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STUDIES ON 3-INDOLEACETIC ACID METABOLISM. II. SOME PRODUCTS OF THE METABOLISM OF EXOGENOUS INDOLEACETIC ACID IN PLANT TISSUES[1,2]

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It has long been known that indoleacetic acid (IAA) is taken up by plants and translocated to various tissues, causing characteristic modifications in the pattern of growth. However the fate of IAA in vivo has received little attention. This is surprising in that IAA and its derivatives are virtually the only materials of known chemical constitution which both exhibit the formative effects of plant growth substances and occur naturally in plants. The fact that the concentrations of endogenous IAA are extremely low is no doubt in part responsible for the neglect of an important field of research. The authors of this paper have attempted to overcome the difficulty by supplying much larger amounts from an ambient solution. They are quite aware, however, that the reactions associated with relatively high concentrations of applied IAA may differ significantly from metabolic reactions as they occur in nature.

Exogenous IAA participates in several biological reactions. Plants are known to contain IAA oxidizing enzymes and part of the IAA administered to plants presumably undergoes oxidative degradation to products which are no longer growth active (8). Hemberg (3) has noted a rise in "bound auxin" in IAA treated corn kernels. Siegel and Galston (6) have reported that IAA administered to excised pea roots becomes attached to proteins. On the other hand we have recently found that IAA administered to pea stems is conjugated with aspartic acid (1). The present communication constitutes a report on the extention of our investigations to other plant species.

MATERIALS AND METHODS

A survey was carried out using 12 plant species belonging to 8 families. The following tissues were investigated: etiolated coleoptiles of oats, corn and barley all harvested just before the emergence of the primary leaf; etiolated epicotyls of peas 7 days old; etiolated hypocotyls of sunflower, cucumber and buckwheat, 7, 4, and 4 days old respectively; etiolated potato sprouts 3 to 5 inches long; stems and petioles of greenhouse grown pea, tomato, and cabbage plants. Immediately after harvesting, the tissue was weighed and cut into pieces aproximately 2 inches long. To each 100 gms of tissue were added 2 liters of M/60 sodium dihydrogen phosphate with or without 60 mg of IAA.[3] The floating tissue was incubated at 24° C for 24 hours in the dark with gentle shaking. After this period the tissue was removed, thoroughly washed, and frozen at −8° until it could be conveniently extracted. The residual IAA in the ambient solutions was measured by the acid-ferric chloride (Salkowski) method (8) and in some cases the solutions were ether extracted and chromatographically analyzed for other Salkowski-positive substances.

To each 150 gm of frozen tissue was added 65 ml of 0.3 *N* sodium bicarbonate solution. The tissue was then ground in a Waring blendor and filtered through cheese cloth. The resulting brei was saturated with ammonium sulfate, infusorial earth was added and the precipitated proteins were removed by filtration on a Buchner funnel. (The unwashed precipitate from pea contained from 10 to 20 % of the Salkowski-positive material. However, when the precipitate was thoroughly washed by redissolving it in water and reprecipitating it with ammonium sulphate, practically all the Salkowski-positive material was in solution. Since the washings contained the same substances as the filtrate and in about the same proportions only the first filtrates were regularly investigated in this survey.) The filtrate (pH about 7.5) was extracted once with 100 ml and twice with 50 ml of peroxide-free ether, acidified to pH 2.5 with phosphoric acid and extracted with ether as before. Then the acidified filtrate was extracted with 100, 50 and 20 ml portions of 1-butanol. The aqueous residue was by this time Salkowski-negative. The acidic extracts were made slightly alkaline either with sodium bicarbonate solution or with dilute ammonia and taken to dryness, the

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[3] Some samples of indoleacetic acid from commercial sources are very impure and contain appreciable amounts of indoleacetamide and other Salkowski reactive substances. The following purification procedure, however, has been found to be effective: Commercial IAA is dissolved in water (about 100 ml/gm) containing an excess of sodium bicarbonate. The aqueous solution is extracted several times with 1-butanol, then several times with ether. The dissolved ether is removed by aeration and the solution is slowly acidified to pH 3.0 with phosphoric acid. About 70 % of the original IAA separates on standing as white, flake-like crystals.

