Plant Physiol. (1972) 50, 507–509

The Binding of Indole-3-acetic Acid and 3-Methyleneoxindole to Plant Macromolecules

Received for publication March 2, 1972

P. S. Basu and V. Tuli
College of Medicine, University of California, Irvine, California 92664

ABSTRACT

Homogenates of pea (*Pisum sativum* L., var. Alaska) seedlings exposed to ^{14}C-indole-3-acetic acid or ^{14}C-3-methyleneoxindole, an oxidation product of indole-3-acetic acid, were extracted with phenol. In both cases 90% of the bound radioactivity was found associated with the protein fraction and 10% with the water-soluble, ethanol-insoluble fraction. The binding of radioactivity from ^{14}C-indole-3-acetic acid is greatly reduced by the addition of unlabeled 3-methyleneoxindole as well as by chlorogenic acid, an inhibitor of the oxidation of indole-3-acetic acid to 3-methyleneoxindole. Chlorogenic acid does not inhibit the binding of ^{14}C-3-methyleneoxindole. The labeled protein and water-soluble, ethanol-insoluble fractions of the phenol extract were treated with an excess of 2-mercaptoethanol. Independently of whether the seedlings had been exposed to ^{14}C-indole-3-acetic acid or ^{14}C-3-methyleneoxindole, the radioactivity was recovered from both fractions in the form of a 2-mercaptoethanol-3-methyleneoxindole adduct. These findings indicate that 3-methyleneoxindole is an intermediate in the binding of indole-3-acetic acid to macromolecules.

An investigation of the biochemical basis of auxin action in higher plants produced the interesting observation that an oxidation product of IAA, MeOx,[1] is an obligatory intermediate in several auxin-mediated responses including stimulation of elongation of pea and mung bean stem segments and of protein synthesis in pea stems (9). The biological properties of MeOx are probably a consequence of its high reactivity with sulfhydryl groups (5). Information concerning its behavior in bacterial model systems has provided important leads to its function in higher plants. As a sulfhydryl reagent, MeOx at low concentrations is capable of desensitizing regulatory enzymes to end-product inhibition both in the test tube and in growing cells; it thus accelerates growth in bacteria which are growth rate limited by excessive sensitivity to such regulation (7). As may be predicted by its affinity for sulfhydryl groups, higher concentrations of MeOx inhibit bacterial growth (2, 5). Stimulation of growth by low concentrations of MeOx and inhibition at higher concentrations is characteristic of the action of its parent compound, IAA, in plants.

It is therefore tempting to speculate that mechanisms similar to those operative in the bacterial model system underlie stimulation and inhibition of plant growth by MeOx. Thus, interactions between the —SH groups of plant informational macromolecules and MeOx were sought. Such interactions would result in the formation of covalently linked adducts of 3-methyleneoxindole and SH-containing macromolecules. The two classes of macromolecules that could be likely targets for MeOx are proteins and nucleic acids, the former due to the presence of SH-containing amino acids, and the latter due to the existence of thio bases in some species. The present communication describes the ability of MeOx to bind to proteins and the obligatory role played by it in the binding of IAA to this class of macromolecules.

[1] Abbreviations: MeOx: 3-methyleneoxide; WS: water-soluble, alcohol-insoluble fraction.

MATERIALS AND METHODS

Plant Material: Treatment and Fractionation. Seven-day-old pea (*Pisum sativum*, var. Alaska) seedlings grown in the dark at 26 C on moistened vermiculite were used throughout. About 100 g of seedlings were rinsed with distilled water, and their roots were immersed in 100 ml of an aqueous solution of 10 μM indole-3-acetic acid-methylene-^{14}C (12.5 μc) or 1 μM 3-methyleneoxindole-methylene-^{14}C (12.5 μc) contained in a 1-liter Pyrex beaker. The seedlings were incubated at 26 C at a distance of 2 feet from a bank of eight cool white fluorescent lamps 3 feet in length. After 16 hr of incubation the seedlings were rinsed for 1 hr with tap water and homogenized in a chilled blender with 100 ml of 20 mM tris-HCl buffer, pH 7.4, containing 0.20% sodium dodecyl sulfate. Subsequent manipulations were carried out at 4 C. The homogenate was filtered through eight layers of cheesecloth and centrifuged at 37,000*g*. The clarified extract was dialyzed overnight against 100 volumes of 20 mM tris-HCl buffer, pH 7.4. A small portion of the dialyzed extract was treated with three volumes of chilled anhydrous ethanol. The resultant precipitate was redissolved in 20 mM tris-HCl buffer, pH 7.4, to yield a final volume equal to the original extract. This is referred to as the "macromolecule fraction."

