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MALONYLTRYPTOPHAN IN HIGHER PLANTS[1,2]

NORMAN E. GOOD AND W. A. ANDREAE
SCIENCE SERVICE LABORATORY, CANADA DEPARTMENT OF AGRICULTURE, LONDON, ONTARIO, CANADA

During the course of our study of indoleacetic acid metabolism (1, 6), we investigated the effect of exogenous tryptophan on the synthesis of indoleacetylaspartic acid in excised pea epicotyls. Tissues bathed for 24 hours in solutions containing 50 to 200 mg/l tryptophan accumulated considerable amounts of a substance which closely resembled indoleacetylaspartic acid in chromatographic mobility in several solvents, in acid strength, and in color reactions with the Ehrlich (*p*-dimethylaminobenzaldehyde) and Salkowski (acid-ferric chloride) reagents (fig 1). Consequently, in discussing metabolic precursors of indoleacetic acid at the 1956 Annual Meeting of the American Society of Plant Physiologists at Storrs, Connecticut, we reported erroneously the conversion of tryptophan into indoleacetylaspartic acid. Differences soon became apparent. The unknown substance, which occurred in several families of higher plants, had a slightly higher R_f in most solvents than had indoleacetylaspartic acid. Furthermore, although the color produced on paper with the Salkowski reagent was the purple of indoleacetylaspartic at very low concentrations, the color at higher concentrations was brown; in this respect the substance resembled acetyltryptophan. Basic hydrolysis of the unknown yielded tryptophan, not indoleacetic acid.

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ISOLATION

In order to assign a structure to the tryptophan derivative its isolation in a relatively pure state was necessary. This isolation was a somewhat laborious procedure since the solubilities of the derivative were similar to those of the bulk of the plant acids. However, it was possible to take advantage of the fact that it was a very strong acid and a considerable purification was achieved by partitioning the plant

acids between water and *n*-butyl alcohol, or water and ether, with careful pH regulation; fortunately it was a simple matter to determine, rapidly and quantitatively, the distribution of the tryptophan derivative between the organic and aqueous phases by use of the above mentioned Ehrlich reagent.

The methods ultimately adopted were as follows: two kilograms of spinach leaves, purchased at the grocers', were incubated with a tryptophan solution (100 mg/l) in large crocks oxygenated with a stream of air bubbles. A coarse screen and weights kept the leaves submerged. After 24 hours the fully turgid and apparently healthy leaves were removed, washed, ground in a Waring blendor with a little water and enough sodium bicarbonate to make the brei 0.25 M. The brei was saturated with ammonium sulfate and the aqueous phase was removed by filtration on a Büchner funnel with the aid of a generous amount of infusorial earth. The filtrate, which was free of chlorophyl and quite clear, was acidified to pH 4.3 with phosphoric acid and extracted four times with ether. This ether was discarded. The filtrate was then acidified again, this time to pH 2.5 and extracted 5 times with ether. The extract was made alkaline with a few drops of concentrated ammonium hydroxide solution and the ether was removed on a steam bath in a stream of air. The residue was taken up in about 100 ml of 0.25 *N* sodium bicarbonate solution. The bicarbonate solution was acidified to pH 5.6 and extracted twice with 15-ml lots of *n*-butyl alcohol. This butyl alcohol was set aside for reworking. The aqueous phase was further acidified to pH 3.2 and extracted four times with *n*-butyl alcohol in 15-ml lots. This time the alcohol phase contained most of the tryptophan derivative. The butyl alcohol was extracted three times with 0.25 *N* sodium bicarbonate solution (total volume 70 ml). The bicarbonate solution was acidified to pH 4.3 and extracted 3 times with 30 ml of ether. This ether was set aside for reworking. Then the aqueous phase was further acidified to pH 2.5 with phosphoric acid and extracted 6 times with ether. The aqueous phase was set aside for reworking and the ether phase, containing about half the original tryptophan derivative, was taken almost to dryness in an air stream (heat avoided).

