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]

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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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[2] Contribution No. 104, Canada Department of Agriculture, Science Service Laboratory, London, Ontario.

which resulted in a brown background with lighter areas over the substances. The 2,4-D derivatives were also detected by spraying the chromatograms with riboflavin and viewing them under ultraviolet light; 2,4-D and some of its derivatives are oxidized by photoactivated riboflavin (3) with the concomitant reduction of the flavin to its non-fluorescent leuco form so that the compounds show as dark spots.

The use of radioactive benzoic acid and 2,4-D facilitated quantitative evaluation of the reactions under investigation. Samples of the ambient solution before and after the incubation period, of the incubated tissues, and of the various extracts and residues of the tissues were collected. Aliquots containing not more than 36 mg of carbon were transferred with 2 gm potassium dichromate to a boiling tube. The boiling tube was provided with a side arm and was fitted with a two-holed stopper. Through one hole sulfuric acid saturated with potassium dichromate was added while a gentle stream of CO_2-free air entered by the other hole and bubbled through the digestion mixture. This air stream then passed through the side arm into a Pettenkofer tube containing 40 ml of 2.5 % barium hydroxide solution, 3 ml of *n*-butyl alcohol (to eliminate foaming) and a few drops of phenolphthalein solution. After 25 ml of the sulfuric acid had been added to the boiling tube, the digestion mixture was slowly heated over a period of about 15 minutes until the sulfuric acid just began to reflux. Then a stream of CO_2 was passed through the apparatus until all the barium had been converted into the carbonate (disappearance of the pink color). This carbonate was transferred as a methanol slurry to planchets, dried and counted at "infinite thickness" using an end window Geiger counter.

Results

Paper Chromatography of the Indole Acids and Their Derivatives: Figure 1 (*upper*) illustrates the chromatographic behavior of the synthetic substances with the isopropyl alcohol–concentrated ammonium hydroxide–water solvent. As can be seen in figure 1 (*upper*), increasing the length of the side chain increases the solubility of the indole derivatives in the non-aqueous phase and hence their mobility. On the other hand, odd numbers of carbons in the side chain seem to increase the polarity of the compounds (compare the vastly different water solubilities of the odd and even carbon dicarboxylic acids such as malonic vs oxalic and succinic acids). Consequently indoleacetic acid is considerably more mobile than indoleformic acid (effects additive), while indolepropionic acid and its derivatives are only slightly more mobile than the corresponding acetic compounds (effects opposite). Indolebutyric acid and its derivatives are again conspicuously more mobile (effects additive).

Indoleformic acid gives a pink color with the Ehrlich reagent. All the other substances give characteristic purple colors which gradually change to blue and blue-grey on long standing.

