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Properties of a Solubilized Microsomal Auxin-binding Protein from Coleoptiles and Primary Leaves of *Zea mays*[1]

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ABSTRACT

An auxin-binding protein can be solubilized from microsomal membranes of *Zea mays* using either Triton X-100 extraction of the membranes or buffer extraction of the acetone-precipitated membranes. This paper describes the properties of the binding protein solubilized by these two methods. The binding is assayed by gel filtration chromatography in the presence of naphthalene [2-^{14}C]acetic acid. Binding is rapid and reversible with an optimum at pH 5. Both preparations show similar molecular weights by gel filtration (80,000 daltons) at pH 7.6 and 0.1 molar NaCl, and both aggregate at low ionic strength. They appear to be the same active molecular species. The binding activity is destroyed by trypsin, pronase or *para*-chloromercuribenzoic acid, but not significantly reduced by phospholipase C, DNase, RNase, or dithioerythritol. Since saturating amounts of naphthalene acetic acid protect the molecule from inhibition by *para*-chloromercuribenzoic acid, it is concluded that the binding protein has a sulfhydryl group at the binding site, or protects such a group in its binding conformation. The dissociation constant of the protein for naphthalene acetic acid is 4.6×10^{-8} molar with 30 picomoles of sites per gram of tissue fresh weight. Binding constants were estimated for 13 other natural and synthetic auxins by competition with naphthalene[2-^{14}C]acetic acid. Their dissociation constants are in general agreement with published values for their binding to intact membranes and their biological activity, although several exceptions were noted. A supernatant factor from the same tissue changes the apparent affinity of the protein for naphthalene acetic acid. This factor may be the same one as has been previously reported to alter the affinity of intact microsomes for auxin.

The molecular basis for the action of auxin is not yet well understood. We assume that the initial event in the process must involve the recognition of the hormone by cellular receptors specific for the active molecule. These receptors must then somehow signal the cell to begin the physiological response typical for auxin in that tissue. Therefore, it is important to our understanding of the mechanism of auxin action to identify and isolate such receptors from auxin-sensitive tissue.

Hormone receptors are typically identified on the basis of their ability to bind the hormone with an affinity and molecular specificity characteristic of the hormonal response. Natural and synthetic auxin have, in fact, been found to bind to sites in the microsomal membranes of *Zea mays* (2, 17–19). Furthermore, these auxins are bound with relative affinities roughly correlated to their ability to produce shoot elongation in the same tissue (2, 19). These sites are most concentrated in microsomes of coleoptiles and primary leaves (which respond to auxin by elongation), while they are fewer in microsomes from roots (which respond differently to auxins), and are nearly absent in tissue from the coleoptilar node (which has no known auxin growth response) (19). These findings suggest that the auxin-binding sites in these membranes represent auxin receptors involved in the action of this hormone.

Since the auxin-binding sites are associated with membranes, they must first be solubilized before they can be purified for study. The first solubilization of these sites was by Dohrmann and Ray (6, 7) who used the nonionic detergent Triton X-100, and later by Venis who used buffer extraction of acetone-precipitated microsomes (22). The purpose of this paper is to determine whether these two methods result in the solubilization of the same auxin-binding molecule, and to establish whether its properties are altered by the process of solubilization.

MATERIALS AND METHODS

Chemicals. The 1-naphthalene [1-^{14}C]acetic acid (98%) was obtained from Amersham/Searle. Unlabeled 1-naphthalene acetic acid, 2-naphthalene acetic acid, 1-naphthol, indole-3-propionic acid, indole-3-butyric acid, indole-3-ethanol, indole-2-carboxylic acid, indole-3-acetaldehyde, tryptophan, 5-hydroxyindole-3-acetic acid, 2,4-dichlorophenoxyacetic acid, benzoic acid, phenylmethylsulfonylfluoride, *p*-chloromercuribenzoic acid, trypsin, phospholipase C (*Clostridium welchii*, type I), DNase (bovine pancreas), 2-mercaptoethanol, Triton X-100, apoferritin, catalase, aldolase, serum albumin, and ovalbumin were from Sigma. Pancreatic RNase was a gift of W. F. Thompson. Pronase was from Calbiochem. Ultra Pure Enzyme Grade sucrose was obtained from Schwarz/Mann. Bio-Gel A-1.5m was from Bio-Rad Laboratories, while Sephadex G-25 and blue dextran 2000 were products of Pharmacia. Dinitramine (N^3,N^3-diethyl-2,4-dinitro-6-trifluoromethyl-1,3-benzenediamine), 99+% pure, was a gift of W. G. Woods, U.S. Borax Research Corp., Anaheim, Calif. Indole-3-acetic acid (Calbiochem) had a light pink hue, and so was recrystallized twice from hot chloroform to obtain a colorless product. The indole-3-propionic acid was recrystallized three times from hot chloroform-isopropyl alcohol (about 20/1, v/v). All other chemicals were used without further purification. Chemicals not specified were reagent grade. Since many reagents contain traces of insoluble material which may gradually block Sephadex columns (Ultra Pure sucrose, for instance), all buffers were filtered through a Reeve Angel binderless glass fiber filter.

