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Inhibitory Oxidation Products of Indole-3-Acetic Acid: 3-Hydroxymethyloxindole and 3-Methyleneoxindole as Plant Metabolites[1]

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Summary. Extracts of pea seedlings (*Pisum sativum*, variety Alaska) oxidize indole-3-acetic acid to a bacteriostatic compound which has been identified as 3-hydroxymethyloxindole. At physiological pH this compound is readily dehydrated to 3-methyleneoxindole, another bacteriostatic agent. The extracts of pea seedlings also contain a reduced triphosphopyridine nucleotide-linked enzyme which reduces 3-methyleneoxindole to 3-methyloxindole, a non-toxic compound.

These enzymatic reactions also take place in intact seedlings; thus, a pathway of indole-3-acetic acid degradation via oxindoles appears to be pertinent to plant metabolism.

The significance of such metabolism lies in the fact that a key intermediate of this pathway, 3-methyleneoxindole, is a sulfhydryl reagent capable of profound effects on metabolism and growth.

The use of *Escherichia coli* as a biological model system revealed that IAA, though inactive itself, can be oxidized to 3-methyleneoxindole which has important effects on bacterial growth. At micromolar concentrations it inhibits (3) and at even lower levels it stimulates growth (10). Methylene-oxindole forms adducts with sulfhydryl compounds, including sulfhydryl enzymes (8), a property which readily accounts for its bacteriostatic action. This reactivity is also responsible for its ability to accelerate bacterial growth. Like other sulfhydryl reagents 3-methyleneoxindole can desensitize several regulatory enzymes to feedback inhibition, however, in contrast to the other reagents, 3-methyleneoxindole can desensitize such an enzyme in vivo: Bacterial growth prevented by excessive sensitivity of a regulatory enzyme to an internal feedback inhibitor can be restored by 3-methyleneoxindole (10), indicating that the desensitization observed in the test tube also takes place in the growing cell.

The dual activities of 3-methyleneoxindole, stimulation at low concentrations and inhibition at higher levels, invite comparison with the effects of the parent compound, IAA, on higher plants. This has prompted us to consider the possible involvement of 3-methyleneoxindole in plant metabolism. Accordingly the experiments described here were addressed to the question of whether plants carry out reactions analagous to the model system which convert IAA to oxindole derivatives. The reactions of the model system (fig 1) are: i) photo-oxidation of IAA, either by visible light with riboflavin as a catalyst (4) or directly with ultraviolet light (7), to 3-hydroxymethyloxindole (3); ii) dehydration of 3-hydroxymethyloxindole to 3-methyleneoxindole (3); and iii) reduction of 3-methyleneoxindole to 3-methyloxindole, a non-toxic compound, by a bacterial, DPNH-linked menadione reductase of incomplete substrate specificity (8).

Methods and Materials

Plant Materials. Pea seedlings (Alaska variety), unless otherwise stated were grown in continuous darkness without intermittent watering at 26° for 7 days on vermiculite moistened with de-ionized water. Extracts of the seedlings were prepared by homogenization for 2 minutes in a blendor (100 g fr wt of tissue and 30 ml of de-ionized water). Both harvesting and homogenization were carried out in room light. Following clarification by centrifugation extracts were dialyzed against de-ionized water for 18 hours at 4°.

Bacteria. Escherichia coli W in the exponential phase of growth in a mineral salts-glucose medium was used for the bioassay and for the localization of 3-methyleneoxindole and 3-hydroxymethyloxin-

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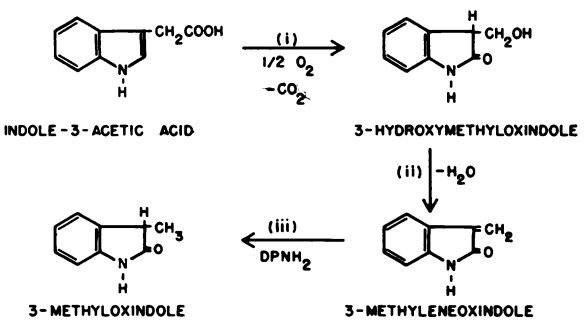

FIG. 1. The oxindole pathway of IAA degradation in a bacterial model system.

dole on chromatograms by bioautography (3). The medium for the latter procedure was modified by using 0.7 % ion agar No. 2 (Consolidated Laboratories). *E. coli* responds directly only to 3-methyleneoxindole and indirectly to 3-hydroxymethyloxindole as the latter compound is rapidly converted to the methylene derivative in the culture medium. Thus, the bioassay does not distinguish between these compounds unless they are first separated by chromatographic or other procedures.