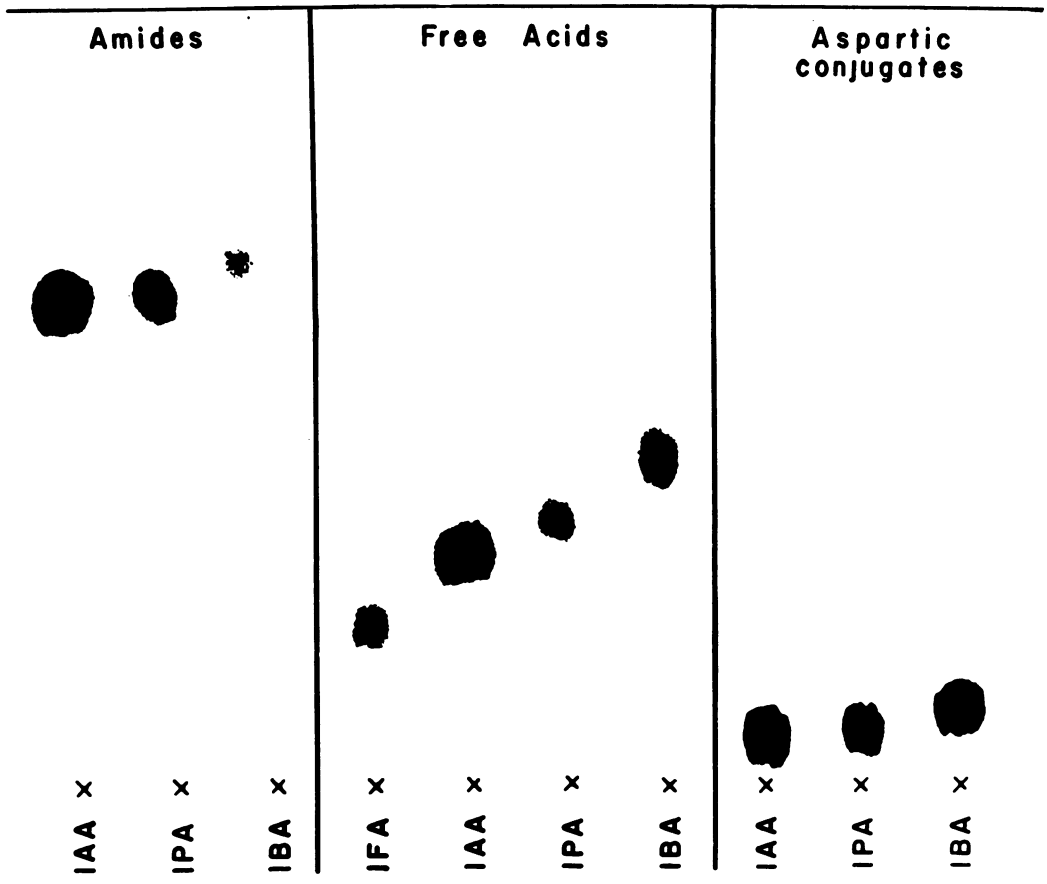
Conjugation Reactions with Indole Acids in Vivo: Chromatograms of ether extracts from tissues treated with indoleacetic acid and its homologues (30 mg/l) are shown in figure 1 (*lower*). Markers of the corresponding synthetic free acids and their amides and aspartic derivatives are included, except in the case of indoleformic acid, the derivatives of which were not prepared. The chromatogram (fig 1 B) of indoleacetic acid treated tissues has already been described in a previous publication (6) and is included for comparison. Since there were no ether-soluble indole compounds which gave a reaction with the Ehrlich reagent in ether extracts of control tissue all spots illustrated in figure 1 (*lower*) probably represent substances derived from the applied indole acids.

Indoleformic Acid (Indole-3-Carboxylic Acid) (fig 1 A): Epicotyl sections treated with indoleformic acid accumulated two Ehrlich reactive substances both of which gave the pink color associated with indoleformic acid. One of these substances was acidic and chromatographically indistinguishable from unchanged indoleformic acid (R_f 0.23). Extraction from water into ether of the second substance (R_f 0.56) was difficult; a similar solubility relation had been noted for the amide of indoleacetic acid (fig 1 B). The properties of this indoleformic acid derivative viz neutrality, relative water solubility and mobility in the isopropanol-water solvent, are those one would expect of indoleformamide.

Indoleacetic Acid (fig 1 B): Indoleacetic acid treated tissues contained, as already reported (6), indoleacetamide (R_f 0.66), free indoleacetic acid (R_f 0.35), and indoleacetylaspartic acid (R_f 0.10), as well as an unidentified spot (pH 4.6, R_f 0.27) just below IAA.

Indolepropionic Acid (fig 1 C): Pea epicotyls incubated with indolepropionic acid accumulated several indole compounds. A mobile compound (R_f 0.65) was chromatographically indistinguishable from indolepropionamide and gave the same color reaction with the Ehrlich reagent (purple) and Salkowski (acid-ferric chloride) reagent (brown). The most abundant substance (R_f 0.36) was an acid, extracted at pH 4.6, which corresponded in every respect with unchanged indolepropionic acid. A stronger acid (extractable at pH 2.6) moved more slowly (R_f 0.10) and was chromatographically similar to indolepropionylaspartic acid. This substance was hydrolyzed in 3 *N* barium hydroxide solution at 100° C for 3 hours, yielding indolepropionic acid and aspartic acid. Since the acidic substance had the same mobility and the same products on hydrolysis as synthetic indolepropionylaspartic acid, the two substances were considered identical. An unidentified spot was observed (pH 4.6) with an R_f of 0.26.