Preparation of Solubilized Microsomal Extracts. Corn seedlings (*Zea mays*, Wf9 × Bear 38, Bear Hybrid Seed Co., Decatur, Ill.) were grown as previously described (18). Four-day-old shoots were cut at the coleoptilar node and placed on ice. All subsequent operations were conducted in a cold room at 5 C or on ice. Both the coleoptiles and the primary leaves were used as starting material, since both contain auxin-binding sites (19). Tissue was chopped briefly with electrically driven razor blades (18), and was then homogenized in an Ultra Turrax homogenizer for about 20 sec in short bursts. This method produces very thorough homog-

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enization. The procedure does not change the recovery of auxin-binding sites compared to the previous procedure employing a mortar and pestle (unpublished results). The homogenization buffer was 50 mM Tris · HCl, 1.0 mM Na_2EDTA, 0.10 mM $MgCl_2$, 14 mM 2-mercaptoethanol, 0.25 M sucrose (pH 8.0), normally saturated before use with phenylmethylsulfonylfluoride to retard proteolysis (9). The fluid was pressed through nylon cloth and microsomes were prepared by sequential sedimentation at 10,000*g* (10 min) and 48,000*g* (60 min) as previously described (5). The microsomal pellet was washed by resedimentation after resuspension in 10 mM Na citrate-citric acid, 0.5 mM $MgCl_2$, 0.25 M sucrose (pH 5.5) (5). To obtain a detergent-solubilized preparation, this fraction was mixed with the appropriate amount of a concentrated solution of Triton X-100 in resuspension buffer, and centrifuged at 145,000*g* (60 min). The supernatant fraction was removed carefully to avoid stirring the pellet.

The following modification of the procedure of Venis was adopted to solubilize the auxin-binding protein without detergent. The washed microsomal suspension was rapidly added to a centrifuge bottle containing 20 volumes of rapidly stirred acetone at −15 to −20 C. During the addition, the temperature of the acetone increased about 5 C. The precipitate was then pelleted at 5,000 rpm in the Sorvall GSA rotor. The supernatant was decanted, and the drained pellet was dried briefly under a stream of filtered air. To remove residual acetone, the bottle was then evacuated to less than 500 μm of Hg for 1 to 2 min. The pellet was homogenized with resuspension buffer minus sucrose at a ratio of 1 ml/5 g of fresh weight of original tissue, and after stirring for several hr, the suspension was centrifuged at 27,000*g* (20 min), and the supernatant carefully removed.

Binding of Auxin by Solubilized Extracts. Binding of [^{14}C]NAA[4] by solubilized extracts was measured by gel filtration chromatography of the extract on a column of Sephadex G-25 (4 × 0.55 cm), normally preequilibrated with 5×10^{-8} M [^{14}C]NAA as previously described (5, 7). Radioactive ligand was not normally added to the sample to be assayed, but (as indicated in the text) similar results were obtained when both sample and column were equilibrated with [^{14}C]NAA. The assay value was the difference in radioactivity between the void volume of the column containing the binding activity and the radioactivity contained in an equal volume of assay buffer (minus any necessary correction for the binding of auxin by detergent micelles) (5).

This assay for auxin binding depends on several variables. (*a*) The binding must occur quite rapidly, since no [^{14}C]NAA is added to the sample before assay and the excluded material must pick up ligand as it passes through the gel. (*b*) The binding must be rapidly reversible, since several of the experiments to be described involve treatment of the sample with ligands or inhibitors before assay on a column without these molecules present. (*c*) Finally, the entire peak of excluded binding material should be collected, but none of the following trough, which is due to the omission of [^{14}C]NAA from the sample, and the removal of the labeled ligand from the gel by the excluded material (7).