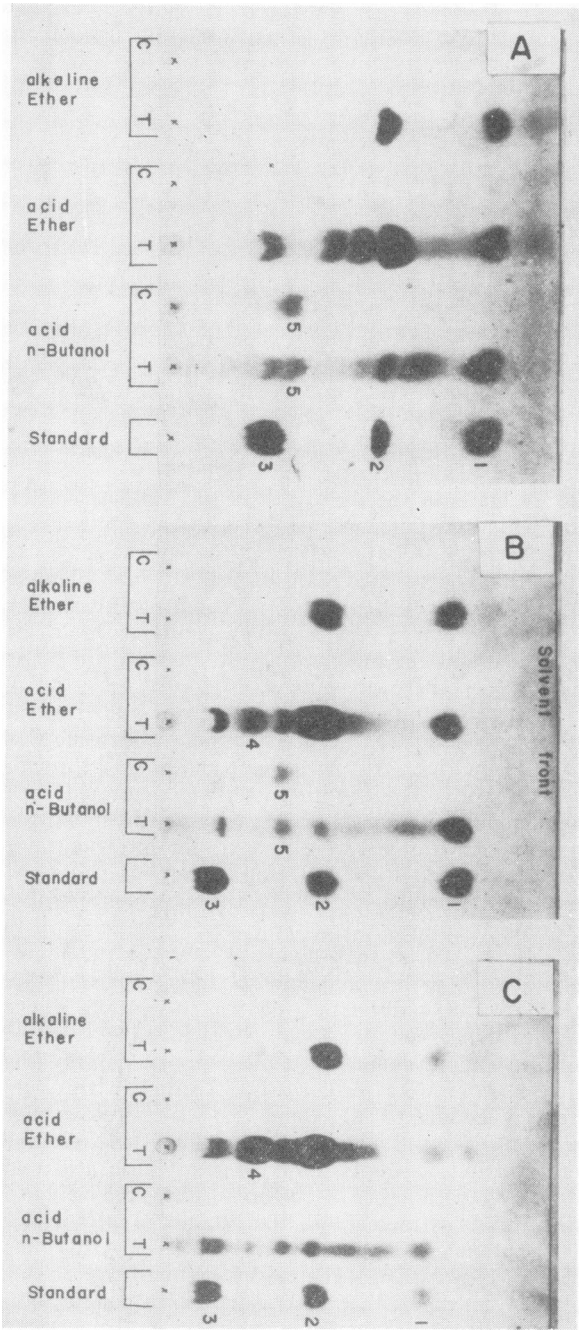

FIG. 1. Typical chromatograms of extracts of IAA treated (T) and untreated (C) plant tissues (sunflower hypocotyls) obtained by successive alkaline-ether, acid-ether and acid-butanol extractions. East spot represents the extract from 3 gm fresh wt of tissues. Chromatogram (A) was developed with an isopropanol–ammonium acetate–acetic acid solvent and sprayed with the Ehrlich reagent (*p*-dimethylaminobenzaldehyde–HCl). Chromatograph (B) was developed with an isopropanol-ammonia solvent and sprayed with the Ehrlich reagent. Chromatogram (C) was developed with the same isopropanol-ammonia solvent but sprayed with the Salkowski reagent (ferric chloride–perchloric acid). Spot 1 is synthetic indoleacetamide, spot 2 is indoleacetic acid and spot 3 is synthetic indoleacetylaspartic acid, each 0.05 micromoles. Spot 4 is an unknown acidic substance described in the text, and spot 5 is tryptophan.

ether extracts in an air stream and the butanol extracts by distillation at reduced pressure. The solvent-free residues were taken up in 1.0 ml of dilute aqueous ammonia or bicarbonate solution. An aliquot (20 μl) was immediately chromatographed on paper and the remainder was stored at $-8°$ C.