Indolebutyric Acid (fig 1 D): Tissues treated in a like manner with indolebutyric acid contained a number of indole compounds. A highly mobile, neutral substance (R_f 0.73) was chromatographically indistinguishable from synthetic indolebutyramide. An acidic substance (R_f 0.48) was chromatographically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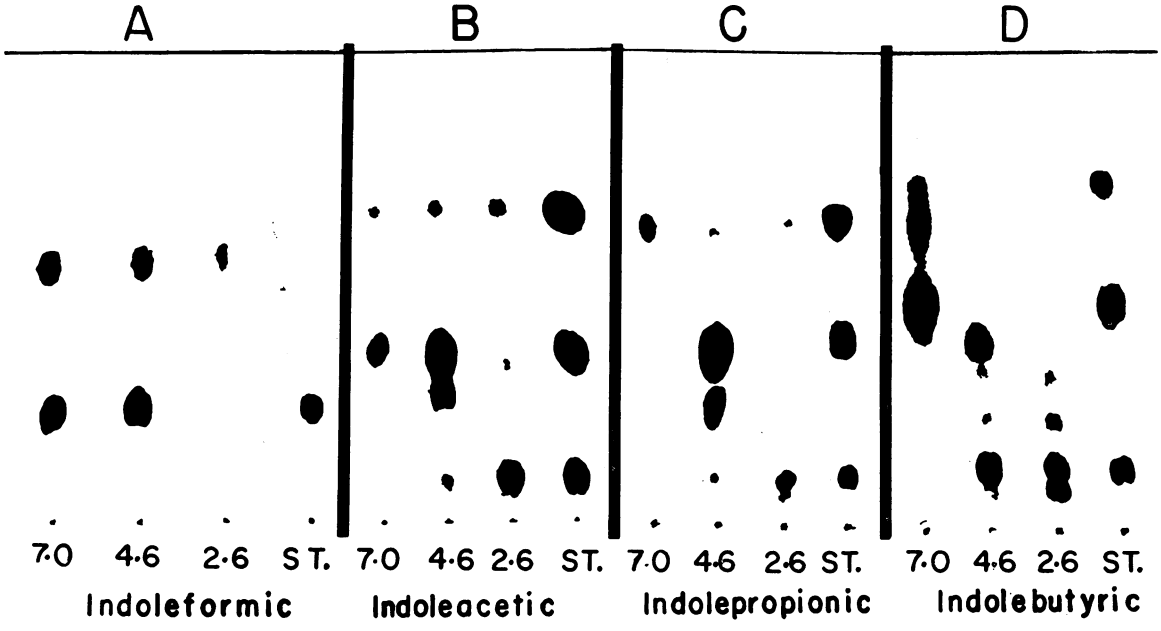
A
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7·0 4·6 2·6 ST.
Indoleformic
Indoleacetic
Indolepropionic
Indolebutyric

TABLE I

DISTRIBUTION OF THE RADIOACTIVE CARBON OF C^{14}-CARBOXYL LABELED BENZOIC ACID AND 2,4-D IN PEA TISSUES

		2,4-D		BENZOIC ACID	
		CPM	% OF ORIGINAL	CPM	% OF ORIGINAL
(A)	Original solution	85,000	100	79,400	100
(B)	Solution after incubation period	76,000	89.5	10,300	13
(C)	Lost from solution (A - B)	9,000	10.5	69,000	87
(D)	Found in tissue	6,250	7.4	21,000	26
(E)	Not accounted for. Probably lost as CO_2 (C - D)	2,750	3.2	48,100	61
(F)	Insoluble in saturated $(NH_4)_2SO_4$ solution (cell walls, proteins, etc.)	2,700	3.2	2,000	2.5
(G)	Soluble in saturated $(NH_4)_2SO_4$ solution	3,900	4.6	20,400	26
(H)	Soluble in acidified, saturated $(NH_4)_2SO_4$ solution. Insoluble in ether and *n*-butyl alcohol	165	0.2	2,650	3.3
(I)	Extracted from acidified saturated $(NH_4)_2SO_4$ solution by ether and *n*-butyl alcohol (G - H)	3,735	4.4	17,800	22

Etiolated pea epicotyl sections (10 gm) incubated in 100 ml M/60 NaH_2PO_4 solution containing 2.0 mg of labeled 2,4-D or benzoic acid (approx. 12 μc per expt).

indistinguishable from indolebutyric acid and like that acid was completely extracted by ether at pH 7.0. Two spots (pH 7.0, R_f 0.65 and pH 4.6, R_f 0.40) were not identified.

