^2
malonate	2×10^2	3×10^2	negligible

TABLE VIII - The amount of 3-nitropropionic acid (3 NPA) in leaf, root and stem of indigo plants fed various substrates.

substrate	3-nitropropionic acid mg per sample leaf	root	stem	mg / g dry wt.
	leaf	root	stem	Total mg/ g dry wt.
	mg per sample			
acetate	0.50	1.00	2.20	0.42
carbonate	3.48	0.94	3.06	1.09
diethyl malonate	4.60	1.30	0.94	0.86
malonate	4.70	8.00	3.30	1.32

TABLE IX - Distribution of radioactivity in organic acid fraction of water extract of intact plants from preliminary experiments.

(dpm /g dry wt.)

substrate	total	3 NPA *	malate	malonate
sodium acetate	7.10×10^4	8.22×10^2	17.7×10^2	30.9×10^2
sodium carbonate	0.63×10^4	3.64×10^2	1.45×10^2	5.34×10^2
diethyl malonate	41.50×10^4	8.80×10^2	18.00×10^2	23.8×10^4

* 3 NPA - 3-nitropropionate

PRECURSOR EXPERIMENTS

Malonic acid stem feeding

In experiment 1 and 3, malonic acid-2-^{14}C in 5 ml of water was fed to the cuttings, immediately followed by nutrient medium for the remainder of the two day run. The amount of labelled material fed per tube was 0.37 μc in experiment 1 and 2.60 μc in experiment 3.

The labelled acid feeding in experiment 2 was followed by 0.01 M malonic acid in nutrient solution. Here the levels of radioactivity were 0.95 μc and 1.89 μc per tube. Results from these experiments are listed in Tables X and XI.

Hydroxamic acid stem feeding

The monohydroxamate of malonic acid was fed in water at two different levels of radioactivity, 0.81 μc per tube and 1.62 μc per tube, followed by nutrient medium. In a later similar experiment 1.52 μc was in each tube. Results of these experiments are shown in Tables XII and XIII. The concentration of the monohydroxamate was 0.01 M in water.

Sodium carbonate stem feeding

Sodium carbonate-^{14}C was fed in water at a level of 7.3×10^5 dpm/tube or 0.33 μc/tube, followed by nutrient medium for two days. A similar pulse of sodium carbonate was then given followed by nutrient medium for two more days. The organic acid fraction of these samples was found to contain approximately 2.5×10^4 dpm / g dry wt. of sample. No counts were found in the 3-nitropropionic acid fraction.

TABLE X - Amount of 3-nitropropionic acid in water extract samples from stem feeding experiments where malonic acid -2-^{14}C was fed as precursor.

treatment and level of radioactivity per tube	number of samples	mean and range mg 3 NPA per g dry wt.
pulse 0.371 μc	5	1.26 (0.81 - 1.70)
0.946 μc +0.01 M malonic acid	4	2.55 (1.63 - 3.30)
1.892 μc 0.01 M malonic acid	2	2.52 (2.12 - 2.93)
pulse 2.61 μc	4	1.84 (0.35 - 4.19)

TABLE XI - Incorporation of ^{14}C into various organic acids from stem feedings of malonate -2-^{14}C.

(dpm / g dry wt.)

treatment and level of radioactivity per tube	total organic acids dpm x 10^{-5}	malate dpm x 10^{-2}	3 NPA dpm x 10^{-2}	malonic acid dpm x 10^{-5}
Experiment 1				
pulse 0.371 μc and nutrient only	1.30	11.4	2.02	1.17
	1.42	15.9	2.38	1.11
	1.76	11.1	0.55	1.64
	2.00	16.7	2.68	1.90
	2.02	----	1.87	1.87
Experiment 2				
pulse 0.946 μc + 0.01 M malonate + nutrient	2.74	18.0	1.39	2.20
	4.17	----	3.08	3.74
	4.12	9.5	4.87	3.80
	3.34	9.7	4.70	2.88
pulse 1.892 μc + 0.01 M malonate + nutrient	4.75	29.4	2.80	4.40
	5.70	21.8	5.20	5.02
Experiment 3				
pulse 2.61 μc + nutrient	9.46	89.5	14.9	7.35
	9.40	80.7	21.3	7.08
	10.30	67.0	15.9	7.15
	7.50	59.0	15.0	6.37