The residue was taken up in a small volume of alcohol, transferred as streaks to four sheets of Whatman No. 1 filter paper (46 × 38 cm) and chromatographed in an isopropyl alcohol–concentrated ammonium hydroxide–water solvent (80 : 10 : 15, v/v). The tryptophan derivative was located by spraying strips from the developed chromatograms with the Ehrlich reagent (1 % *p*-dimethylaminobenzaldehyde dissolved in equal volumes of alcohol and concentrated HCl). The appropriate regions were then cut out as carefully as possible using the fluorescence of various contaminants in ultra-violet light as precise markers. In order to achieve maximum purification a considerable loss was deliberately introduced in this operation. The tryptophan derivative was eluted from the paper with 1 *N* ammonium hydroxide solution. The eluate was acidified to pH 2.5 with phosphoric acid and extracted 12 times with ether. The ether was then removed in an air stream as above and the chromatographic procedure was repeated twice more—first in a solvent consisting of pyridine, *t*-butyl alcohol and water (35:35:30, v/v) and then in the alcoholic phase from a mixture of *n*-butyl alcohol, acetic acid and water (80:20:10, v/v). The final chromatogram was also eluted with 1 *N* ammonium hydroxide solution and the eluate was again acidified to pH 2.5 and extracted with ether. The tryptophan derivative, about 40 mg, crystallized out of the water residue left after removal of the ether.

Identification

These crystals melted about 80° C, were slightly deliquescent and obviously impure. The ultraviolet absorption spectrum of an alcoholic solution of the crude material was almost indistinguishable from that of acetyltryptophan. Assuming that practically all the absorption at 280 and 288 mμ was due to the tryptophan part of the molecule and that the molecule contained only one tryptophan residue, it was possible to calculate a molecular weight of 306 for the compound. The impurities present in unknown amounts, obviously made this value too high—by how much we could not know. A single determination of the neutralization equivalent, using 8.3 mg of the crystals, gave a value of 144. Clearly the tryptophan derivative was a dibasic acid. The titration curve obtained on neutralization of the acid also suggested two dissociation constants, one (pK_1 about 3.2) being definitely stronger and the other (pK_2 about 4.2) being somewhat weaker than that of acetyltryptophan (pK about 3.8). From these data it was possible to predict that the unknown part of the molecule was itself a dibasic acid, molecular weight not greater than 120, attached to the amino group of tryptophan through one of its carboxyls. Moreover the acid had to be very strong because the first dissociation constant of the condensed compound (pK_1 3.2) presumably reflected the ease of ionization of the remaining free carboxyl; since the tying up of one carboxyl of a dibasic acid usually decreased the dissociation constant of the remaining carboxyl, the first pK of the parent dicarboxylic acid was presumably lower than 3.2. Very few acids could meet these requirements. In fact the only ones to do so were oxalic (pK_1 1.32, MW 90), malonic (pK_1 2.83, MW 104), maleic (pK_1 1.92, MW 116) and fumaric (pK_1 3.02, MW 116). Succinic acid (MW 118) was improbable because of its relative weakness (pK_1 4.19).

Samples of the crude crystalline material were hydrolysed in 3 *N* barium hydroxide solution in a steam autoclave at 20 lb pressure per square inch for three hours. Carbon dioxide was bubbled through the cooled and diluted hydrolysate until the excess barium had been precipitated as carbonate. The carbonate was removed by filtration and washed. The filtrate and washings were then taken to dryness and the residue taken up in 70 % alcohol containing an

FIG. 1 (*left*). Paper chromatogram of ether extracts of tryptophan treated pea epicotyls. Developing solvent isopropyl alcohol–concentrated ammonium hydroxide–water solvent (80 : 10 : 10, v/v). The chromatogram was sprayed with Ehrlich's reagent (*p*-dimethylaminobenzaldehyde) in alcohol-aqueous HCl. Numbers refer to the extraction pH. St. is synthetic indoleacetylaspartic acid. Extracts from 3 gm fresh weight of epicotyl tissue were applied to the chromatogram.