Chemicals. 3-Bromooxindole-3-acetic acid was synthesized by the method of Hinman and Bauman (5). In water this compound is rapidly converted to 3-methyleneoxindole (5). IAA and 2,4-dichlorophenol were purchased from Eastman Organic Chemicals Company and chlorogenic acid from Aldrich Chemical Company. Other chemicals were of reagent grade.

Analytic Procedures. Chlorogenic acid was isolated and estimated by the method of Dear and Aronoff (2). IAA was estimated with a modified Salkowski reagent (9).

Table I. *Production of an Inhibitor from IAA by Pea Seedling Extracts*

Oxidation of IAA was measured in reaction mixtures containing 10 mM potassium phosphate buffer, pH 6.2; 1 mM $MnSO_4$; 0.001 mM 2,4-dichlorophenol; 0.3 mM IAA; and extracts of roots (12 mg of protein) or stems (10 mg of protein). The mixtures were incubated at 22° for 45 minutes. Disappearance of IAA during oxidation was followed colorimetrically (9). Bacteriostatic compounds in the filtrates were estimated by tube dilution assays with *E. coli* as the test organism. The reaction mixtures were chilled to 0° in order to stop the reaction, clarified by centrifugation and sterilized by filtration. This procedure does not distinguish 3-methylene-oxindole from 3-hydroxymethyloxindole as the latter is converted to the former rapidly and non-enzymatically in the medium used for the assay. 3-Hydroxymethyloxindole was isolated from the reaction mixtures and characterized using previously described procedures (3, 8).

	Root	Stem
	μmoles	
IAA oxidized	14.0	12.0
Inhibitor produced:		
As 3-methyleneoxindole or 3-Hydroxymethyloxindole by bioassay	*ca* 2.7	*ca* 1.3
As 3-hydroxymethyloxindole by isolation	1.4	

Results

Oxidation of Indole-3-acetic Acid to 3-Hydroxymethyloxindole. Bacteriostatic compounds are important products of the oxidation of IAA by extracts of the stems and roots of pea seedlings (table I). The inhibitory material in the case of the root extract is equivalent to 2.7 μmoles of 3-hydroxymethyloxindole and/or 3-methyleneoxindole or to 20 % of the IAA oxidized. Half of the bacteriostatic material in the reaction mixture was recovered following extraction with organic solvents and chromatography. Identification of the inhibitor from pea seedlings as 3-hydroxymethyloxindole was based on a comparison of its chemical and physical properties with those described for this compound (3, 6, 8). More detailed discussion of these criteria will be presented in connection with the identification of 3-hydroxymethyloxindole as a product of IAA metabolism in intact seedlings.

Reduction of 3-Methyleneoxindole to 3-Methyloxindole by Extracts of Pea Seedlings. At neutral pH 3-methyleneoxindole is a product of a non-enzymatic dehydration of 3-hydroxymethyloxindole. Enzymatic acceleration of this reaction has not yet been observed; therefore, the enzymatic origin of 3-methyleneoxindole is still in question. On the other hand this compound is metabolized by plant extracts (table II); 3-methyleneoxindole accelerates the oxidation of TPNH by the extracts but has little if any effect on the oxidation of DPNH. This indicates the presence of a TPNH-linked 3-methyleneoxindole reductase. The most obvious product of such a reduction is 3-methyloxindole. This compound was indeed recovered from a reaction mixture containing 3-methyleneoxindole, a TPNH generating system, and an extract of seedlings (table II). The failure to obtain complete conversion of 3-methyleneoxindole to 3-methyloxindole has been attributed tentatively to interfering reactions in the crude extracts as highly purified preparations of the reductase catalyze a nearly quantitative conversion (Unpublished observations of H. S. Moyed).

Oxidation of IAA to 3-Hydroxymethyloxindole by Intact Seedlings. To determine whether the enzymatic reactions observed in extracts also occur in the plant itself, 7-day old, etiolated seedlings from which the cotyledons had been removed were incubated with 0.1 mM IAA. About 100 g fresh weight of seedlings were immersed in a vertical position to just above the roots in 500 ml of an aqueous solution of IAA contained in a 1000 ml

Table II. *TPNH-linked Reduction of 3-Methyleneoxindole by Pea Seedling Extracts*

TPNH oxidation was followed by recording the decrease in absorbancy at 340 mμ of a mixture containing 0.1 M potassium phosphate buffer, pH 6.5; 0.44 mM TPNH; 0.4 mM 3-methyleneoxindole; and an extract of either the stems or roots of pea seedlings. 3-Methyloxindole was estimated after isolation from mixtures containing 33 mM potassium phosphate buffer, pH 6.5; 0.0133 mM TPN; 0.67 mM DL-isocitrate; 3-methyleneoxindole as indicated; and an extract of seedling roots (240 mg of protein) or an extract of seedling stems (207 mg of protein). The identification and isolation procedures are described in fig 5 and in the text.

