The Biosynthesis of 3-Nitropropionic Acid in Creeping Indigo (*Indigofera spicata*)*

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ABSTRACT: A study of the biosynthesis of 3-nitropropionic acid in *Indigofera spicata* (Jacq.) Forsk. was undertaken by feeding ^{14}C-labeled precursors to intact plants or to cuttings. An organic acid fraction containing 3-nitropropionic acid was obtained by extraction of the plant material and separation by column chromatography.

The 3-nitropropionic acid and other major organic acids were separated and analyzed quantitatively by gas–liquid partition chromatography. No evidence was obtained to suggest that Krebs cycle acids were precursors of 3-nitropropionic acid. Malonic acid or a derivative of malonate appears to be a fairly immediate precursor since ^{14}C was found in 3-nitropropionic acid when malonate-2-^{14}C or malonyl monohydroxamate-2-^{14}C were fed as precursors.

3-Nitropropionic acid was first identified as a component of hiptagin, a glycoside from *Hiptage mandablota* Gaertn. (Carter and McChesney, 1949). The glycoside hiptagin was isolated from the above plant at a considerably earlier date (Gorter, 1920). The same glycoside was also isolated from the seed of *Corynocarpus laevigata* (Carter, 1943) which when heated in acidic medium decomposed to give hiptagenic acid (3-nitropropionic acid), carbon dioxide, ammonia, and a sugar residue (glucose or mannose). The toxic compound of trailing or creeping indigo (*Indigofera endecaphylla* and *Indigofera spiccata*) was identified as 3-nitropropionic acid (Morris *et al.*, 1954).

In lower organisms, particularly fungi, 3-nitropropionic acid has been isolated from cultures of *Aspergillus flavus-oryzae* (Bush *et al.*, 1951), *Aspergillus oryzae* (Nakamura and Shimoda, 1954), and *Penicillium atrovenetum* (Raistrick and Stössl, 1958). Krebs cycle acids have been reported to be precursors for the synthesis of this nitro acid in *Penicillium atrovenetum* G. Smith (Birkenshaw and Dryland, 1964; Gatenbeck and Forsgren, 1964; Shaw and Wang, 1964; Shaw and McCloskey, 1967; Birch *et al.*, 1960). It has been demonstrated (Shaw and McCloskey, 1967) that reduced nitrogen (NH_4^+) is much more effective in the synthesis of the nitro acid in *Penicillium* than is NO_3^-. It was shown that ^{18}O was not incorporated into the NO_2 of the nitro acid when KNO_3 ^{18}O was supplied as the source of nitrogen, thus further indicating that complete reduction of nitrogen appears necessary. Hydroxylamine appeared to have an indirect effect. It was again shown that aspartate could furnish the carbon skeleton. Shaw (1967) has also shown that *Penicillium* possesses the ability to reduce 3-nitroacrylic acid to 3-nitropropionic acid.

In contrast, no Krebs cycle acids have, to the best of our knowledge, been found to act as precursors in the biosynthesis of 3-nitropropionic acid in creeping indigo. We now wish to report the successful incorporation of a ^{14}C-labeled precursor in this acid. Both ^{14}C-labeled malonic acid and malonylmonohydroxamic acid were supplied as precursors either to stem cuttings or intact plants of creeping indigo and a nonrandom incorporation of these was demonstrated by degradation of the labeled 3-nitropropionic acid.

Methods

Feeding Experiments with Stem Cuttings. Creeping indigo plants were grown in pots in the greenhouse at 75–80°F. Cuttings (12–14 in.) were taken from the runners from well-established plants. As the common name infers, the creeping indigo plant produces numerous runners after about 3–5 months. This characteristic can be encouraged by periodic "pruning" which results in a profusion of fresh runner growth which when cut are very suitable for use directly or for induction of roots using a rooting hormone. The nutrient solution used was 0.01 M in $CaCl_2$, in $MgSO_4$, in NH_4Cl, and in NaCl and 0.0015 M in KH_2PO_4. This solution was adjusted to pH 6.5 with NaOH when necessary. Plastic tubes (50 ml) 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°.