Exchange of ^{14}C-Label from the Ammonium Sulfate Precipitable Fraction by 2-Mercaptoethanol. The pea seedlings were exposed to ^{14}C-IAA or ^{14}C-MeOx and extracted in the manner described above. The supernatant resulting from centrifugation of the extract at 37,000*g* was made 1% with respect to streptomycin sulfate and centrifuged to remove the nucleic acids. The supernatant was brought to 80% of saturation with ammonium sulfate by addition of solid ammonium sulfate. The precipitated material was redissolved in and dialyzed against 20 mM tris-HCl buffer, pH 7.4, for 18 hr at 4 C. The dialysate was treated with an equal volume of 2 M 2-mercaptoethanol at room temperature for 16 hr for the release of bound ^{14}C-MeOx in the form of an adduct with 2-mercaptoethanol. This treatment was found to release more than 90% of ^{14}C-MeOx bound to macromolecules. The bulk of the macromolecules were removed by passing the reaction mixture through a Diaflo XM 50 filter and washing with 20 mM tris-HCl buffer, pH 7.4, until no further radioactivity could be detected in the effluent. The filtrate which contained the suspected ^{14}C-MeOx-mercaptoethanol adduct was concentrated to a small volume under

Table I. *Incorporation of 2-^{14}C-indole-3-acetic acid and Methylene-2-^{14}C-3-methyleneoxindole*

Preparation of the "macromolecule" fraction, concentrations of ^{14}C-IAA and ^{14}C-MeOx used and their application to pea seedlings are described in the text. Aliquots of 0.1 ml of the macromolecule and protein fractions were used for measuring the radioactivity. The alcohol-insoluble material was collected on 24-mm glass fiber filters and counted by the scintillation method after drying.

Additions	Radioactivity in Various Fractions		
	Nondialyzable macromolecules	Phenol-insoluble acid-insoluble proteins	Water-soluble alcohol-insoluble
	dpm/ml of extract		
^{14}C-IAA	7350	6100	650
^{14}C-IAA + MeOx	5000	3100	600
^{14}C-MeOx	5000	4000	530
^{14}C-MeOx + IAA	5100	4200	540

Table II. *Effects of Chlorogenic Acid on the Incorporation of 2-^{14}C-indole-3-acetic Acid and Methylene-2-^{14}C-3-methyleneoxindole*

Chlorogenic acid (0.80 mM) was added to the IAA or MeOx solutions fed to the seedlings. Other details are the same as in Table I.

Additions	Fractions		
	Non-dialyzable macro-molecules	Phenol-insoluble acid-insoluble protein	Water-soluble alcohol-insoluble (WS)
	dpm/ml of extract		
^{14}C-IAA	7350	6100	650
^{14}C-IAA + chlorogenic acid	3820	2860	460
^{14}C-MeOx	5000	4000	600
^{14}C-MeOx + chlorogenic acid	7300	5300	1200

Table III. *Release of Radioactivity from the Phenol-insoluble Acid-insoluble Protein Fraction by Limited Digestion with Trypsin*

The interphase protein fraction obtained after phenol treatment was extracted with ether, dried under nitrogen, and digested with 1 mg of trypsin as described by Ingram (3). Total volume of reaction mixture was 1 ml. After digestion for 90 min at 37 C, the reaction was stopped with 5% trichloroacetic acid. The radioactivity in acid precipitated materials was measured as described in Table I for alcohol-insoluble material.

Additions	Radioactivity in acid precipitate	
	−Trypsin	+Trypsin
	dpm/ml	
^{14}C-indole-3-acetic acid (IAA)	2450	1170
^{14}C-IAA + chlorogenic acid	1280	640
^{14}C-3-methyleneoxindole (MeOx)	2000	1090
^{14}C-MeOx + chlorogenic acid	2775	1530

vacuum and banded onto Whatman No. 3MM filter paper for descending chromatography with 5% isopropanol in water. A strip was removed from the developed chromatogram and cut into 1-cm segments for measurement of radioactivity by scintillation counting to locate the radioactive band.