Two other substances in the indolebutyric acid treated tissues were of particular interest. These had the mobility, acid strength, and color reactions with the Salkowski and Ehrlich reagent characteristic of indoleacetic acid (pH 4.6, weak spot R_f 0.34) and indoleacetylaspartic acid (pH 2.6, R_f 0.10). Clearly, indolebutyric acid undergoes conversion, apparently by β-oxidation, to indoleacetic acid which is then conjugated with aspartic acid. There was no evidence of similar β-oxidation of indolepropionic acid to indoleformic acid.

THE METABOLISM OF 2,4-D AND BENZOIC ACID: Ten grams of pea epicotyl sections were incubated for 24 hours in 100 ml M/60 sodium acid phosphate solutions containing 2 mg of carboxyl-labeled benzoic acid or carboxyl-labeled 2,4-D (about 12 microcuries C^{14} per experiment). The radioactivity of the ambient solution was measured before and after the incubation period. Since no new radioactive compounds were detected in the bathing solutions it is probably safe to equate the disappearance of radioactivity to the amount of acid taken up by the tissues. The total radioactivity of the tissues was then determined. This was always less than the radioactivity lost from solution. Presumably the difference represents loss of carboxyl carbon in the form of CO_2. No attempt to trap the respiratory CO_2 was made, however. The tissues were ground in sodium bicarbonate solution, the brei was saturated with ammonium sulfate and the insoluble fraction, containing among other things the cellulose and precipitated proteins, was separated by filtration and washed with saturated ammonium sulfate solution. Aliquots of the soluble and insoluble materials were oxidized as described in Methods and the radioactivity of each aliquot was measured as barium carbonate. Finally the filtrate was acidified and extracted with ether and *n*-butyl alcohol and the radioactivity of the residual aqueous phase was again determined.

Table I presents the results of these determinations. Unlike indoleacetic acid (2) and benzoic acid described below, 2,4-D is not readily taken up by the pea tissue. Only 10.5 % of the 2,4-D was lost from the incubation solution. However most of this (7.4 %) was found in the tissues. Apparently during a period of 24 hours there was little decarboxylation. Of the tissue radioactivity, about 40 % was in the fraction containing the cell walls and proteins while about 60 % was in the filtrate. Acetone extraction of the cellulose-protein fraction yielded considerable amounts of 2,4-D but no other radioactive substance. The radioactivity in the filtrate was nearly all ether- or butanol-soluble and considered predominantly (over 95 %) of unchanged 2,4-D (fig 2). There was no 2,4-dichlorophenoxyacetamide (R_f 0.86). Small amounts of three other substances, all acids, were detected. The most abundant and slowest moving of these (R_f 0.15) agreed with 2,4-dichlorophenoxyace-

FIG. 1 (*upper*). Chromatograms of synthetic indoleformic (indole-3-carboxylic) acid (IFA), indoleacetic acid (IAA), indolepropionic acid (IPA), indolebutyric acid (IBA), and the corresponding amides, and the aspartic derivatives. Partitioning solvent: isopropyl alcohol, concentrated ammonium hydroxide, water (80:10:10, v/v).

FIG. 1 (*lower*). Conjugation reactions involving indole acids. Chromatograms of the ether soluble substances found in pea epicotyls which had been incubated with indoleformic, indoleacetic, indolepropionic or indolebutyric acid solutions. The numbers refer to the pH of the aqueous plant extracts shaken with ether. St. refers to standard solutions containing 1 mg/ml of the corresponding free acid, amide and aspartic acid derivatives. (For their relative positions see figure 1 (*upper*).)