TABLE XII - Amount of 3-nitropropionic acid (3 NPA) in water extract samples from stem feeding experiments where malonylmonohydroxamate -2-^{14}C was fed as precursor.

level of radioactivity per tube	number of samples	mean and range mg 3 NPA per g dry wt.
0.81μc	5	2.20 (1.51 - 2.74)
1.62μc	7	2.17 (1.29 - 3.11)
1.52μc	7	2.17 (0.74 - 3.72)

TABLE XIII - Incorporation of ^{14}C into various organic acids from stem feedings of malonylmonohydroxamate-2-^{14}C.

(dpm / g dry wt.)

level of radioactivity per tube	organic acid dpm x 10^{-5}	malate dpm x 10^{-2}	3 NPA * dpm x 10^{-2}	malonic acid dpm x 10^{-5}
0.81 μc	4.82	138.0	6.70	4.08
	5.42	50.7	2.98	4.27
	4.25	26.7	3.06	3.71
	5.30	32.5	2.98	3.57
	4.35	57.7	6.22	3.82
1.62 μc	5.44	32.6	4.78	4.65
	4.55	34.0	4.15	3.40
	4.10	27.8	3.83	3.60
	4.23	29.8	4.56	3.93
	4.59	46.1	2.31	3.26
	5.85	46.0	4.88	5.37
	5.05	45.3	9.50	4.65
1.52 μc	7.03	131.0	22.8	6.02
	8.95	71.0	21.0	6.86
	6.38	51.3	20.1	4.54
	9.50	116.5	26.4	7.28
	7.40	41.5	12.6	6.25
	6.93	51.9	17.4	5.65
	6.45	73.0	15.4	5.50

* 3-nitropropionic acid

Intact plant feedings

Malonic acid -2-^{14}C was fed at the level of 3.49μc per jar or 7.75×10^6 dpm. Malonylmonohydroxamate-2-^{14}C was fed at the level of 3.21μc per jar or 7.13×10^6 dpm. The results are tabulated in Tables XIV and XV.

Degradation of samples

Stem and root samples of the final experiment were used to collect labelled methylated 3-nitropropionic acid. Results of degrading these samples are shown in Table XVI.

TABLE XIV - Amount of 3-nitropropionic acid (3 NPA) in water extract samples of stems and roots from intact plant feeding experiments.

precursor	plant portion	number of samples	mean and range mg 3 NPA per g dry wt.
malonate	stem	3	1.53 (0.95 - 2.31)
malonate	root	2	2.47 (2.21 - 2.73)
hydroxamate	stem	6	1.49 (0.66 - 2.34)
hydroxamate	root	2	2.51 (2.38 - 2.64)

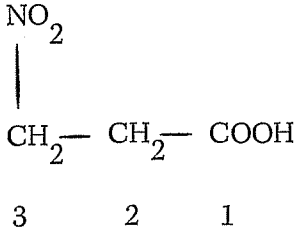
TABLE XV - Incorporation of ^{14}C into various organic acids from intact plant feeding experiments.

(dpm / g dry wt.)

treatment	organic acid dpm x 10^{-5}	malate dpm x 10^{-2}	3 NPA dpm x 10^{-2}	malonic acid dpm x 10^{-5}
malonic acid stem	0.104	1.64	2.15	0.0635
	0.106	0.38	1.38	0.0783
	0.111	1.01	1.76	0.0450
hydroxamate stem	0.240	2.78	1.08	0.177
	-----	13.60	2.37	0.302
	0.147	4.57	1.52	0.090
	0.750	----	7.05	0.508
	0.147	2.30	1.25	0.118
	00.430	7.90	4.80	0.253
malonic acid root	1.077	----	9.10	0.604
	1.915	12.4	4.75	1.210
	2.050	27.8	17.6	1.120
hydroxamate root	2.20	26.5	14.8	1.15
	1.65	36.4	13.8	0.97
	2.88	12.7	17.8	1.82

TABLE XVI - Degradation of labelled 3-nitropropionic acid * obtained from feeding experiments.