FIG. 2 (*right*). Paper chromatogram of malonic acid (R_f 0.65), tryptophan (R_f 0.37), and the hydrolysis products of the ether soluble tryptophan derivative from tryptophan treated spinach leaves. Developing solvent: Alcoholic phase from a mixture of *n*-butyl alcohol, formic acid and water (30 : 6 : 45, v/v). The acids were located by their inhibition of mercurochrome fluorescence. 1. A mixture of 0.7 micromoles tryptophan and 0.7 micromoles malonic acid. 2. Tryptophan derivative (approximately 0.7 micromoles) after hydrolysis with 3 *N* barium hydroxide for 3 hours in an autoclave. 3. A mixture of malonic acid and tryptophan (both 0.7 micromoles) after similar treatment with barium hydroxide.

excess of formic acid to decompose the barium salts. The hydrolysis products in alcohol were applied to Whatman No. 1 paper and chromatographed in a *n*-butyl alcohol–formic acid–water solvent (30 : 6 : 45, v/v, alcoholic phase used). Acids were detected on the dried chromatograms by their effect in preventing the fluorescence of mercurochrome (dibromohydroxymercurifluorescein) in ultra-violet light. Figure 2, a chromatogram sprayed with a 0.02 % mercurichrome solution, dried and photographed with the visible light emitted on ultraviolet irradiation, shows that the hydrolysis products were tryptophan and malonic acid. Oxalic, maleic, fumaric and succinic acids have quite different mobilities.

Oxalytryptophan, malonyltryptophan and succinyltryptophan were synthesized by the action of the acid chlorides on tryptophan suspended in dry ether (3). Rigorous identification of the products was not