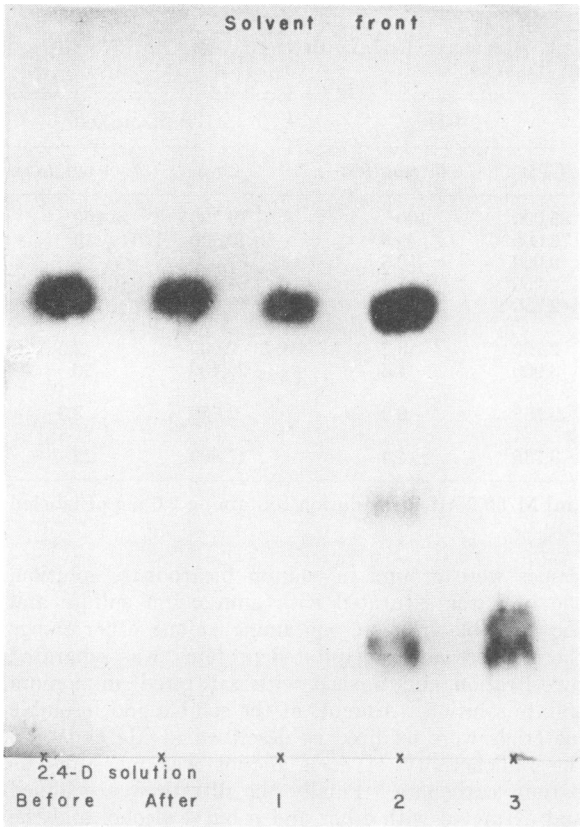

FIG. 2. Metabolites of C^{14}-carboxyl-labeled 2,4-D from pea epicotyls. Radioautographs of chromatograms of 2,4-D solutions before and after incubation and of tissue extracts: 1) ether extract at pH 7.0; 2) ether extract at pH 2.6 and 3) *n*-butyl alcohol extract at pH 2.6. The slowest moving substance in 2 and 3 is probably 2,4-dichlorophenoxyacetylaspartic acid.

tylaspartic acid in mobility and acid strength. Furthermore, this substance yielded 2,4-D on hydrolysis. The amounts involved were so small that no attempt was made to identify the aspartic acid moiety. 2,4-D which had accumulated in tissues during a 24-hour incubation did not noticeably decrease during a subsequent 24-hour period in a moist chamber.

The metabolism of benzoic acid presented a very different picture. As much as 87 % of the radioactivity disappeared from the ambient solution during the incubation period (table I). Most of this radioactivity was not in the tissues. Apparently pea tissue can readily degrade benzoic acid at least to the stage of decarboxylation. Approximately one quarter of the radioactivity lost from the solution did accumulate in the tissue, nearly all in the ether soluble fraction. Of this ether soluble material by far the greater part (90 to 95 %) was chromatographically indistinguishable from benzoylaspartic acid (fig 3, R_f 0.14) and yielded benzoic and aspartic acids on hydrolysis with barium hydroxide. Small amounts of other radioactive substances occurred. These included a trace of free benzoic acid and somewhat more of a neutral substance chromatographically indistinguishable from benzamide (R_f 0.75).

DISCUSSION

The ability of plant tissue to condense applied indoleacetic acid with aspartic acid or ammonia is not restricted to indoleacetic acid, for it is shown by a variety of carboxylic acids. Thus indolepropionic acid, indolebutyric acid and even benzoic acid are con-