Expt 1	Addition	TPNH oxidized mμmoles min × mg protein	
		Stem	Root
	None	0.33	0.28
	3-Methyleneoxindole	0.82	0.60

Expt 2	Extract	3-Methyleneoxindole added	3-Methyloxindole recovered
		μmoles	
	Stem	2.0	0.88
	Root	2.2	0.89

pyrex beaker. The seedlings were incubated for 16 hours at 25° at a distance of 2 feet from a bank of 8 cool white fluorescent lamps 3 feet in length. The seedlings were then rinsed with water, blotted dry, and homogenized with ether-ethanol-water (50:47.5:2.5) using 10 ml of the solvent per g fresh weight of seedlings. The volume of the extract was reduced by about two-thirds with a stream of air. The extract was brought to its original volume by addition of ethanol and homogenized. Following removal of insoluble material by centrifugation the sample was concentrated under vacuum to near dryness. After the addition of 50 ml of water insoluble materials were removed by centrifugation, and the sample was extracted 5 times using 5 volumes of ethyl ether each time. The organic phases from the successive extractions were pooled, and evaporated under a stream of air. The aqueous phase remaining after the evaporation was extracted with 2 volumes of petroleum ether (b.p. 30°–60°). The aqueous phase was again retained and evaporated to dryness under vacuum. The residue was dissolved in ethyl ether and applied in a band to Whatman No. 3 MM paper for descending chromatography. The solvent systems were: A) isopropanol-water (5:95) and B) saturated $(NH_4)_2SO_4$–0.1 M potassium phosphate buffer, pH 7.0-water-isopropanol (50:38:10:10). A test strip was cut from the developed chromatogram and placed on the surface of a glucose-mineral salts-agar medium that had been seeded with *E. coli* W. After 12 hours of incubation at room temperature zones of inhibition were detected and marked.

The results of such a chromatographic procedure are shown in figure 2. It has been possible to extract from the seedlings an inhibitor with the same mobility as 3-hydroxymethyloxindole in the 2 solvent systems. In addition a trace of another inhibitor corresponding in R_F to 3-methyleneoxindole was detected on the chromatogram developed with solvent A. (The problems affecting the isolation and detection of 3-methyleneoxindole and the interpretation of such detection will be considered in a separate section).

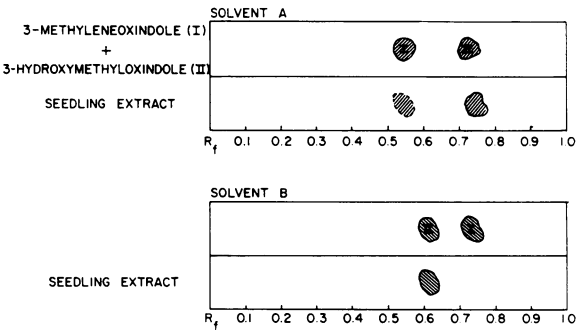

FIG. 2. Bioautography of 3-hydroxymethyloxindole, 3-methyleneoxindole and an inhibitory metabolite of IAA extracted from seedlings. Shaded areas correspond to zones of inhibition observed in a mineral salts-glucose-agar medium seeded with *E. coli* W following incubation with a developed chromatogram on the surface. The seedlings were exposed to 0.1 mM IAA in the light as described in the text.

The major inhibitory compound which corresponded to an ultraviolet quenching band was eluted from the chromatogram with water; however, its absorption spectrum closely resembled that of 3-methyleneoxindole though its R_F was that of 3-hydroxymethyloxindole. This disparity could easily arise from the conversion of 3-hydroxymethyloxindole to 3-methyleneoxindole on the chromatogram during handling, particularly drying. To diminish the opportunity for such a conversion the ultraviolet quenching band corresponding to 3-hydroxymethyloxindole was eluted before the chromatogram had dried. The spectrum of the compound eluted in this manner (fig 3 A) corresponded to that reported for 3-hydroxymethyloxindole (3, 8). The spectrum recorded an hour later indicated that a transition was underway. After 16 hours at room temperature, conversion to a compound with the double absorption maxima of 3-methyleneoxindole was clearly visible. Another more concentrated sample had the clearer 3-methyleneoxindole-like spectrum shown in figure 3 B. Addition of 2-mercaptoethanol resulted in the obliteration of the double absorption maxima indicating the formation

of a mercaptan, a reaction characteristic of 3-methyleneoxindole (8). Bacteriostatic activity of the plant product, its R_F in 2 solvent systems and ultraviolet absorption spectrum, together with a characteristic conversion to 3-methyleneoxindole support its identification as 3-hydroxymethyloxindole.