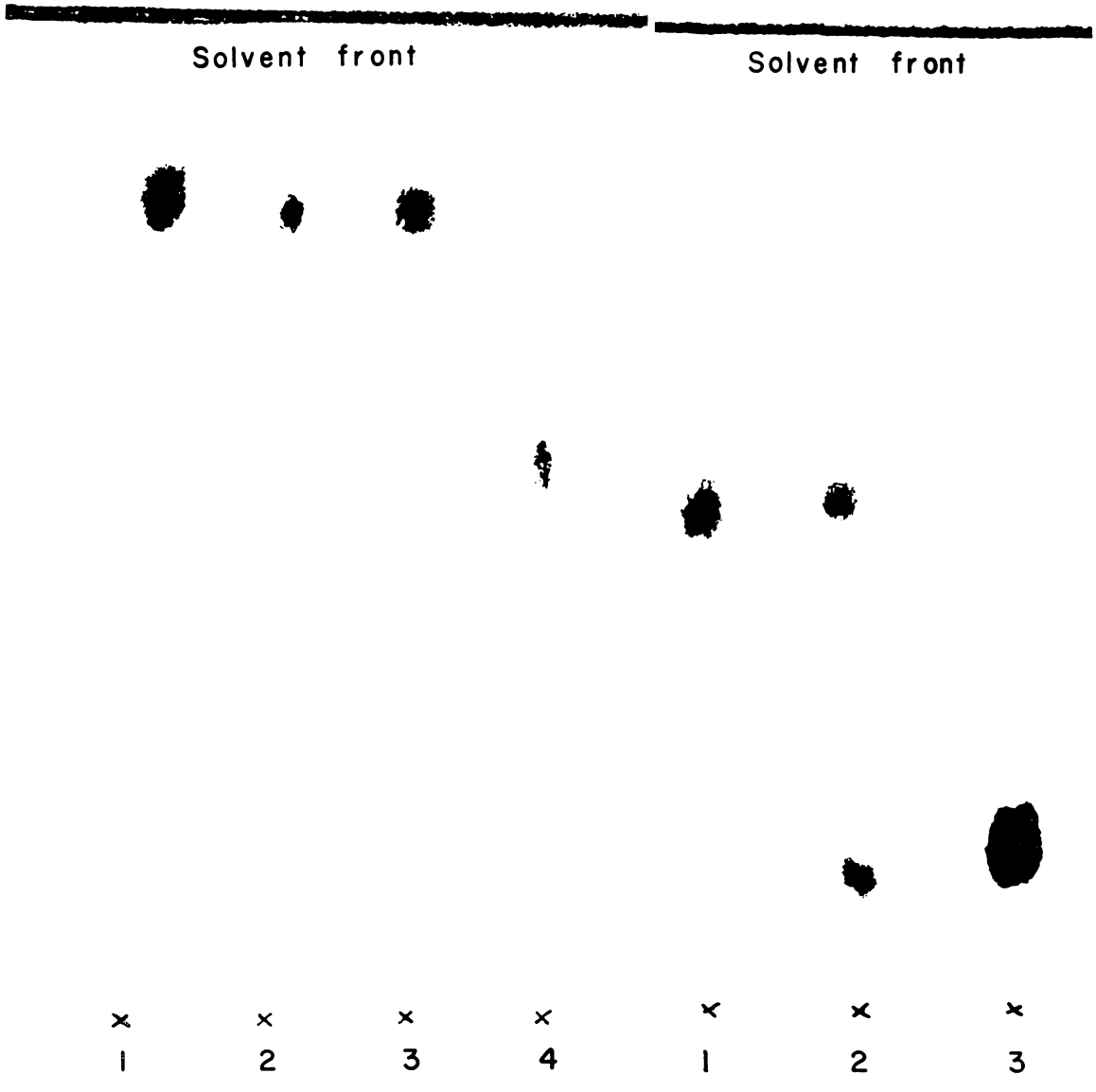

FIG. 3 (*left*). Paper chromatogram of tryptophan derivatives, developed with a solvent consisting of the alcoholic phase from a mixture of *n*-butyl alcohol, acetic acid and water (80 : 20 : 10, v/v), and sprayed with Ehrlich's reagent. 1. Synthetic succinyltryptophan. 2. Synthetic malonyltryptophan. 3. Extract of tryptophan treated spinach leaves. 4. Synthetic oxalyltryptophan.

FIG. 4 (*right*). Paper chromatogram of heated and unheated samples of the tryptophan derivative from tryptophan treated spinach leaves. Chromatogram developed with an isopropyl alcohol, ammonium hydroxide, water solvent (80 : 10 : 10, v/v) and sprayed with Ehrlich's reagent. 1. An acetyltryptophan marker. 2. Tryptophan derivative heated for about 2 minutes at 145° C. 3. Tryptophan derivative before heating.

undertaken. Malonyl chloride was prepared by the action of phosphorous pentachloride on malonic acid (4). The chromatographic mobilities of the synthetic tryptophan derivatives were compared with the mobility of the unknown in the solvents described above. In each case the unknown was very much more mobile than the oxalyl derivative, slightly but consistently less mobile than the succinyl derivative and indistinguishable from the malonyl derivative (fig 3).

Finally it occurred to us that a monoamide of malonic acid, such as malonyltryptophan, should decarboxylate on heating to yield CO_2 and acetyltryptophan. Both synthetic malonyl tryptophan and the biological material, when heated on a melting point block evolved gas at temperatures above 125° C and as can be seen in figure 4, acetyltryptophan was formed.

Physiological Significance

The malonyltryptophan observed in tryptophan treated pea and spinach tissues can scarcely have resulted from the metabolic activities of bacteria contaminating the incubation medium. Malonyltryptophan was formed consistently, regardless of the conditions of tryptophan application. In several experiments the tissues were carefully surface sterilized with NaOCl and large amounts of penicillin and streptomycin were added to the sterile incubation medium. After removal of the tissues these media were still absolutely clear. Nevertheless the usual amount of malonyltryptophan synthesis had taken place. Malonyltryptophan also seems to be widely distributed, albeit in very small amounts, in plants which have never been exposed to an exogenous source of tryptophan; consequently it is virtually certain that the plants themselves have the requisite enzyme systems for the synthesis.