The concentrated extracts were chromatographed on 15″ × 15″ sheets of Whatman #1 filter paper for 16 hours at 24° C, using the ascending solvent techniques. The principal partitioning solvents were:

(A) 80 parts of 2-propanol to 20 parts of 8 *N* ammonium hydroxide solution (v/v).

(B) 80 parts of 2-propanol containing 1.25 % acetic acid to 20 parts of a 20 % aqueous solution of ammonium acetate (v/v).

Equivalent extracts from the untreated and the IAA treated tissues were chromatographed as adjacent spots. The dried chromatograms were sprayed either with a modified Salkowski reagent (0.05 M $FeCl_3$ in 35 % $HClO_4$) or with the Ehrlich reagent (1 % *p*-dimethylaminobenzaldehyde in 50 % alcohol–50 % concentrated HCl). See figure 1. In some cases regions on unsprayed chromatograms corresponding to the colored regions on sprayed chromatograms were eluted with water or dilute aqueous ammonia and rechromatographed. The resulting purified material was again eluted, its ultraviolet absorption spectrum determined and its hydrolysis products tested for IAA, amino acids and other substances. When the survey was completed the frozen acid-ether and butanol extracts were thawed and chromatographed simultaneously (see fig 2).

RESULTS

The Salkowski and Ehrlich-reactive IAA derivatives were similar in the various plants grown under different conditions. Figure 1 shows typical chromatograms of successive extracts, in this case of sunflower hypocotyls. Similar chromatograms were made with extracts from each species. Tissues which had not been treated with IAA (C) were devoid of Salkowski-reactive material and the only Ehrlich-positive substance, which occurred alike in treated and untreated plants, was tryptophan (spot 5). This was true of all 12 plants examined. For purposes of comparison the extracts of the 12 plant species were chromatographed together to form the composite picture shown in figure 2; space limitation did not permit inclusion of the controls. There were 4 major Salkowski-reactive substances and only these have been studied in detail. A description of the numerous minor components will entail a more intensive study of the tissue extracts.

3-INDOLEACETAMIDE: All the extracts of IAA

treated plants, with the possible exception of cucumber, contained neutral rapid-moving substances chromatographically identical with 3-indoleacetamide (spot 1, figs 1 and 2). These materials, like synthetic indoleacetamide, gave the purple color typical of IAA with the Ehrlich reagent and a slowly developing weak color with the Salkowski reagent. A sample from corn coleoptiles had the ultraviolet absorption spectrum of indole compounds with maxima at 280 and 288 mμ. It yielded IAA and ammonia (as determined by the Nessler reaction) in approximately equimolar amounts on hydrolysis. Apparently indoleacetamide occurs in particularly large amounts in IAA treated grasses (fig 2).