treatment and type of experiment	1st carbon dpm	2nd carbon dpm	3rd carbon dpm
hydroxamate			
stem feeding	none	15	none
stem feeding	none	100	none
intact plant	none	37	54
intact plant	none	20	28
malonate			
intact plant	none	11	36

* 

DISCUSSION

The biosynthesis of 3-nitropropionic acid was studied by feeding various labelled precursors to cuttings and intact plants of creeping indigo. Radiotracers were used as concentrations of the labelled compound can be kept very small. This makes it possible to carry out the experiment using concentrations of precursors which would likely be encountered by the growing plant. When stimulation techniques were used, the concentration of the substrate is usually much higher than would occur in natural surroundings. Conclusions from such experiments may not indicate the pathway followed under normal conditions.

The demonstrated usefulness of gas-liquid chromatography in organic acid analysis of plant material suggested its application to similarily extracted samples of Indigofera spicata. When good resolution of the methylated 3-nitropropionic acid peak was obtained and a comparison with the previously used colorimetric method of nitrite determination was made, the decision to use gas-liquid chromatography was thought to be of value for several reasons:

(1) The quantitative results were as accurate as with the colorimetric procedure.

(2) Other organic acids could be examined at the same time yielding more information from a single procedure than previously used techniques.

(3) the 3-nitropropionic acid peak was sufficiently isolated to allow collection, which led to ease of counting the samples in the liquid scintillation counter.

(4) Collected samples could be used in degradation studies.

(5) In this method the plant extract is divided into acid, basic and neutral portions. In these experiments, only the organic acid fraction was examined, but the other two fractions were in a form that could be easily examined.

The preliminary experiments with aspartic acid led to the conclusion that a different pathway for 3-nitropropionic acid biosynthesis was followed in Indigofera spicata than in the mould Penicillium atrovenetum (Birkenshaw and Dryland 1964; Gatenbeck and Forsgren 1964; Shaw and Wang 1964). No radioactivity was incorporated into 3-nitropropionic acid when ^{14}C labelled aspartic acid was fed as a precursor. As there was a high count in malic acid, used as the tricarboxylic acid cycle representative, there seemed no reason to believe that 3-nitropropionic acid was derived from the tricarboxylic acids directly. This did not eliminate the possibility that some of these acids might not have been precursors via some pathway other than the TCA cycle. Birkenshaw and Dryland seem inconsistent in their conclusions with regard to the involvement of the tricarboxylic acid cycle in 3-nitropropionic acid production. Their experiments with radioactive acetate and pyruvate led them to conclude that these compounds were incorporated into 3-nitropropionic acid by Penicillium atrovenetum by way of the tricarboxylic acid. An additional conclusion was that

the unequal distribution of radioactivity in C_2 and C_3 of 3-nitropropionic acid derived from pyruvate-2-^{14}C indicated that fumaric acid (another TCA cycle acid) owing to its symmetry, could not be a direct precursor.

The stimulation experiments carried out by Unrau seemed to indicate a relationship between aspartic acid and 3-nitropropionic acid. However, the apparent stimulation of production may have resulted from an aspartate induced stimulation of metabolism in general.

Preliminary feeding experiments (Table V) seemed to indicate a stimulatory effect of malonic acid on 3-nitropropionic acid production, so radioactive malonic acid was used in a stem feeding experiment and some incorporation was obtained. About this time, Doxtader (1966) published results of his experiments with heterotrophic microorganisms and concluded that malonylmonohydroxamic acid might be a precursor of 3-nitropropionic acid in these organisms. Labelled malonylmonohydroxamic acid was prepared from malonic acid-2-^{14}C and fed to cuttings and plants of creeping indigo. In these experiments an incorporation of label into 3-nitropropionic acid was obtained (Tables XI, XIII and XV).

In the experiments where malonate-2-^{14}C and malonylmonohydroxamate -2-^{14}C feedings were compared, the initial level of radioactivity in hydroxamate fed was slightly lower than the initial level of radioactivity in malonate fed (Table XVII). The mean incorporation of label into 3-nitropropionic acid was greater when hydroxamate was fed than when malonate was fed. However, the difference was not statistically significant.