To test our knowledge of, and control over, these variables, several tests were run. Initially, assay columns of Sephadex G-25 were calibrated roughly for their void volumes by the addition of 0.250 ml of resuspension buffer containing blue dextran. Then complete elution profiles were run as described in Dohrmann and Ray (7), to determine the proper cut-off point to avoid overlap of the binding peak and trough (not shown). Simple tests of rapidity and reversibility of binding were then run in order to verify that the proper samples were being collected from the columns.

Table I shows the results of some of these tests. First, the addition of 2×10^{-7} M [^{14}C]NAA to the sample several min before chromatography does not significantly alter the measured binding. This indicates that the distance of passage of sample through the column is sufficient to attain equilibrium. Furthermore, since the amount of labeled ligand added to the sample was greater than the concentration in the column (5×10^{-8} M), it is clear that the excess of auxin does not appear in the fractions taken for the measurement of radioactivity. Finally, when a sample of buffer alone without either solubilized extract or [^{14}C]NAA is chromatographed, the sample representing the void volume does not show any reduction in its radioactivity compared to an equal volume of column buffer (Table I). This proves the lack of significant overlap of the void volume fraction and the fractions containing the following trough. These experiments together with the original column elution profiles verify that our assay constitutes a valid measure of the auxin-binding activity present in the solubilized material.

Table I. Rapidity and Reversibility of Solubilized Auxin-Binding Activity.
Solubilized auxin-binding activity was prepared by the method of acetone precipitation and buffer extraction and assayed for auxin-binding activity on a column of Sephadex G-25 pre-equilibrated with 5×10^{-8} M [^{14}C]NAA as described in the text. Samples of the solubilized preparation were mixed with an equal volume of buffer containing the indicated additions immediately before assay. Final concentrations of the additions are listed.

Experiment	Addition to sample	dpm bound (1)	dpm bound (2)	dpm bound average	Percentage of control
A 1.	None	1745	1684	1714	(100)
	2×10^{-7} M [^{14}C]NAA	1755	1484	1684	95
2.	None	1803	1860	1831	(100)
	2×10^{-7} M [^{14}C]NAA	1668	1771	1719	94

		Replicate	dpm in 0.500 ml sample of void volume
B 1.	Resuspension buffer alone chromatographed (without added [^{14}C]NAA)	1	3417
		2	3313
2.	Same volume of [^{14}C]NAA-column buffer	1	3353
		2	3421
		3	3433

Agarose Chromatography. The solubilized material was mixed with a solution containing blue dextran 2000 and 50% sucrose and was chromatographed on a column (0.9 × 50 cm) of Bio-Gel A-1.5m equilibrated with the indicated buffer. The column was eluted at 4 ml/hr^{-1} with a peristaltic pump, and fractions of about 1.5 ml were collected. Fractions were analyzed for auxin binding as described above, and for protein by a modification of the method of Lowry (12), or by measurement of the *A* at 280 nm. Sucrose was determined by refractometry. The column was calibrated with proteins of known wt: apoferritin horse spleen (475 kdaltons), catalase (bovine, 240 kdaltons), aldolase (rabbit, 145 kdaltons), serum albumin (bovine, 68 kdaltons), ovalbumin (chicken, 43 kdaltons). Proteins were labeled with fluorescamine (3) before chromatography and relative fluorescence was estimated with a Perkin-Elmer MPF 3 L spectrofluorimeter (excitation 390 nm, emission 475 nm, 10 nm slits). This prelabeling does not affect the mol wt of treated proteins appreciably (16), nor does it affect the elution profile of serum albumin under our conditions (not shown).

Preparation of Supernatant Factor. A crude preparation of supernatant factor was prepared from the microsomal (58,000*g*, 60 min) supernatant by adjusting the solution to pH 4.5 with HCl and bringing the resulting suspension to a boil. The heavy precipitate was filtered off with Whatman No. 4 filter paper, and the resulting brown solution was brought to pH 5.5 with NaOH and chilled. Before use it was filtered again through a Reeve Angel glass fiber filter.