Factors Affecting the Formation of Inhibitory Oxindoles. The extraction procedures described above for the isolation of 3-hydroxymethyloxindole were also employed for its quantitative assay. The final residue, which would have been used for chromatography, was dissolved in water instead of in ether. This solution was sterilized by filtration, and samples of it were added to tubes containing 2.0 ml of a glucose-mineral salts medium. Graded amounts of a solution of authentic 3-methyleneoxindole were added to a similar set of tubes to provide standards. Each tube was inoculated with approximately 2×10^6 cells of *E. coli* W in the exponential phase of growth and incubated for 16 hours at room temperature. The inhibitory effect of the sample extracted from plants was compared with that obtained with 3-methyleneoxindole. Because of rapid conversion of 3-hydroxymethyloxindole to 3-methyleneoxindole in the bacteriological medium this assay does not distinguish between the 2 compounds, but since it is unlikely that the extraction procedure would have resulted in the recovery of 3-methyleneoxindole the results shown in table III are expressed in terms of 3-hydroxymethyloxindole. The production of 3-hydroxymethyloxindole is dependent both on the concentration of IAA and a source of light. Only a small portion, about 1 %, of the IAA utilized by seedlings in the light can be recovered as 3-hydroxymethyloxindole (table III). This is a minimal value for the conversion as quantitatively significant losses probably occur at several stages during these experiments: 3-Hydroxymethyloxindole undergoes a spontaneous dehydration with the consequence that it has a chemical half-life of about 10 hours under the conditions of the experiment (3); such an experiment, including the incubation period and extraction, requires about 30 hours. The dehydration product, 3-methyleneoxindole, is in turn metabolized to mainly unidentified compounds by pea seedlings; and, in addition, 3-methyleneoxindole undergoes chemical decomposition, particularly during concentration of aqueous solutions. Thus, isolation of 3-methyleneoxindole from aqueous solutions by currently available extraction procedures is probably precluded.

The stimulatory effect of light on the formation of 3-hydroxymethyloxindole might result from enhanced uptake of IAA. Alternatively, it is possible that the increased inhibitor production might be the result of photooxidation of IAA. Chlorogenic acid

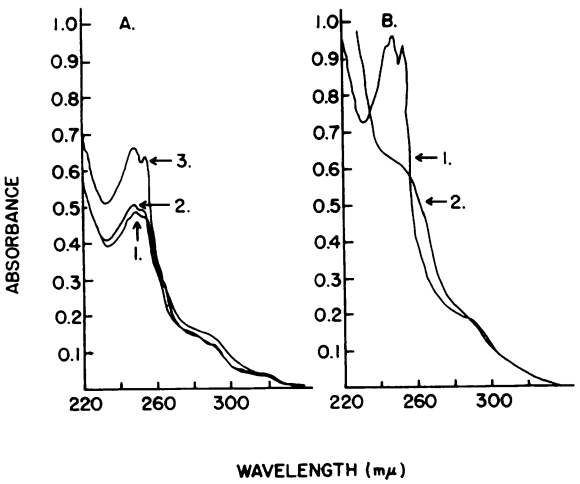

FIG. 3. Absorption spectra and reactions of an inhibitory metabolite of IAA isolated from pea seedlings. The isolation procedure is described in the text. A) Absorption spectra of inhibitor immediately after elution with water from paper chromatogram developed with solvent A (curve 1); after 1 hour at 25° (curve 2); after 16 hours at 25° (curve 3). B) Absorption spectrum of a higher concentration of inhibitor after 16 hours at 25° (curve 1); absorption spectrum immediately after addition of 0.03 mM 2-mercaptoethanol (curve 2).