Intact Plant Feeding Experiments. Stem cuttings were rooted in moist sand, then moved to boxes containing a modified Hoagland nutrient medium. For feeding experiments the plants were transferred to bottles containing the same nutrient medium as that used for stem cutting experiments. Labeled precursors were added to the nutrient medium and aeration was provided during the feeding period. The plants were dried and appropriate analyses were carried out on roots, stems, and leaves.

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TABLE I: Incorporation of ^{14}C into 3-Nitropropionate of Plants of Creeping Indigo Fed Malonate-2-^{14}C.

Expt	No. of Samples	Malonate-2-^{14}C Fed/Sample		3-Nitropropionate		
		μmoles	μCi	mg/g dry wt	Sp Act. (dpm × 10^{-2}/mg)	% Incorp
1[a]	5	0.05	0.37	1.26	1.50	0.036
2[a]	4	0.23	2.60	1.84	9.15	0.047
3[a]	4	0.01	0.95	2.55	1.38	0.017
4[b]	3	350.00	3.49	1.66	1.78	0.038

[a] Cuttings. [b] Intact plants.

TABLE II: Incorporation of ^{14}C into 3-Nitropropionate of Plants of Creeping Indigo Fed Malonylmonohydroxamate 2-^{14}C.

Expt	No. of Samples	Malonylmonohydroxamate-2-^{14}C Fed/Sample		3-Nitropropionate		
		mmole	μCi	mg/g dry wt	Sp Act. (dpm × 10^{-2}/mg)	% Incorp
5[a]	5	0.20	0.81	2.20	1.90	0.032
6[a]	7	0.20	1.62	2.17	2.24	0.018
7[a]	7	0.20	1.52	2.17	8.95	0.081
8[b]	6	0.35	3.21	1.64	2.90	0.067

[a] Cuttings. [b] Whole plant.

Sample Preparation. Ground samples (approximately 2 g) were extracted with 50 ml of distilled water by heating under pressure. The water extract obtained by filtering the hydrolyzed mixture was separated into basic, acidic, and neutral fractions by ion-exchange chromatography. The organic acid fraction, containing the 3-nitropropionic acid, was methylated using diazomethane and analyzed by gas chromatography (Canvin, 1965). Separation was achieved on a column (4 ft × 0.25 in.) packed with 5% Versamid 900 on silanized Chromosorb W (60–80 mesh). Compounds corresponding to identified peaks (identified by comparison with authentic samples) were collected and counted in a liquid scintillation counter using a dioxane scintillator mixture (6 g/l. of 2,5-diphenyloxazole).

Degradation of Labeled 3-Nitropropionic Acid. The gas chromatographic effluent containing the methyl ester of 3-nitropropionic acid was collected in water. The sample was hydrolyzed by refluxing for at least 1 hr with 3 ml of 2 N NaOH, after which was added 400 mg of unlabeled 3-nitropropionic acid and the degradation continued as follows.

The Nef reaction (as described by Birkenshaw and Dryland, 1964) was used to degrade the nitro acid to acetaldehyde dinitrophenylhydrazone whereby the first carbon is liberated as CO_2. The CO_2 was trapped in 3 ml of a 1:2 mixture of ethanolamine–ethylene glycol monoethyl ether. Ethyl Cellosolve–toluene (containing 6 g/l. of 2,5-diphenyloxazole) was added followed by liquid scintillation counting.

Acetaldehyde dinitrophenylhydrazone was degraded to sodium acetate by the Kuhn–Roth oxidation (Stickings and Townsend, 1961) after which the Schmidt reaction (Colowick and Kaplan, 1962,a,b) was used to degrade the acetate. The CO_2 evolved was trapped and counted as described above.

Precursors. Malonic acid-2-^{14}C (0.1 mCi/1.4 mg) was obtained from New England-Nuclear Corp. Malonylmonohydroxamic acid was synthesized from malonic acid-2-^{14}C, by first preparing malonic acid monochloride (Colowick and Kaplan, 1962a,b) and converting to malonylmonohydroxamate by adding neutralized hydroxylamine (Lipmann and Tuttle, 1945).