RESULTS

Comparison of Methods of Solubilization. We have previously reported that solubilized binding activity prepared with Triton X-

[4] Abbreviations: PMSF: phenylmethylsulfonylfluoride; NAA: naphthalene acetic acid; DTE: dithioerythritol; pCMB: *para*-chloromercuribenzoate.

100 had an approximate mol wt of 90,000 daltons when chromatographed on a column of Bio-Gel A-1.5m equilibrated with 10 mM Na citrate-citric acid, 5 mM $MgCl_2$, 0.25 M sucrose, 0.05% Triton X-100 (w/v), (pH 5.5) (5). Venis, however, reported a mol wt of about 50,000 for his predominant species on Sephadex G-100 (21). We have attributed this discrepancy (5) to the protection against proteolysis afforded by our inclusion of the phenylmethylsulfonyl fluoride in the homogenization buffer. This compound is a potent inhibitor of proteases found in homogenates of grass seedlings (9).

To compare the two preparations we have solubilized auxin-binding material by both methods in parallel, starting from the same PMSF-treated homogenate. The results (Fig. 1) show only a single chromatographic peak from either solubilized extract. Both activities chromatograph with the same relative volume of buffer. Furthermore, calibration of the columns with proteins of known mol wt indicates that the mol wt of the binding substance is in either case 80,000 daltons (Fig. 2). These chromatograms were run under conditions of moderate ionic strength and neutral pH. However, lowering the ionic strength (pH 7.6) results in aggregation of the binding molecule to an apparent mol wt of about 200,000 (Fig. 3). Similar results were obtained with the detergent-solubilized preparation (not shown). The chromatographic similarity of the two preparations under our conditions implies that they contain the same active molecule. Since the acetone-buffer extract can be assayed with far less difficulty, it was chosen for further study.

pH Optimum. Auxin binding was measured in the buffer-extracted acetone precipitate prepared as described under "Materials and Methods." The optimum activity (Fig. 4) was obtained with Na-citrate buffer at pH 5 with either 5×10^{-8} M or 2×10^{-7} M [^{14}C]-NAA. Most measurements reported here were made at pH 5.5 and 5×10^{-8} M [^{14}C]NAA since these conditions have been used in most reported assays for auxin binding to membranes (2, 18) and solubilized membrane proteins (6, 22).

Binding Affinity for 1-Naphthylene Acetic Acid. Using measurements on intact membranes, Ray *et al.* (18, 19) have reported finding only a single class of high affinity binding sites for NAA ($K_d = 8 \times 10^{-7}$ M). However, Batt *et al.* (1, 2) have reported finding two classes of high affinity sites in both microsomes and in solubilized material from microsomes ($K_d = 1.4 \times 10^{-7}$ M and 1.7×10^{-6} M). We have, therefore, examined the binding kinetics of our acetone-buffer-solubilized preparation, paying particular attention to the range of concentrations in which we would expect to see evidence for low affinity sites. Figure 5 shows that in a double reciprocal plot only a single class of NAA-binding sites are detected. The plot is a single straight line with no break at higher concentrations of NAA. Such a break would be expected if there were low affinity sites. The affinity of the sites detected is much higher ($K_d = 4.6 \times 10^{-8}$ M) than has been previously reported by other workers. We observe 32 pmol of sites/g of original tissue fresh weight in this experiment, and 25 to 40 pmol in other experiments not shown. This compares with 40 to 50 pmol/g observed by Ray *et al.* (18) and by Batt *et al.* (2) for high affinity sites in intact membranes. These differences in the number of sites reported and their affinities for NAA may be because of the different genetic material used by Batt *et al.* (*Zea mays*, var. Kelvedon 33 [2, 22]) proteolysis in their material and that of Ray *et al.* (18) or perhaps because of modification of the receptor sites by the PMSF in our preparation. Loss of sites in our preparation might also be due to denaturation caused by acetone. One test for degradation or modification is to measure the affinity of this material for IAA and for synthetic auxin analogs besides NAA.