Both malonate and malonylmonohydroxamate were absorbed by the root system as well as taken up by stems. The 3-nitropropionic acid obtained from the roots contained more label than that of the stem and leaf portion (Table XVII). These findings seem to indicate that the roots contain the enzyme system necessary for 3-nitropropionic acid formation.

Levels of 3-nitropropionic acid found in the various experiments conducted here were much lower than were found in plants grown for other experiments conducted in Australia and Hawaii (Murray and Moore 1965; Unrau 1965). The generally low level of 3-nitropropionic acid found in these plants would indicate that the environmental factors were not optimum for the production of this compound. The last experiment conducted in our greenhouse (the root feeding experiment and the last reported stem feedings) showed much higher incorporation of label into the nitro acid than the previous experiments, although the initial levels of radioactivity of the compounds fed were nearly identical.

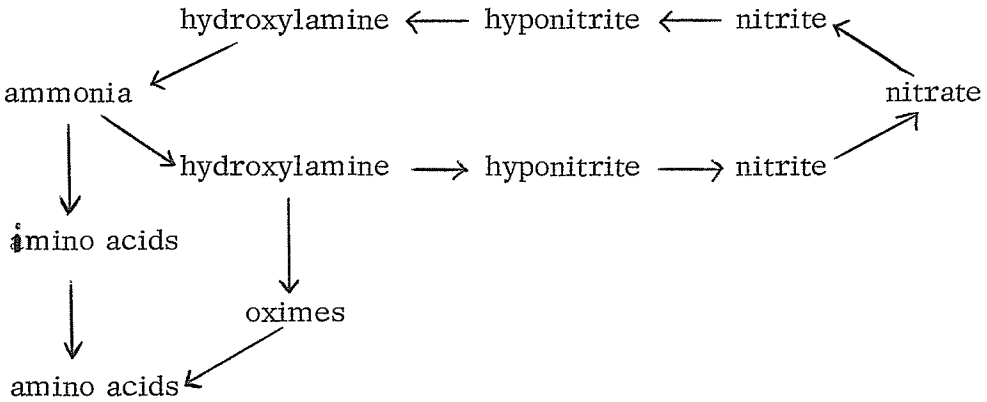
The level of labelling of 3-nitropropionic acid is not high in any of the experiments reported here. There are several reasons which can explain these low incorporation levels. The precursors found may not be immediate precursors. There could be several pathways using the precursor and only one of these leads to 3-nitropropionic acid or there could be many intermediates between the precursor and the nitro acid. If the young leaves produce most of the new 3-nitropropionic acid and there is not a rapid turnover of the compound, this could also account

for the low level of labelling obtained. With the root feeding experiments there was a lower incorporation of labelled compounds into the organic acid fraction than with the stem feeding experiments, but the level of 3-nitropropionic acid labelling remained much the same in the two cases. This would indicate that the maximum rate of production was reached in both cases for the prevailing conditions as higher levels of radioactivity did not produce correspondingly higher levels of radioactivity in the 3-nitropropionic acid produced.

McKee (1962) reported that nitrate but not gaseous nitrogen induced adaptive nitrate and hydroxylamine reductases in Azotobacter agile. The conclusion was reached that hydroxylamine seems to be related to nitrate assimilation, not fixation. No labelled oxidized nitrogen compounds were detected in Azotobacter agile supplied with N^{15}. McKee proposes a scheme for nitrogen utilization to account for various results obtained in connection with nitrogen and hydroxylamine metabolism studies. Hydroxylamine is toxic to plants, but is very reactive with carbonyl compounds and could be utilized by the plants in combined form such as hydroxamic acids which are known intermediates in reactions such as the following:

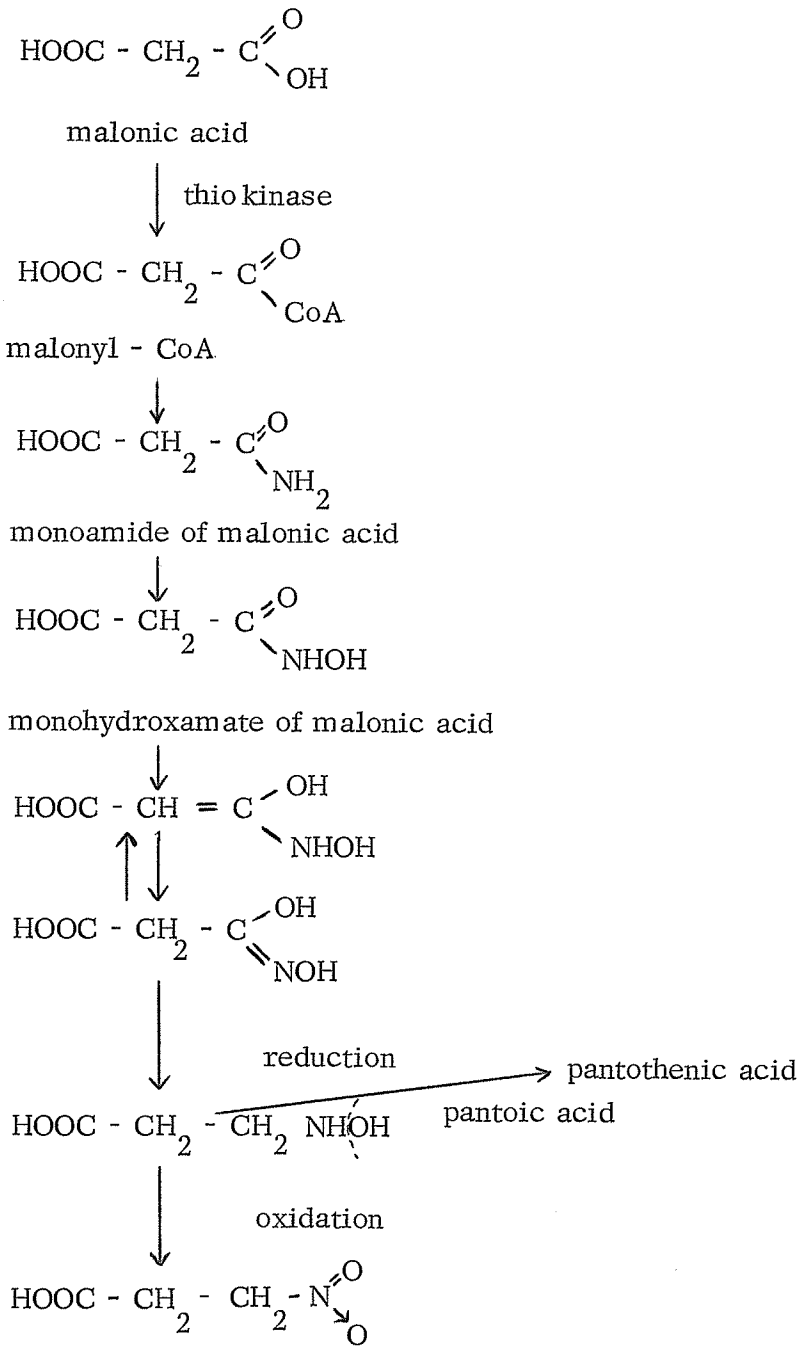
$$\underset{\text{glutamic acid}}{HOOC-(CH_2)_2-\overset{NH_2}{\overset{|}{C}}HCOOH} + NH_2OH + ATP \longrightarrow HOOC-\overset{NH_2}{\overset{|}{C}}H(CH_2)_2-\underset{\text{hydroxamate}}{CONHOH} + ADP + PO_4^{\equiv}$$

The scheme McKee proposes is shown below:



This scheme explains why the nitrogen of aspartic acid and β-alanine may not be incorporated into the nitrite of 3-nitropropionic acid and yet both 3 NPA and β-alanine could be formed from hydroxamate.

A possible scheme for 3-nitropropionic acid production from malonic acid is shown below:



This scheme would explain the retention of label in position two of 3-nitropropionic acid and includes the monohydroxamate of malonic acid in the pathway from malonic acid to 3-nitropropionic acid. The reduction or oxidation necessary at the end of this mechanism would have to include the slow step in the sequence as the labelled monohydroxamate was not incorporated significantly more than the labelled malonic acid.