Results

Malonic Acid Stem Feeding. In expt 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 2-day run. The amount of labeled material fed per tube was 0.37 μCi in expt 1 and 2.60 μCi in expt 3.

The labeled acid feeding in expt 2 was followed by 0.01 M malonic acid in nutrient solution. Here the levels of radioactivity were 0.95 and 1.89 μCi per tube. Results from these experiments are listed in Table I.

Malonylmonohydroxamate. The monohydroxamate

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

Intact Plant Feedings. Malonic acid-2-^{14}C was fed at the level of 3.49 μCi per jar or 7.75×10^6 dpm. Malonylmonohydroxamate-2-^{14}C was fed at the level of 3.21 μCi/jar or 7.13×10^6 dpm. The results are tabulated in Tables I and II.

Degradation of Samples. Results from sequential degradation of ^{14}C-labeled 3-nitropropionic acid are given in Table III.

TABLE III: Degradation of Labeled 3-Nitropropionate Derived from Malonate-2-^{14}C or Malonylmonohydroxamate-2-^{14}C.[a]

Source of Material	Radioactivity in 3-Nitropropionate (dpm/sample) C-1	C-2	C-3
Malonyl Monohydroxamate-2-^{14}C Feeding			
Cuttings	0	15	0
Cuttings	0	100	0
Intact plant	0	37	54
Intact plant	0	20	28
Malonate-2-^{14}C Feeding			
Intact plant	0	11	36

[a] As collected from samples obtained in expt 2, 4, 7, and 8.

Discussion

The biosynthesis of 3-nitropropionic acid was studied by feeding various labeled precursors to cuttings and intact plants of creeping indigo. Radiotracers were used as concentrations of the labeled 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 are used, the concentration of the substrate is usually much higher than would occur in natural surroundings. Conclusions from such experiments may not validate the pathway followed under normal conditions.

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-labeled aspartic acid was fed as a precursor. The major portion of label appeared in malic acid and since this acid is a component of the tricarboxylic acid cycle, there was no direct evidence to indicate that 3-nitropropionic acid was derived directly from the tricarboxylic acids. This of course does not eliminate the possibility that some Krebs cycle acids may have been precursors *via* some pathway other than the tricarboxylic acid cycle.

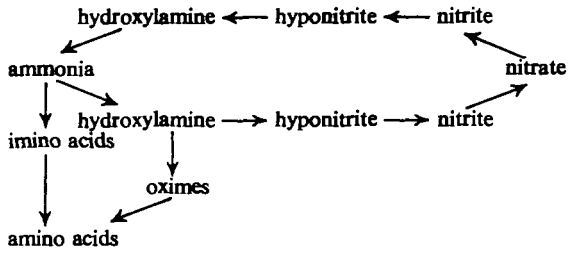
Feeding experiments indicated a stimulatory effect of malonic acid on 3-nitropropionic acid production. Consequently radioactive malonic acid was used in stem feeding experiments and some label incorporation into 3-nitropropionic acid was obtained. At this juncture Doxtader (1966) reported results of experiments with heterotrophic microorganisms and concluded that malonylmonohydroxamic acid might be a precursor of 3-nitropropionic acid in these organisms. We prepared labeled malonylmonohydroxamic acid from malonic acid-2-^{14}C and the derivative was fed to cuttings and intact plants of creeping indigo. In these experiments an incorporation of label into 3-nitropropionic acid was again obtained (Table II).

Both malonate-2-^{14}C and malonylmonohydroxamate-2-^{14}C were absorbed by the root system and by stems at roughly similar levels. The 3-nitropropionic acid obtained from the roots contained more label than that of the stem and leaf portion. These findings gave an indication that the root tissue contains the major proportion of the enzyme system necessary for 3-nitropropionic acid formation.