Exchange of ^{14}C-Label from Alcohol Dehydrogenase-^{14}C-MeOx Complex. A reaction mixture consisting of 0.40 mg of alcohol dehydrogenase (Calbiochem), ^{14}C-MeOx (8 μM; 0.135 μc) and 20 mM tris buffer, pH 7.4, in a total volume of 1.0 ml was incubated for 2 hr at 37 C to allow the binding of MeOx to the −SH groups on the enzyme. The reaction mixture was cooled on ice, and the unreacted ^{14}C-MeOx was removed by passing through a Diaflo XM 50 filter and washing with 20 mM tris buffer, pH 7.4. The material retained by the filter was suspended in the same buffer and reacted with an equal volume of 2 M mercaptoethanol. Procedures used for obtaining the ^{14}C-mercaptoethanol adduct and its chromatography were similar to those described in the preceding section.

Exchange of ^{14}C-Label from WS of Phenol Extracts. The supernatant resulting from centrifugation of the extract at 37,000*g* was centrifuged for 90 min at 176,000*g*. Approximately 250 ml of this supernatant was treated with an equal volume of 90% phenol in a manner analogous to that used by Kirby (4) for the extraction and precipitation of RNA. The precipitate was dissolved in 50 mM acetate buffer, pH 5.1, and reacted with an equal volume of 2 M 2-mercaptoethanol for 16 hr at room temperature. After concentration of the reaction mixture to a small volume, it was chromatographed and the localization of the radioactive band was determined as in the case of the ammonium sulfate precipitate.

Preparation of ^{14}C-MeOx. ^{14}C-MeOx was prepared by riboflavin catalyzed photooxidation of indole-3-acetic acid-methylene-^{14}C, (specific radioactivity 14.2 mc/mmole) purchased from Schwarz Bioresearch. The acetone-toluene mixture in which the chemical was shipped was evaporated under a stream of nitrogen. The residue was dissolved in water to yield an 80 μM solution of ^{14}C-IAA containing 12.5 μc. The solution which

Table IV. *Chromatographic Mobility of the Exchange Products following Treatment with 2-Mercaptoethanol*

Methods for obtaining 2-mercaptoethanol adducts are described in the text. Reaction of IAA with 2-mercaptoethanol does not result in the formation of an adduct. Since an excess of 2-mercaptoethanol was used for obtaining the exchange products, the unreacted mercaptoethanol gave a discrete spot. Nonradioactive compounds were detected by their ultraviolet quenching or fluorescent properties. Radioactive compounds gave ultraviolet quenching spots with coincidental radioactivity.

Sample	Retardation Factor in Isopropanol: Water (5:95)
	R_F
2-Mercaptoethanol	0.852
Indole-3-acetic acid	0.910
3-Methyleneoxindole	0.548
3-Methyleneoxindole-mercaptoethanol adduct	0.758
^{14}C-3-Methyleneoxindole-mercaptoethanol adduct exchanged from ^{14}C-3-methyleneoxindole-alcohol dehydrogenase complex	0.763
^{14}C-3-Methyleneoxindole-mercaptoethanol adduct from protein fraction of seedlings exposed to ^{14}C-indole-3-acetic acid	0.765
^{14}C-3-Methyleneoxindole-mercaptoethanol adduct from protein fraction of seedlings exposed to ^{14}C-3-methyleneoxindole	0.760
^{14}C-3-Methyleneoxindole-mercaptoethanol adduct from WS fraction of seedlings exposed to ^{14}C-indole-3-acetic acid	0.758
^{14}C-3-Methyleneoxindole-mercaptoethanol adduct from WS fraction of seedlings exposed to ^{14}C-3-methyleneoxindole	0.765

also contained 1 μg/ml riboflavin was irradiated for 90 min as previously described (2). This procedure resulted in almost complete oxidation of IAA with 3-hydroxymethyloxindole as the major product. The resultant solution was diluted to 1 μM before being administered to the seedlings. 3-Hydroxymethyloxindole is readily dehydrated to MeOx under physiological conditions (5, 6, 8).