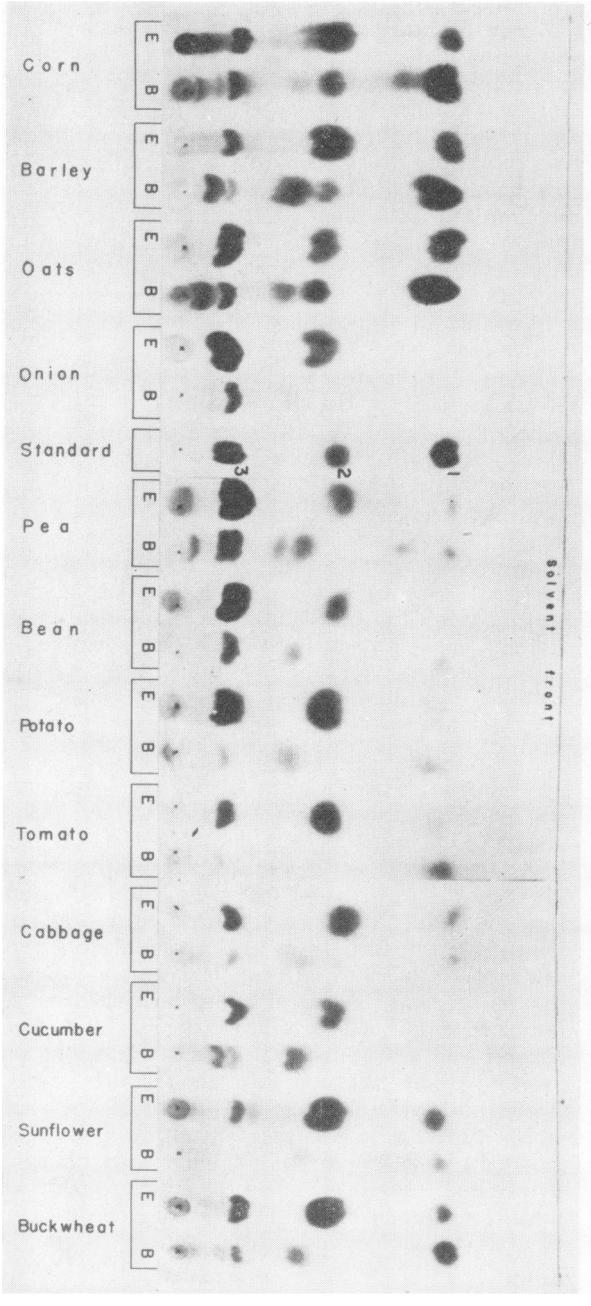

FIG. 2. Chromatograms of acid-ether (E) and acid-butanol (B) extracts from the IAA treated tissues of 12 plant species. Each spot represents the extract from 3 gm fresh wt of tissue. The chromatograms were developed with an isopropanol-ammonia solvent and sprayed with the Ehrlich reagent. Spot 1 is synthetic indoleacetamide, spot 2 is indoleacetic acid, and spot 3 is synthetic indoleacetylaspartic acid, each 0.05 micromoles.

3-INDOLEACETIC ACID: Each extract of IAA treated plants contained an acidic substance chromatographically identical with IAA (spot 2, figs 1 and 2).

3-INDOLEACETYLASPARTIC ACID: The acid-ether and butanol extracts of all the IAA treated plants contained a slow-moving acidic substance chromatographically identical with the indoleacetylaspartic acid (spot 3, figs 1 and 2) previously reported in pea (1). The earlier report stressed the facts that indoleacetylaspartic acid was not extracted by ether and that it did not occur in barley. Both of these assertions must be qualified. Because of its increased acid strength, presumably due to the presence of an acylated amino group adjacent to one of its carboxyls, indoleacetylaspartic acid is only extracted by ether from strongly acidic solutions (about pH 3.0) and thus can be separated easily from IAA. Moreover indoleacetylaspartic acid or some very similar substance does occur in barley but in such small amounts that its presence was missed until highly concentrated extracts were examined (fig 2).

AN UNIDENTIFIED ACIDIC SUBSTANCE: Another acidic material occurred in the acid-ether extracts of many IAA treated plants, particularly in the extracts of sunflower (spot 4, fig 1 C), tomato, bean, cucumber and pea. This substance appeared approximately midway between IAA and indoleacetylaspartic acid on chromatograms developed with the 2-propanol-ammonia solvent. There was some evidence that two compounds may have been involved since the R_f value of the material from sunflower was considerably higher than that of the comparable material from cucumber. The substance (or substances) gave an immediate bright pink or scarlet color with either acid–ferric chloride (Salkowski) or acid-nitrite sprays and could be readily distinguished from IAA by the speed of the color development. It gave a very weak, atypical (grey) color with the Ehrlich reagent. Since none of it was extracted by ether from weakly basic or neutral solutions, it is probably a stronger acid or its salts are less soluble in ether than is the case with IAA. The undissociated form, however, was almost quantitatively removed by ether from acidified aqueous solutions. A sample obtained from sunflower hypocotyls was unstable to boiling with either acid or base but no IAA or other Salkowski-positive material could be found after the treatment. The substance could not be detected among the products of IAA oxidation when the oxidation was brought about enzymatically by oxygen or non-enzymatically by perbenzoic acid, hypochlorite or permanganate.

None of the compounds described above appeared in the ambient solution during the incubation of the plant tissues and therefore it is unlikely that the metabolism of inadvertently introduced bacteria could have contributed any of these derivatives.