After examining the various known mechanisms for malonic acid metabolism (Bonner and Varner 1965), incorporation of label into the three-position of 3-nitropropionic acid (Table XVI) cannot be easily explained. The only known mechanism which can locate the label in this manner is

$$HOOC\text{-}\underset{*}{CH_2}\text{-}\overset{O}{\overset{\|}{C}}\text{-}COOH \xrightarrow{\text{oxaloacetate decarboxylase}} CO_2 + HOOC\text{-}\underset{*}{CH_2}\text{-}COOH$$

oxaloacetic acid malonic acid

which would then have to proceed by the introduction of the nitro group and reduction of the carbonyl group.

$$HOOC\text{-}\underset{*}{\overset{NO_2}{\overset{\searrow}{CH}}}\text{-}\overset{O}{\overset{\|}{C}}\text{-}COOH \longrightarrow O_2N\text{-}\underset{*}{CH_2}\text{-}CH_2\text{-}COOH$$

Oxaloacetate decarboxylase is most reactive in the roots according to Bonner and Varner. Addition of malonate to the medium would force the reaction in the opposite direction, assuming reversibility. In these experiments the label appeared in carbon 3, only when intact plants were fed. If the roots were mainly responsible for this conversion, it would explain why the stem feedings showed no indication of oxaloacetate as a precursor. There was also no evidence of oxaloacetate labelling when D-L-aspartate -3-^{14}C was fed to stem cuttings. If the oxaloacetate decarboxylase was confined to the roots, the involvement of the Krebs cycle acids would not be evident in these findings. There is also the possibility that the oxaloacetate utilized in this malonate conversion is in a separate pool from the one containing the Krebs cycle acids.

Why 3-nitropropionic acid is in the plant is a question with many possible answers. The experiments reported here would implicate the nitro acid in fatty acid metabolism and it could result from a utilization of nitrogen under conditions differing from those which ordinarily produce β-alanine. In other words, 3-nitropropionic acid could be an alternative pathway for the toxic hydroxylamine when either because of saturation, inhibition or other reasons, the normal β-alanine pathway cannot be followed.

The nitro compound under study can yield HCN on gentle treatment. There may be an involvement with the production of indolyl-3-acetonitrile which is a growth substance. A nitro compound produced by Aspergillus aventii (Burrows, Mills, et al 1965), I-amino-2-nitrocyclopentane carboxylic acid, causes unusual morphological changes in higher plants. This might imply a role of some special significance for 3-nitropropionic acid or it may just be a non-specific electron acceptor for the flavoprotein enzyme system.

These experiments have shown that gas liquid chromatography can be used for detecting and collecting 3-nitropropionic acid in Indigofera spicata. The precursor experiments have revealed the involvement of malonic acid and malonylmonohydroxamic acid in 3-nitropropionic acid biosynthesis in this legume.

SUMMARY

(1) Gas Liquid Chromatography provides a quantitative method for the determination of 3-nitropropionic acid in Indigofera spicata (Jacq.) Forsk. and allows for easy collection of methylated derivatives of the organic acids.

(2) β -alanine and aspartic acid do not produce 3-nitropropionic acid in creeping indigo.

(3) The tricarboxylic acid cycle does not seem to be directly involved in 3-nitropropionic acid biosynthesis in Indigofera spicata.

(4) Malonylmonohydroxamate seems a slightly better precursor of the nitro acid than malonic acid.

(5) A mechanism for the biosynthesis of 3-nitropropionic acid is proposed involving the precursors found in these experiments.

APPENDIX

TABLE XVII - Statistical analysis results

groups compared	mean	df	s	t	% probability
0.946μc malonate and 1.892μc malonate	3.51 4.00	4	1.645	0.34	0.75
Experiment 2					
0.81μc hydroxamate and 1.62μc hydroxamate	4.39 4.57	10	2.1	0.382	0.70
malonate treatments Experiment 2 and hydroxamate treatments Experiment 0.81μc and 1.62μc	3.67 4.50	14	1.98	1.000	0.35
malonate 2.61μc and hydroxamate 1.52μc	16.8 19.4	9	3.9124	1.07	0.35
malonate root fed and hydroxamate root fed	1.76 3.01	7	1.10	1.61	0.15

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