Table III. *Factors Affecting the Conversion of IAA to 3-Hydroxymethyloxindole by Pea Seedlings*

The incubation of the seedlings with IAA, the extraction of 3-hydroxymethyloxindole from the seedlings, and the estimation of 3-hydroxymethyloxindole are described in the text. 3-Hydroxymethyloxindole was detected only within seedlings exposed to IAA. Neither this oxindole nor other inhibitors were detected in the incubation fluid at the beginning or the end of the experiment or in control fluids incubated without seedlings or without IAA. Chlorogenic acid, when used, was added to the incubation fluid.

	IAA added	IAA utilized		Chlorogenic acid added	3-Hydroxymethyloxindole formed	
		Dark	Light		Dark	Light
		μmoles			μmoles	
	1.0 mM	Not determined		None	0.06	2.04
	0.1 "	"	"	None	0.01	0.29
	0.1 "	"	"	0.1 mM	...	0.02
*	0.1 "	21	30	None	0.01	0.17

* 60 g instead of 100 g fr wt of pea seedlings were used.

was used to distinguish between these alternative explanations. Chlorogenic acid is a well known inhibitor of the enzymatic oxidation but not of the photooxidation of IAA; therefore, the finding that 0.1 mM chlorogenic acid reduced the synthesis of 3-hydroxymethyloxindole by 85 % in the light (table III) tends to eliminate photooxidation of IAA as a possible explanation.

Chlorogenic Acid Content and the Formation of 3-Hydroxymethyloxindole. The inhibitory effect of exogenous chlorogenic acid on the production of 3-hydroxymethyloxindole prompted an examination of the relationship of endogenous chlorogenic acid to this process. The chlorogenic acid content of the cotyledons and of the seedlings from which the cotyledons had been removed was compared with the ability of such seedlings to oxidize IAA to 3-hydroxymethyloxindole (fig 4). The cotyledons have considerably higher levels of chlorogenic acid than the seedlings. A significant transfer of chlorogenic acid from cotyledon to seedling, together with the inhibitory effect of this compound on the formation of 3-hydroxymethyloxindole, could account for the fact that removal of the cotyledons generally

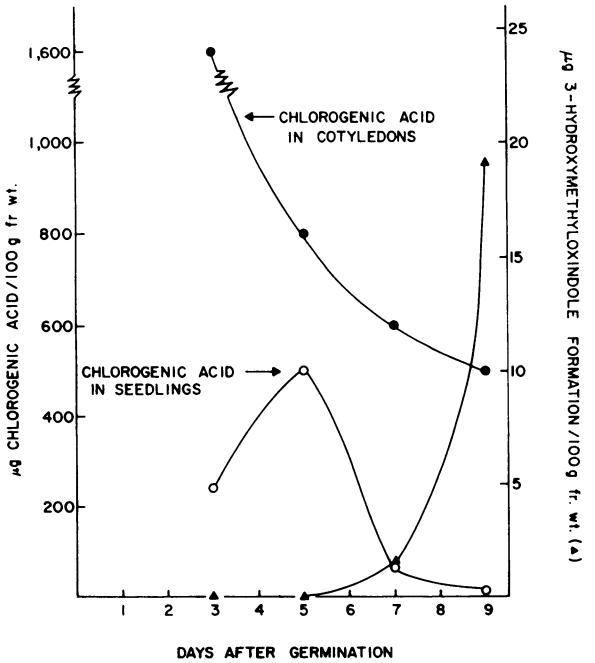

FIG. 4. Chlorogenic acid content and ability of pea seedlings to oxidize IAA to 3-hydroxymethyloxindole. The measurement of 3-hydroxymethyloxindole formation followed the procedure described in the text under *Oxidation of IAA to 3-Hydroxymethyloxindole by Intact Seedlings* except that dark grown seedlings of the indicated ages were employed. The IAA concentration was 0.1 mM. ●—●, chlorogenic acid content of cotyledons; ○—○, chlorogenic acid content of seedlings from which the cotyledons had been removed; ▲—▲, capacity of seedlings from which the cotyledons had been removed to convert IAA to 3-hydroxymethyloxindole.