Relatively little of the IAA lost from solution could be found in the tissue extracts by means of the color tests used. Table I shows the quantitative discrepancy between the disappearance of IAA and the accumulation of extractable Salkowski-reactive material in the plants. An attempt to identify the missing unreactive substances has been made, without success to date. Alkaline hydrolysis of the tissue proteins did not release IAA or recognizable degradation products. Sprays containing nitrous acid, diazotized sulphanilic acid, diazotized *p*-nitroaniline, and 2,4-dinitrophenylhydrazine did not reveal significant amounts of new substances on the chromatograms of tissue extracts. Similarly, unspecific oxidants such as ferric chloride–ferricyanide mixtures detected only the spots already observed with the aid of the conventional indole reagents. However, the precipitated proteins, the aqueous residue after butanol extraction and the ambient solution have not yet been examined for Salkowski- and Ehrlich-negative IAA derivatives. It would seem that the investigation of these more elusive substances must await the development of improved methods for their detection.

TABLE I

THE CONVERSION OF IAA BY PLANT TISSUES INTO SUBSTANCES LESS CHROMOGENIC WITH THE SALKOWSKI REAGENT

TISSUE	SALKOWSKI COLOR ** DEVELOPED WITH COMBINED TISSUE EXTRACTS AND EXPRESSED AS AMOUNT OF IAA REQUIRED TO GIVE THE SAME COLOR	LOSS OF IAA FROM THE AMBIENT SOLUTION	DECREASE IN SALKOWSKI REACTION OF THE AMBIENT SOLUTION ACCOUNTED FOR BY THE REACTION OF THE TISSUE EXTRACTS
	mg	*mg*	*%*
Corn	6	52	11.5
Barley	6	64	9.5
Oats *	2.5	54	4.5
Onion	5	50	10
Pea	12.5	70	18
Bean	7	20	3.5
Tomato	2.5	15	16.5
Potato	4	34	12
Cabbage	2.5	0 (?)	?
Cucumber ...	1	21	5.0
Sunflower	3	15	20
Buckwheat ..	4	16	25

The data refer to 150 gm fresh wt of tissue incubated for 24 hrs at 24° C in 3 liters of M/60 NaH_2PO_4 solution containing 90 mg IAA.

* 75 gm fresh wt.

** IAA in the ambient solution and Salkowski reactive materials in the tissue extracts were determined by the method of Tang and Bonner (8) except that the readings were taken 2 hrs after the addition of the Salkowski reagent (1).

DISCUSSION

It is not known which compounds derived from exogenous IAA are also formed from endogenous IAA and consequently the pertinence of our observations to the problems of the normal metabolism of plant growth hormones has not been established. Other workers have reported a number of Salkowski-reactive substances from plant extracts. Among these were indoleacetic acid (2), indolepyruvic acid (7), the ethyl ester of indoleacetic acid (5), and indoleacetonitrile (4). Indoleacetylaspartic acid and indoleacetamide have not yet been reported as naturally occurring and judging by the published information there is no reason to believe that they have been encountered without being recognized.

In spite of the large number of Salkowski-positive substances found in the treated tissues (at least 7 in sunflower) most of the IAA lost from the ambient solution was converted into Salkowski- and Ehrlich-negative substances, or at least, into substances which give very much weaker colors with both these reagents. Since the Ehrlich reagent as used in the experiments reacts fairly generally with 3-substituted indoles, it is probable that the degradation involves an early ring cleavage. Radioactive indoleacetic acid with C^{14} in the aromatic nucleus is being synthesized and will be employed in future studies to locate these less easily recognized substances.

SUMMARY

Tissues from twelve plant species have been incubated with indoleacetic acid. Extracts of these tissues have been examined for IAA derivatives.

A large number of substances which give characteristic colors with the Salkowski or Ehrlich reagents were found in all the IAA treated tissues. Those occurring most generally and in largest amounts were, on the basis of chromatographic behavior and hydrolysis products, indoleacetamide, unchanged indoleacetic acid, and indoleacetylaspartic acid. An unknown acidic compound which reacts with the Salkowski reagent but not with the Ehrlich reagent occurred in several plants.