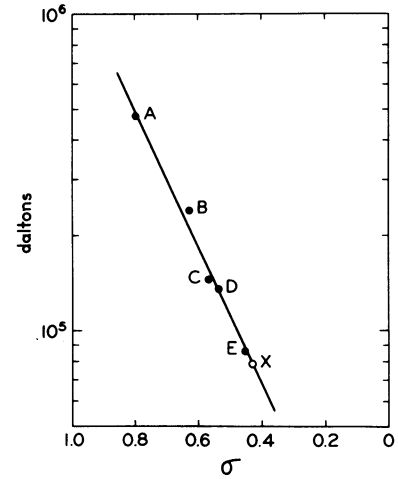

FIG. 2. Mol wt of solubilized auxin-binding activity. Mol wt of standard proteins are plotted as logarithms *versus* value of $\sigma = (V_s - V_i)/(V_e - V_i)$. A: apoferritin, 475 kdaltons; B: catalase, 240 kdaltons; C: aldolase, 145 kdaltons; D: serum albumin dimer, 136 kdaltons; E: chicken ovalbumin dimer, 86 kdaltons; X: auxin-binding protein, prepared using PMSF and solubilized by acetone precipitation and buffer extraction. Chromatography as in Figure 1.

Affinity of Binding for Natural Auxin and for Synthetic Auxin Analogs. Thirteen substances have been tested for interaction with the acetone-buffer-solubilized binding material by competition with [^{14}C]NAA (5×10^{-8} M). The substances were tested by 3-fold increments over a range of concentrations from 10^{-8} to 10^{-3} M,

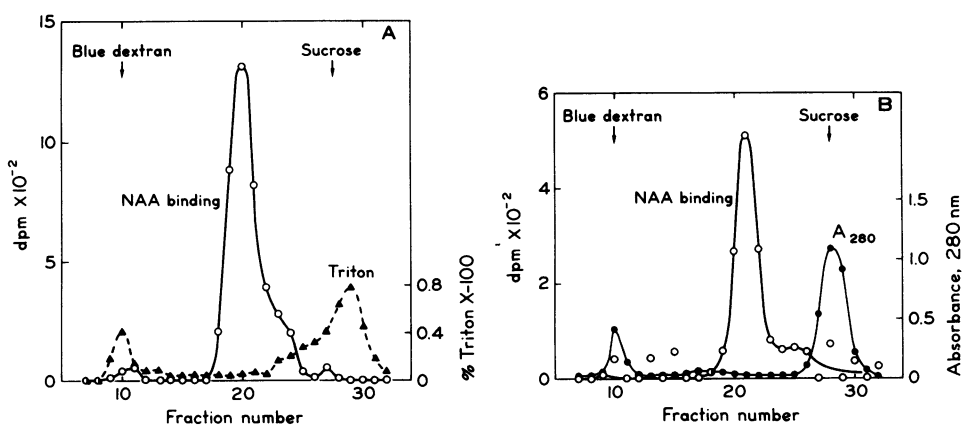

FIG. 1. Chromatography on Bio-Gel A-1.5m of solubilized auxin-binding activity. Auxin-binding activity was solubilized from corn microsomes either by extraction with Triton X-100 (0.5%, w/v, or 0.5 mg/g fresh wt) (A) or by acetone precipitation of the membranes followed by buffer extraction of the dried precipitate (B) as described under "Materials and Methods." Extracts were prepared by homogenization with buffer saturated with PMSF. Chromatography was in 0.100 M NaCl, 0.010 M Tris·HCl (pH 7.6).

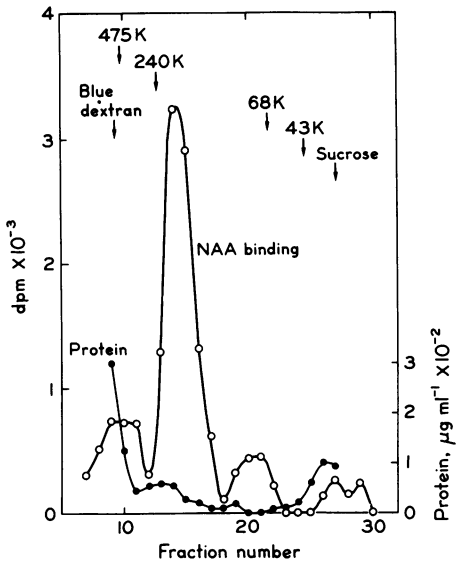

FIG. 3. Chromatography on Bio-Gel A-1.5m of solubilized auxin-binding activity at low ionic strength. Auxin-binding protein was prepared using PMSF and solubilized by acetone precipitation and buffer extraction as described under "Materials and Methods." Chromatography was performed using a column preequilibrated with 10 mM Tris·HCl (pH 7.6) and eluted with the same. Similar results were obtained using detergent-solubilized material.