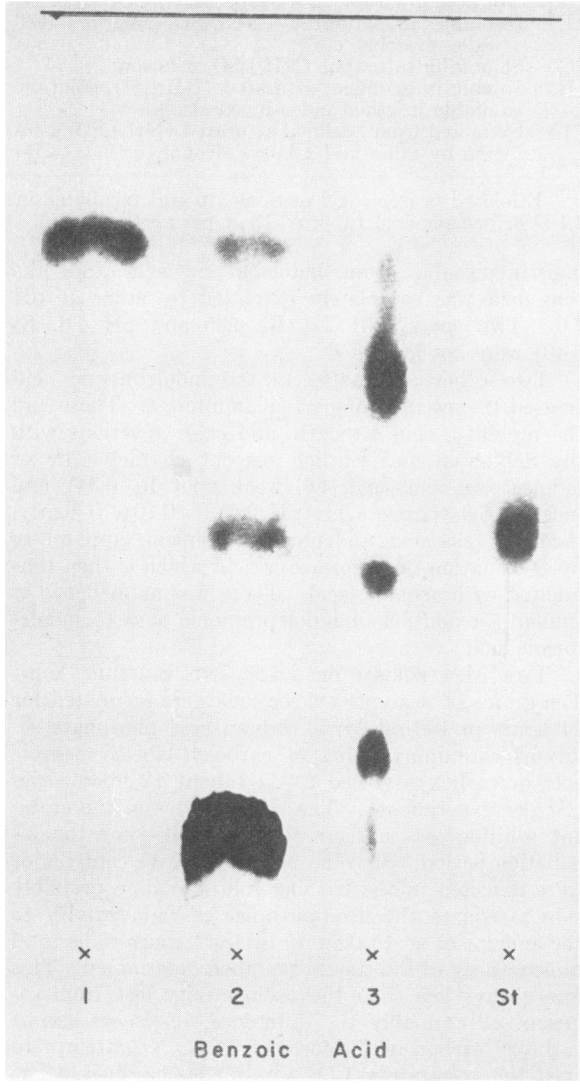

FIG. 3. Radioactive metabolites of C^{14}-carboxyl-labeled benzoic acid from pea epicotyls. Radioautographs of chromatograms of tissue extracts: 1) ether extract at pH 7.0; 2) ether extract at pH 2.6; 3) *n*-butyl alcohol extract at pH 2.6. (St.) is a marker of radioactive benzoic acid. The mobile, neutral substance (R_f 0.75 in 1 and 2) is chromatographically indistinguishable from benzamide. The slow moving acidic substance (R_f 0.14 in 2) is benzylaspartic acid.

verted, in greater or lesser degree, to the amides and to derivatives of aspartic acid. However indoleformic acid is converted only to a neutral substance, probably its amide, and no indoleformylaspartic acid is found. 2,4-D is outstanding in that it is conjugated to a very limited extent and up to 95 % of the 2,4-D taken up can be recovered unchanged.

Previous workers have shown that complexes of unknown composition are produced in the metabolism of 2,4-D by the plant. Weintraub et al (11) found that 2,4-D underwent several transformations in dormant plant tissue during a period of several months. Jaworski and Butts (7) recovered two major derivatives other than 2,4-D in the 80 % alcohol extract of treated bean stems. They considered that one of the unidentified products might be a glycoside containing 2,4-D as the aglycon. It is, however, hazardous to attempt a direct comparison of the results of these earlier studies with those reported in the present paper because of the great differences in experimental techniques such as the method of application and duration of the incubation period.

In the present paper, the metabolism of 2,4-D is compared to that of the other acids. Relative to these acids 2,4-D is hardly metabolized at all. In contrast, indoleacetic acid is, over a wide range of concentrations, so rapidly degraded that only 20 % of that taken up accumulates in the tissue as recognizable indole compounds (2). At concentrations below about 20 mg/l, practically all of the indoleacetic acid which escapes degradation is conjugated with aspartic acid to form the much less active indoleacetylaspartic acid. In spite of the fact that indoleacetic acid is removed from the ambient solution at least three times as fast as is 2,4-D, the level of free 2,4-D in plant tissue greatly exceeds that of indoleacetic acid (except at very high and toxic levels of applied indoleacetic acid). It is tempting to suggest that this difference is one reason for the potent herbicidal activity of 2,4-D for, being resistant both to degradation and to conjugation, 2,4-D remains in the plant tissues as the free acid is able to exert its physiological action long after growth substances such as indoleacetic acid have been metabolized.

The appearance of indoleacetylaspartic acid in tissues treated with indolebutyric acid is an indication of β-oxidation, a reaction which has been previously demonstrated by Fawcett et al (4) in the phenoxyalkylcarboxylic acids. It seems probable that this β-oxidation of indolebutyric acid involves the intermediate formation of indolebutyryl-CoA and indoleacetyl-CoA. Probably the conjugation of indoleacetic acid with aspartic acid also involves CoA. These observations therefore lend some support to the hypothesis that indoleacetyl-CoA is involved in the metabolism of indoleacetic acid (10, 13).

Summary

1. Pea epicotyls were incubated for 24 hours in solutions of various carboxylic acids. After the incubation period the tissues were extracted and the metabolic products of these acids were identified chromatographically.

2. Indoleformic acid (indole-3-carboxylic acid) was in part converted into a neutral substance which is probably indoleformamide. No indoleformylaspartic acid was found.

3. Indolepropionic acid yielded indolepropionylaspartic acid and a neutral substance chromatographically indistinguishable from indolepropionamide.

4. Indolebutyric acid treated tissues accumulated substances which were presumably indolebutyramide and indolebutyrylaspartic acid. In addition considerable amounts of indoleacetylaspartic acid and traces of indoleacetic acid were found.