No convincing qualitative differences in the products of IAA metabolism were noted among the plants investigated. However, indoleacetamide predominated in the grasses and indoleacetylaspartic acid predominated in the legumes and in onion.

Most of the indoleacetic acid which disappeared could not be accounted for by substances reacting with either of the reagents.

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STUDIES ON 3-INDOLEACETIC ACID METABOLISM. III. THE UPTAKE OF 3-INDOLEACETIC ACID BY PEA EPICOTYLS AND ITS CONVERSION TO 3-INDOLEACETYLASPARTIC ACID[1,2]

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In two earlier publications (2, 5) it was reported that 3-indoleacetic acid (IAA) administered to plant tissues is taken up and converted in part to derivatives which still give positive Salkowski (acid-$FeCl_3$) reactions. The two major derivatives which react with this reagent were identified as 3-indoleacetylaspartic acid and 3-indoleacetamide. Indoleacetamide predominates in grasses while indoleacetylaspartic acid is by far the most abundant Salkowski reactive derivative accumulating in legumes.

The present paper deals with factors affecting the uptake of IAA and its conversion to Salkowski reactive derivatives in pea epicotyls. Paper chromatographic studies have shown that indoleacetylaspartic acid represents about 90 % of the Salkowski reactive IAA derivatives and consequently the present study is essentially one of the formation of indoleacetylaspartic acid. In this investigation IAA and indoleacetylaspartic acid were estimated independently by extracting lyophilized tissues with ether; indoleacetylaspartic acid, which is insoluble in ether under these conditions was then extracted with bicarbonate solution, leaving a Salkowski unreactive residue. For the sake of convenience, the Salkowski positive materials in the ether and bicarbonate fractions will be referred to as "IAA" and "indoleacetylaspartic acid" respectively although in fact both fractions also contained small amounts of unknown IAA derivatives. The procedure used was not sensitive enough to detect the IAA and other Salkowski positive materials which occur naturally in the tissues.

MATERIALS AND METHODS

Pea epicotyl sections were obtained from Alaska peas grown on vermiculite in the dark at 27° C (80° F) and 80 % relative humidity, exposed to red light for 2 hours on the 6th day and harvested on the seventh. The epicotyls, with their terminal buds removed, were cut into approximately 2-inch sections and 10-gram lots were floated on 500 ml of M/60 NaH_2PO_4 solution containing varying amounts of IAA. The pH of the ambient solution was about 4.6. The suspended epicotyls were shaken in the dark at room temperature for intervals of varying duration. Before and after incubation 1-ml aliquots were removed from the ambient solution and treated with 4 ml of the Salkowski reagent (0.001 M $FeCl_3$ in 14 N H_2SO_4). After 20 minutes the intensity of the pink color was determined photometrically as described by Tang and Bonner (9).

After incubation with IAA the tisues were removed from the solution and washed with water. In some experiments they were then immediately frozen and lyophilized; in other experiments the tissues were placed for various intervals of time on glass plates in an atmosphere saturated with water vapor in the dark at room temperature after which they were frozen and lyophilized.

In both cases the lyophilized epicotyls were weighed and ground in a Wiley mill to pass a 40-mesh sieve. Two-hundred-milligram aliquots of the dry powder were then extracted 4 times with 5 ml of water-saturated, peroxide-free ether. The ether extracts were combined, filtered, evaporated to dryness and taken up in 2 ml of 0.1 N $NaHCO_3$. To 1 ml of the concentrated extract was added 4 ml of the Salkowski reagent and the intensity of the color formed determined as described above. The powder, after ether extraction, was suspended in 4 ml of 0.1 N $NaHCO_3$ overnight at 5° C and then centrifuged. To 1 ml of the supernatant solution were added 4 ml of Salkowski reagent and the intensity of the mauve color characteristic of the Salkowski reaction with indoleacetylaspartic acid was determined except that the

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