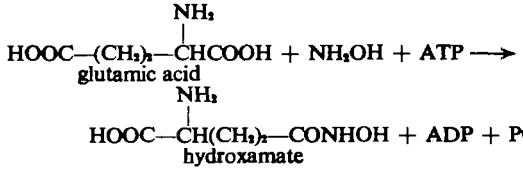
The level of labeling of 3-nitropropionic acid is not high (0.02–0.08%) in any of the experiments reported here; however, label incorporation was specific. There are several reasons which may explain the low incorporation levels observed. The compounds found to be incorporated may still be somewhat distant precursors. There may be several pathways that utilize the administered precursor and only one of these leads to 3-nitropropionic acid. There could be a considerable number of intermediates between the labeled precursor supplied and the nitro acid. In the root feeding experiments a lower incorporation of labeled compounds into the organic acid fraction was observed than in the stem feeding experiments; however, the level of 3-nitropropionic acid labeling remained much the same in the two cases. This would indicate that under the particular conditions, a maximum rate of production was reached in both cases since higher levels of radioactive precursor feeding did not produce correspondingly higher levels of radioactivity in the 3-nitropropionic acid produced.

McKee (1962) has reported that nitrate induced adaptive nitrate and hydroxylamine reductases in *Azotobacter agile* and it was concluded that hydroxylamine appeared to be related to nitrate assimilation, not fixation. No labeled oxidized nitrogen compounds were detected in *A. agile* supplied with ^{15}N. McKee proposed a scheme for nitrogen utilization to account for various results obtained in connection with nitrogen and hydroxylamine metabolism studies. Hydroxylamine, which is rather toxic to plants, is known to be very reactive with carbonyl compounds and could presumably be utilized by the plants in a combined form such as hydroxamic acids. Hydroxamates are known intermediates in reac-

SCHEME I



tions such as the following

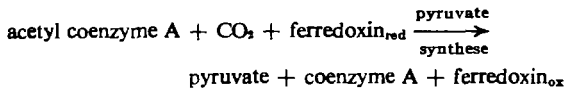


McKee proposes Scheme I. This scheme explains why the nitrogen of aspartic acid and β-alanine may not be incorporated into the NO_2 group of 3-nitropropionic acid and yet both 3-nitropropionic acid and β-alanine could be formed from hydroxamate.

A possible scheme for 3-nitropropionic acid production from malonic acid is shown in Scheme II. 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 labeled monohydroxamate was not incorporated significantly more rapidly than the labeled malonic acid.

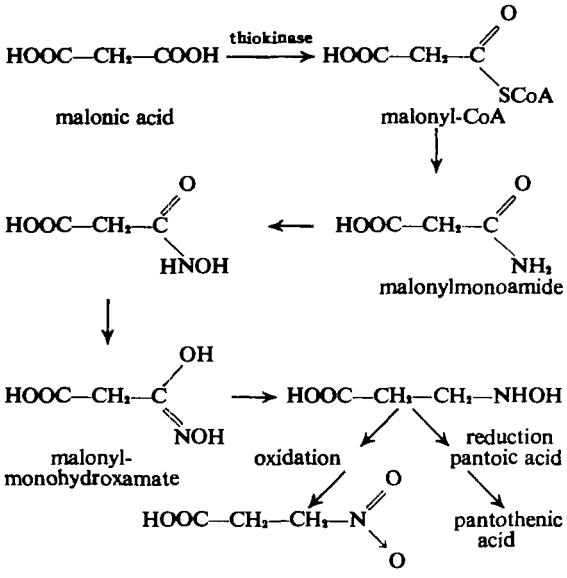
Upon examination of the various known mechanism for malonic acid metabolism, incorporation of label into the 3 position of 3-nitropropionic acid (Table III) cannot be easily explained.

In lower organisms, particularly photosynthetic bacteria, it has been shown (Evans and Buchanan, 1965) that reduced ferredoxin is a component of the carboxylation of acetyl coenzyme A. A similar system involving