5. Benzoic acid was rapidly destroyed but that portion which did accumulate in the tissues was predominantly in the form of benzoylaspartic acid. Benzamide and traces of free benzoic acid were also found.

6. In contrast, very little 2,4-D was destroyed and nearly all of the accumulated acid was in the free form. Traces of an acidic 2,4-D derivative, chromatographically indistinguishable from 2,4-dichlorophenoxyacetylaspartic acid, also accumulated.

7. The metabolism of 2,4-D is compared with the metabolism of indoleacetic acid on the basis of parallel experiments. It is suggested that the persistence of 2,4-D in the tissues, which results not only from its resistance to degradation but also from its resistance to conjugation, is one reason for its efficacy as a herbicide.

The authors gratefully acknowledge the technical assistance of Mr. M. W. H. van Ysselstein. They wish to thank Dr. J. R. Robinson for his advice in all matters pertaining to the use of radioisotopes and for the counting of the barium carbonate samples. They also wish to thank Dr. G. R. Bell who developed the riboflavin method of detecting 2,4-D on chromatograms.

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MOVEMENT OF C^{14}-TAGGED ALPHA-METHOXYPHENYLACETIC ACID OUT OF ROOTS[1]

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Alpha-methoxyphenylacetic acid (MOPA) was reported in 1953 to have marked plant-growth-modifying properties and to be readily translocated by bean and other plants (6). When applied to leaves or stems, this compound was absorbed and translocated both upward and downward within the plant. It moved down into the roots and out of them and was absorbed by adjacent or nearby roots of an untreated plant; then it moved upward in the stem of this plant to partially developed leaves which subsequently became malformed (7). This phenomenon of plant regulators moving out of roots, however, is uncommon (7). MOPA moved from various kinds of bean plants to other bean plants and from other broad-leaved plants to bean, causing malformation and inhibition of new growth. There was no evidence at that time that the acid moved from corn to any other plant.

In studying the translocatability of a compound, it is, of course, necessary to identify the compound by either biological or chemical means after it has been moved through the plant. In a previous study (5) the downward transport and exudation of MOPA were proved by applying approximately 150 μgm of C^{14}-carboxyl-tagged MOPA to the stems of several bean plants. These plants were then grown with their roots immersed in aerated tap water. Three days later the water was found to contain radioactivity. A sample of the water was evaporated and the residue partitioned on paper. This residue was identified as MOPA. If any radioactive metabolites or degradation products were exuded by the roots, these were not detectable. These earlier results indicate that some of the methoxy acid was absorbed by the stems, translocated to the roots and exuded without detectable chemical change.

The present investigation is concerned with the exudation of MOPA as affected by its absorption and translocation, the amount of MOPA applied and environmental conditions.

[1] Received April 18, 1957.

MATERIALS AND METHODS

Carboxyl-tagged alpha-methoxyphenylacetic acid was prepared on a micro scale from benzaldehyde and sodium cyanide-C^{14} by a modification of the standard procedure (1, 8) involving the successive preparation of mandelonitrile and mandelic acid and the conversion of the latter to MOPA by methylation with dimethyl sulfate.

Young plants of Pinto bean, approximately 5 inches (ca 12 cm) tall, were grown in aerated tap water or nutrient solution for these experiments. The plants, grown in pots containing soil, were removed, and after their roots were washed free of soil, they were placed in beakers containing the aerated tap water or nutrient medium.

Five μgm of C^{14}-tagged MOPA dissolved in water was then spread evenly on the upper surface of each primary leaf by means of a thin glass rod, making 10 μgm per plant. Each beaker contained three plants. After preliminary tests for periods up to 300 to 350 hours, it was arbitrarily decided that further experiments would be terminated after approximately 200 hours, as this was long enough to demonstrate the pattern of exudation. During this period, 20-ml portions of the solution were taken at successive intervals of 24 hours, except for the first 48 hours during which samples were taken more frequently, to determine the concentration of radioactive exudate. These aliquots were then evaporated in metal planchets and tested for radioactivity. Solutions in the beakers were always readjusted to their original volume and, whenever necessary, correction for self-absorption was made.

RESULTS

The presence of MOPA in the tap water surrounding the roots was first detected about 5 hours after application of 5 μgm of the acid to each leaf. The amount in the water surrounding the roots (the amount exuded less the amount reabsorbed by the plant through its roots and that adsorbed on the glass)