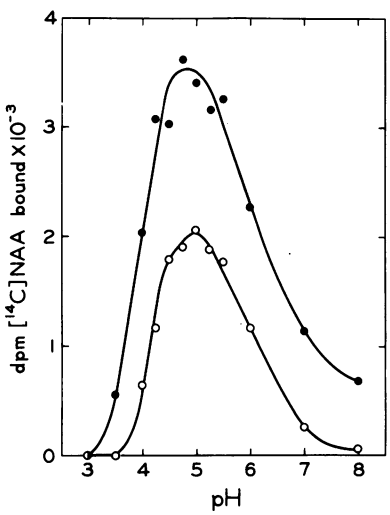

FIG. 4. pH optimum of auxin binding. Auxin-binding activity was solubilized by acetone precipitation of microsomes and buffer extraction of precipitate as described under "Materials and Methods." Binding was assayed on columns of Sephadex G-25 preequilibrated with either 200 nM (●) or 50 nM (○) [¹⁴C]NAA in Na citrate-citric acid (10 mM in citrate) + 5 mM $MgCl_2$, adjusted to the indicated pH.

except for indole-3-ethanol, 1-naphthol, tryptophan, and dinitramine, which were tested by 10-fold increments. The results, expressed as a percentage of the binding in the presence of uncompeted [¹⁴C]NAA at 5×10^{-8} M, are shown in Figure 6. Each binding curve was replicated twice, and the duplicate points are averaged for the figure (except in the case of 5-OH-indole acetic acid, where the replicates were not as close). The concentration of each competing ligand which could displace 50% of the [¹⁴C]NAA from the measured binding was used to calculate the apparent binding constant for that substance. These values are listed in Table II. Since the concentration of [¹⁴C]NAA used to measure binding competition was itself similar to the K_d of the binding material for NAA, a correction (4) was used to calculate the apparent binding constants (Table II). The results indicate that with the exception of NAA itself our solubilized material has affinities for various auxins similar to those reported in the literature (1, 2, 6, 19). Moreover, the dissociation constants are roughly similar to the concentrations giving 50% biological activity, as determined for stimulation of coleoptile elongation in the same variety of corn by Ray (19).

Chemical Nature of Binding Substance. Gel filtration chromatography established that the binding substance has a minimum mol wt of 80,000 daltons under our conditions. However, the

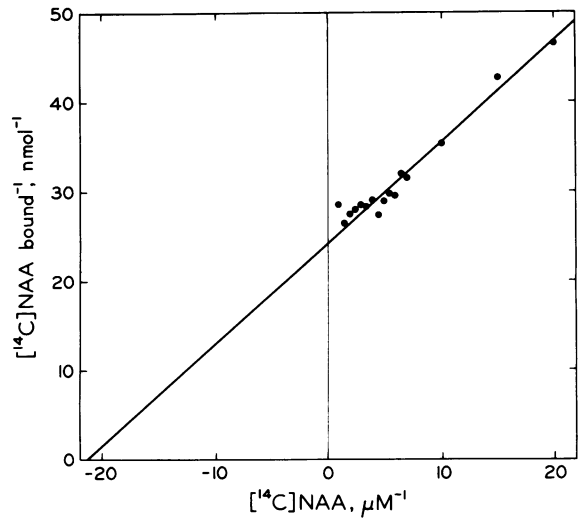

FIG. 5. Binding affinity of solubilized receptors for NAA. Binding by acetone-buffer-solubilized material was assayed as described under "Materials and Methods" using columns of Sephadex G-25 preequilibrated with the indicated concentration of NAA. Linear regression analysis of the double reciprocal plot reveals a single class of binding sites, $K_d = 4.6 \times 10^{-8}$ M, 32 pmol sites/g fresh wt.