A survey of the naturally occurring indole compounds in plants of various species such as tomato, spinach, pea and oats revealed substances present in small amounts in acid-ether extracts, which were chromatographically indistinguishable from malonyltryptophan, gave the same purple color with the Ehrlich reagent and brown color with the Salkowski reagent, and in the case of tomato extracts (fig 5) yielded tryptophan on hydrolysis. On the basis of the color reaction of these acid-ether extracts with the Ehrlich reagent, field grown tomato plants untreated with tryptophan contained about 0.5 mg of malonyltryptophan per kilogram fresh weight while untreated etiolated pea epicotyls contained a great deal less.

Malonyltryptophan is quite stable when laid down in the tissues. Three-week-old tomato plants were sprayed continuously with 100 mg/l of tryptophan for 24 hours in an illuminated chamber during which time they accumulated about 4 mg of malonyltryptophan per kg fresh weight or eight times the endogenous level. The plants were thoroughly washed, transferred to a greenhouse bench and entire plants were sampled every two days for 11 days. There was no decrease in the amount of malonyltryptophan per plant.

In pea epicotyls the formation of malonyltryptophan from applied tryptophan was strongly inhibited by the presence of monobasic acids such as phenylbutyric, di- and trichlorinated phenoxyacetic, napthalene acetic, benzoic and triiodobenzoic. 2,4-D or 2,6-D at 2 mg/l inhibited about 50 % of the malonyltryptophan formation and 20 mg/l inhibited the reaction completely. However, there is no evidence that the conjugation mechanism itself was inhibited since the accumulation of free tryptophan was prevented by these acids.

The authors have no idea what role malonyltryptophan plays in angiosperm physiology. Malonic acid itself seems to be rather widely found in plants often in considerable amounts (2, 5), but studies of its metabolism have been neglected. Malonic acid metabolism has been linked to acetate metabolism in bacteria by Hayaishi who showed a decarboxylation reaction involving the coenzymeA-thioesters of malonic and acetic acids as intermediates (7). If malonyl-CoA is also a metabolic intermediate in higher plants, it

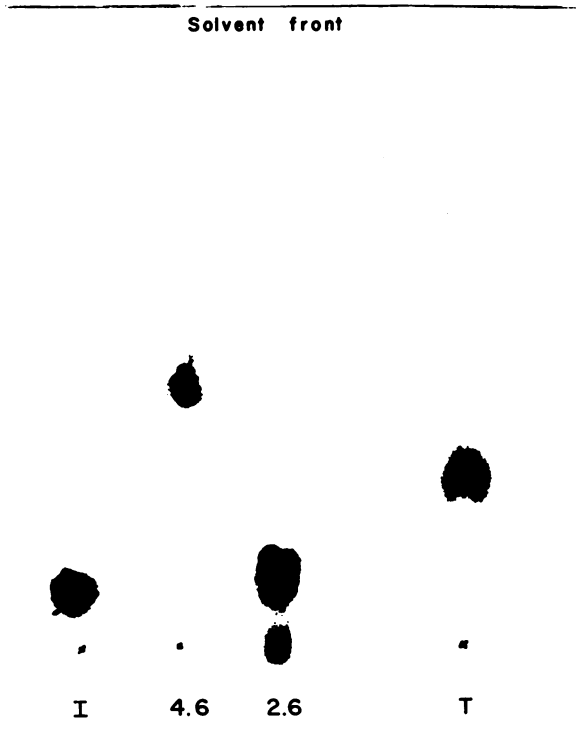

Fig. 5. Paper chromatogram of concentrated ether extracts of field grown tomato plants not treated with tryptophan. Chromatogram developed with isopropyl alcohol, concentrated ammonium hydroxide, water (80: 10 : 10, v/v) and sprayed with Ehrlich's reagent. The numbers refer to the extraction pH. I is synthetic indoleacetylaspartic acid and T is a tryptophan marker. Extracts from approximately 100 gm fresh weight of tissue were applied to the chromatogram. The substance extracted at pH 2.6 (R_f 0.11) yielded tryptophan on hydrolysis.

could well be that this is the activated form of malonic acid which condenses with tryptophan. We have not yet investigated the possibility that other amino acids may be similarly conjugated with malonic acid.