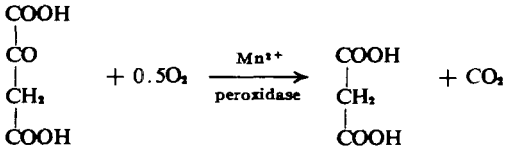
reduced ferredoxin was shown to be capable of carboxylating succinyl coenzyme A (Buchanan and Evans, 1965) to α-ketoglutarate. Since amino acids are the first products of photosynthesis in photosynthetic bacteria, the above findings demonstrated mechanisms whereby alanine and asparate (pyruvate synthese) and glutamate (α-ketoglutarate synthese) are synthesized. A similar carboxylating system might be proposed for malonyl coenzyme A which would give oxalacetate. It is, however, important to note that ferredoxin-dependent CO_2 fixation appears to be characteristic to anaerobic bacteria (Evans *et al.*, 1966) and therefore may not, until

SCHEME II

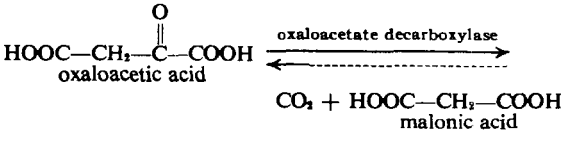


experimental evidence warrants, be extended to higher plants.

Malonic acid is a common constitutent of mature leaves of certain species of Leguminosae and there is evidence that enzymes are present in certain plants which are capable of converting oxalacetate into malonate (Pattee and Shannon, 1965; Shannon *et al.*, 1963) as follows.



Under rather restricted experimental conditions malonyl-dihydroxamate and acetylhydroxamate have been isolated from particulate preparations from bean leaves (Young and Shannon, 1959). Malonyl thiokinase and oxalacetate decarboxylase are generally well represented in plants (Hatch and Stumpf, 1961a,b). It is obvious, however, that although the above systems can locate ^{14}C in the C-2 position of 3-nitropropionic acid, the appearance of label at C-3 cannot be explicated. The only known mechanism which can locate the label in this manner is the following

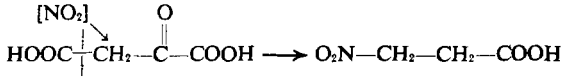


which would then have to proceed by the introduction of the nitro group and reduction of the carbonyl group as shown on the following page.

Oxaloacetate decarboxylase is probably most reactive in the roots since malonate is a major acidic con-

TABLE IV: Distribution of Radioactivity in Aqueous Extract of Plant Material, Using Aspartate-3-^{14}C as Substrate.

Sample	Organic Acid Fraction (dpm/g dry wt $\times 10^{-4}$)	3-Nitropropionic Acid (dpm/g dry wt $\times 10^{-4}$)	Malic Acid (dpm/g dry wt $\times 10^{-4}$)
1	8.65	None	1.73
2	12.50	None	2.50
3	11.60	None	2.15
4	10.00	None	2.05
5	13.00	None	2.81



stituent of certain plants (deVellis *et al.*, 1963). Addition of malonate to the medium could force the reaction in the opposite direction particularly if a system similar to the ferredoxin-mediated carboxylation of acetyl coenzyme A and succinyl coenzyme A in bacteria were operative. In these experiments the label appeared in C-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 labeling when DL-aspartate-3-^{14}C was fed to stem cuttings (Table IV). It follows then that if the oxaloacetate decarboxylase is confined to the root tissue, the involvement of the Krebs cycle acids would not be evident where stem and leaf tissue are involved. The possibility also exists that the oxaloacetate utilized in this malonate conversion is in a separate pool from that involving the Krebs cycle acids. Compartmentation of this type has been shown to exist in plant tissues (MacLennan *et al.*, 1963; Lips and Beevers, 1966). Malate produced by dark fixation carboxylation is in a pool physically separated from malate produced *via* the tricarboxylic acid cycle.

As indicated earlier, 3-nitropropionic acid occurs in only few higher plants. Why creeping indigo accumulates significant quantities of this nitro acid is no more obvious than the reason for the synthesis of apparently innocuous alkaloids by various plants. 3-Nitropropionic acid could be implicated (*via* malonate) in fatty acid metabolism. Conditions of nitrogen utilization other than those which would normally lead to β-alanine could result in elaboration of the analogous β-nitropropionic acid. A suitable precursor of the nitro acid could be the acceptor of hydroxylamine when either because of saturation, inhibition, etc., the normal β-alanine pathway is inoperative.

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