RESULTS AND DISCUSSION

Of the total radioactivity adsorbed by pea seedlings incubated with ^{14}C-IAA or ^{14}C-MeOx, 6.4% and 7.2%, respectively, are recovered in the "macromolecule fraction." Measurement of radioactivity in the "macromolecule fraction" following extraction with phenol indicates that about 90% of the radioactivity is associated with the protein fraction and 10% with the WS fraction (Table I). This pattern of distribution of the radioactivity between the protein and WS fraction is characteristic regardless of whether the label is administered in the form of IAA or MeOx. The similarity in the binding properties of IAA and MeOx to these fractions indicates that the binding of IAA to the macromolecules first involves its oxidation to MeOx, especially since such reactions are known to occur in intact pea seedlings (8). The predictions that arise from this hypothesis are: (*a*) MeOx should be an effective competitor of IAA for the binding sites on protein and WS fractions; and (*b*) blocking the enzymatic oxidation of IAA by chlorogenic acid should cause a reduction in the binding of ^{14}C-IAA to the macromolecules. On the other hand, chlorogenic acid should not affect the binding of MeOx, since it is a product of the blocked reaction. ^{12}C-MeOx is indeed an effective competitor of ^{14}C-IAA, at least for the binding sites on the protein molecules, whereas ^{12}C-IAA has no detectable effect on the binding capacity of ^{14}C-MeOx to the macromolecules (Table I). The presence of chlorogenic acid reduces the level of radioactivity bound to protein by about 50% when the label is applied as ^{14}C-IAA (Table II). The elevated binding of ^{14}C-MeOx to protein and WS fractions observed in the presence of chlorogenic acid is in accord with the earlier finding that chlorogenic acid is an inhibitor of 3-methyleneoxindole reductase (8). Such an inhibition would provide a higher steady state level of MeOx available for binding to these fractions.

Limited digestion of protein fractions with trypsin resulted in a substantial loss of radioactivity (Table III). This finding indicates that ^{14}C-IAA and ^{14}C-MeOx are indeed capable of binding to proteins. The small amount of radioactivity found in the WS fraction is not released by trypsin digestion, indicating that the radioactivity is directly associated with constituents of the WS fraction and does not in fact arise from protein contamination. This was further confirmed by the observation that the ^{14}C-labeled WS fraction failed to yield a positive biuret reaction.

The most direct evidence for the involvement of MeOx in the binding of IAA to macromolecules comes from the results of experiments presented in Table IV. When partially purified plant proteins or the WS fraction labeled with ^{14}C-IAA were reacted with a large excess of 2-mercaptoethanol for the purpose of exchanging the labeled moiety, it was possible to recover an ether-soluble, radioactive mercaptoethanol complex, which had the same chromatographic mobility in 5% isopropanol as the mercaptan formed by reacting MeOx with 2-mercaptoethanol. An addition product with similar chromatographic properties is obtained after exchanging with 2-mercaptoethanol the ^{14}C-MeOx bound to protein and WS fractions from pea seedlings or crystalline alcohol dehydrogenase.

In bacterial model systems, low concentrations of 3-methyleneoxindole are capable of desensitizing regulatory enzymes *in vivo* and *in vitro* (7). Thus, MeOx has the potential for accelerating growth when the sensitivity of a biosynthetic enzyme is made the growth rate-limiting factor. It has not yet been possible to demonstrate conclusively a similar role of MeOx in plants. The binding of MeOx and IAA via prior oxidation to MeOx with protein of plant origin are the minimal requirements for this proposal.

When pea seedlings are incubated with ^{14}C-IAA or ^{14}C-MeOx and extracted with phenol, the bulk of the radioactivity is found associated with proteins and a small portion of the label is bound to a WS fraction, which in either case can be recovered as a MeOx-mercaptoethanol complex. Similar reactions may account for the binding of IAA to plant polysaccharides (1). Our findings indicate that binding of IAA to proteins and the WS fraction first involves the oxidation of IAA to MeOx (Tables I and IV). The biochemical basis for the binding of MeOx to plant proteins is reasonably clear. It is a specific substrate for reductases of plants and must be assumed to bind these enzymes in addition to binding to other proteins via SH-containing amino acids. The consequences of such binding are speculative. The interaction with the reductases would terminate the hormone action of MeOx as the product, 3-methyloxindole, is inert; an encounter between MeOx and an SH-containing regulatory protein might render it insensitive to a negative regulator and thereby accelerate growth.

The incorporation of label into WS from ^{14}C-IAA has been attributed by Davies and Galston (1) to the binding of IAA to the polysaccharide component of the fraction; no radioactivity was detected by them in RNA which is a smaller component of this fraction. Our results show that the incorporation of IAA into WS fraction as well as into protein fractions proceeds via MeOx since the MeOx-mercaptoethanol adduct can be recovered from these fractions of plants exposed to IAA. However, the biochemical basis of the incorporation into the WS fraction is unclear. MeOx is not an effective competitor of ^{14}C-IAA for binding to the WS fraction, nor is there a significant decrease in the amount of label appearing in this fraction when the enzymatic oxidation of IAA is inhibited by chlorogenic acid.

Acknowledgments—The authors are very grateful to Dr. H. S. Moyed for his advice and encouragement. This investigation was supported by grants from the National Science Foundation (GB-15898) and the National Institutes of Health (GM-17521) awarded to H. S. M.

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