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STUDIES ON 3-INDOLEACETIC ACID METABOLISM. IV. CONJUGATION WITH ASPARTIC ACID AND AMMONIA AS PROCESSES IN THE METABOLISM OF CARBOXYLIC ACIDS[1,2]

W. A. ANDREAE and NORMAN E. GOOD

Science Service Laboratory, Canada Department of Agriculture, London, Ontario, Canada

In earlier publications it was reported that pea epicotyls convert applied indoleacetic acid into indoleacetylaspartic acid (1) and, to a much smaller extent, into indoleacetamide (6). In the present paper it will be shown that condensations with aspartic acid or ammonia are not limited to indoleacetic acid. When pea epicotyl sections were incubated in solutions of indoleformic (indole-3-carboxylic), indolepropionic, indolebutyric, benzoic, or 2,4-dichlorophenoxyacetic acids all the corresponding amides except 2,4-dichlorophenoxyacetamide, and all the corresponding aspartic conjugates except indoleformylaspartic acid, were found in the tissues.

Materials and Methods

All experiments were carried out on pea sections grown and treated under conditions previously described (6). In brief, the sections were bathed for 24 hours in M/60 sodium acid phosphate solutions containing 20 to 30 mg per liter of the acid to be investigated. The tissues were then washed and ground in a Waring blendor with sodium bicarbonate solution. The resulting brei was saturated with ammonium sulfate, infusorial earth was added and the solid matter was separated by filtration. The filtrate, about pH 7.0, was extracted several times with ether, then acidified to pH 4.6, and again repeatedly extracted with ether. The filtrate was further acidified to pH 2.6 with phosphoric acid and repeatedly extracted yet again with ether and finally with *n*-butyl alcohol. The butyl alcohol and other extracts were taken to dryness, taken up again in a small volume of alcohol and chromatographed on paper in an isopropyl alcohol, concentrated ammonium hydroxide, water solvent (80 : 10 : 10, v/v).

Identification of the metabolites was based on chromatographic comparisons with synthetic compounds and, in some cases, on the chromatographic identification of the hydrolysis products.

Indolepropionylaspartic acid, indolebutyrylaspartic acid and 2,4-dichlorophenoxyacetylaspartic acid were synthesized by the carbodiimide method as previously published (5). Benzoylaspartic acid was prepared by the action of benzoylchloride on a cold aqueous solution of aspartic acid containing an excess of NaOH. Benzamide was prepared by the action of concentrated aqueous ammonia on benzoylchloride. The amides of 2,4-D, indolepropionic acid and indolebutyric acid were prepared, conveniently, but in rather poor yields, by dehydration of the ammonium salts with dicyclohexylcarbodiimide. The derivatives of indoleformic acid were not prepared; attempts using the carbodiimide were unsuccessful. Indoleformic acid itself was synthesized as the ester by treating indole first with a Grignard reagent and then with ethylchlorocarbonate (9).

The indole derivatives were detected on the paper with the Ehrlich reagent (1 % *p*-dimethylaminobenzaldehyde dissolved in equal volumes of alcohol and concentrated hydrochloric acid). Since benzoic acid and 2,4-D do not give convenient color reactions, C^{14}-carboxyl-labeled preparations were used and the radioactive metabolites of these acids were located by preparing radioautographs of the developed chromatograms. The synthetic derivatives of benzoic acid and 2,4-D were not radioactive and were detected by exposing the developed chromatograms to iodine fumes

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