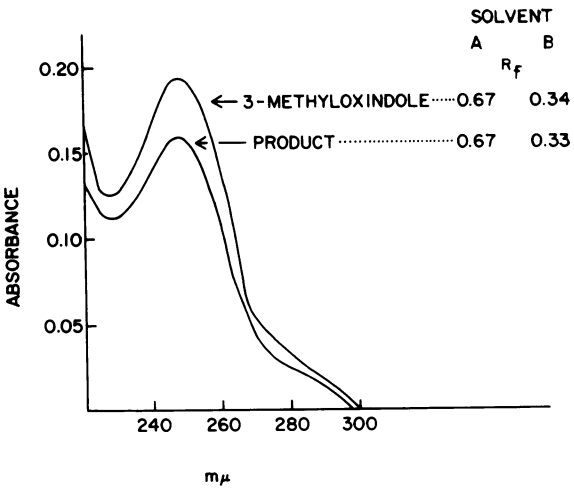

FIG. 5. Chromatographic properties and the absorption spectrum of a product of the metabolism of 3-methyleneoxindole by pea seedlings. About 75 g of 7-day old etiolated seedlings were immersed in a vertical position to just above the roots in 500 ml of an aqueous solution of 0.1 mM 3-methyleneoxindole in a 1000 ml beaker. The seedlings were incubated in the dark at room temperature for 16 hours. The extraction and chromatographic procedures used for 3-hydroxymethyloxindole (described in the text under *Oxidation of IAA to 3-Hydroxymethyloxindole by Intact Seedlings*) were also employed for 3-methyloxindole. The absorption spectrum of the natural product shown was obtained with material eluted from the chromatogram developed with solvent A. 3-Methyloxindole was detected only in seedlings incubated with 3-methyleneoxindole and was not produced in the absence of seedlings.

enhances the ability of the seedlings to produce this oxindole. On the third and fifth days after germination when chlorogenic acid was readily detectable in the seedlings little if any formation of 3-hydroxymethyloxindole was observed, but on the seventh and ninth days when chlorogenic acid had drastically decreased and almost disappeared the capacity to form 3-hydroxymethyloxindole was markedly increasing.

Reduction of 3-Methyleneoxindole to 3-Methyloxindole by Pea Seedlings. A compound with the R_F of 3-methyloxindole in solvents A and B has been recovered from pea seedlings exposed to 3-methyleneoxindole. Its spectrum matches that of 3-methyloxindole (fig 5). However, it only accounts for about 10 % of the 3-methyleneoxindole which was utilized completely by the seedlings. The other metabolites of 3-methyleneoxindole in seedlings remain to be identified.

Discussion

Demonstration of the enzymatic oxidation of IAA to 3-hydroxymethyloxindole and the subsequent reduction of its dehydration product, 3-methyleneoxindole, to 3-methyloxindole in plant extracts

suggests that these reactions might be involved in certain of the effects of IAA on metabolism and growth, particularly as 3-methyleneoxindole is a highly reactive compound capable of profound effects on cell growth (3, 10). The possible relevance of the oxindole pathway of IAA degradation to plant metabolism is strengthened by the detection of the same reactions in intact pea seedlings. It is likely that reactions of this type underlie the conversion of IAA to 3-methyloxindole described in other plant tissues (1). While the non-enzymatic dehydration of 3-hydroxymethyloxindole to 3-methyleneoxindole is reasonably rapid, particularly under physiological conditions of pH and ionic strength (3, 6), the failure, so far, to detect enzymatic acceleration of the dehydration is an important gap in the evidence for an oxindole pathway of IAA degradation. Other examples in metabolism of the enzymatic acceleration of reactions which proceed at easily detectable rates even in the absence of enzymes encourages a continuation of the search for 3-hydroxymethyloxindole dehydrase.

In contrast to the uncertainty concerning the metabolic origin of 3-methyleneoxindole there is definite evidence relating to its metabolic fate. Peas contain at least 1 TPNH-linked enzyme which reduces 3-methyleneoxindole to 3-methyloxindole. A similar reduction in a bacterial model system is catalyzed by a DPNH-linked menadione reductase (8), but a highly purified preparation of the pea 3-methyleneoxindole reductase is incapable of reducing menadione of other acceptors frequently reducible by menadione reductases. This, together with a reasonable substrate constant (Ks = 0.2 mM), suggests that 3-methyleneoxindole is a physiologically significant substrate of the plant reductase (unpublished observations of H. S. Moyed).

Even small fractions of IAA channelled into the oxindole pathway could have a far reaching influence on metabolism and growth as the effects of the key intermediate, 3-methyleneoxindole, would be amplified through its reactions with enzymes. The local concentrations of IAA oxidase and 3-methyleneoxindole reductase, as well as the activators and inhibitors of these enzymes, would be of critical importance as such factors would determine steady state levels of this highly reactive oxindole. It is therefore suggested that these enzymatic reactions might be involved in certain of the physiological responses to IAA, especially inhibition. Furthermore, local changes in the ratio of these activities to each other could have a role in auxin-dependent tropisms.

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