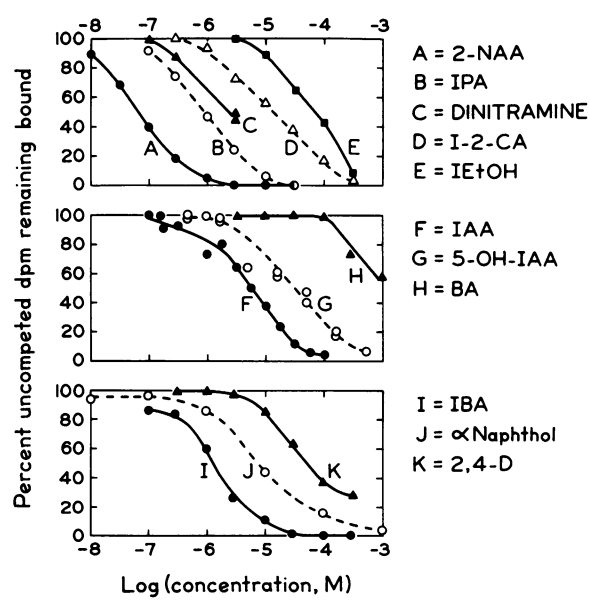

FIG. 6. Competition by auxin analogs and natural auxin for NAA-binding sites. Binding by acetone-buffer-solubilized material was measured as described under "Materials and Methods" using columns of Sephadex G-25 preequilibrated with 50 nM [¹⁴C]NAA plus the indicated concentration of auxin analog. A: 2-naphthalene acetic acid; B: indole-3-propionic acid; C: dinitramine, D: indole-2-carboxylic acid; E: indole-3-ethanol; F: IAA; G: 5-hydroxy-indole-3-acetic acid; H: benzoic acid; I: indole-3-butyric acid; J: α-naphthol; K: 2,4-dichlorophenoxyacetic acid.

results of Dohrmann and Ray (7, 18) indicated that binding is not only sensitive to destruction by protease, but also by phospholipase action. Moreover, these enzymes might be contaminated by nuclease activity. Therefore, a more complete study of the inactivation of binding by enzymes and inhibitors was undertaken. The results (Table III) demonstrate that the soluble binding activity is inactivated to a significant degree only by heating, protease, and by the sulfhydryl-specific organomercurial, pCMB. The table shows that trypsin produced a 71% reduction in binding after 1 hr at 25 C, but more lengthy incubations (not shown) resulted in complete inactivation. These data indicate that the binding is due to a sulfhydryl-dependent protein. Contrary to the results of Dohrmann and Ray (7, 18), there is no significant reduction in activity when phospholipase C is applied to the preparation. However, since Dohrmann and Ray did not include PMSF in their buffer during the initial extraction of the plant material, it is

Since Dohrmann and Ray (7) have reported that the binding activity is inhibited by DTE and restored by oxidation, it is surprising that we do not find any inhibition by DTE (Table III).

Table II. Binding Constants of Auxin Analogues.
Dissociation constants (K_d) of auxin analogues were measured by competition with [^{14}C]NAA as described in the legend to Fig. 6 using an acetone-buffer-solubilized preparation. Data are presented as negative logarithms.

Auxin analogue	pK_d A	B	C	D	pC_{50} E
naphthyl-1-acetic acid	7.3	---	6.1	6.8/5.8	5.3
naphthyl-2-acetic acid	7.1	6.8	5.9	---	5.6
1-naphthol	5.0	4.7	4.4	---	---
indole-3-acetic acid	5.2	4.9	5.4	5.1/5.6	5.4
indole-3-propionic acid	6.0	5.7	5.2	---	5.7
indole-3-butyric acid	5.8	5.5	5.0	---	4.7
indole-3-ethanol	4.8	4.5	---	---	---
indole-2-carboxylic acid	4.8	4.5	3.5	---	---
indole-3-acetaldehyde	NC*	NC	---	---	---
tryptophan	NC	NC	---	---	---
5-hydroxyindole-3-acetic acid	4.4	4.1	---	---	---
2,4-dichlorophenoxyacetic acid	4.3	4.0	4.0	5.3/4.9	5.2
dinitramine	5	4.7	---	---	---
benzoic acid	< 3	< 2.7	2.1	---	< 3

A: apparent K_d from Fig. 6.
B: data from column A, corrected for the concentration of ^{14}C NAA used in measuring binding. The corrected K_d = K_d (apparent) x (1 + [NAA]/K_d(NAA)) where [NAA] = 5 x 10^{-8} M and K_d(NAA) = 4.6 x 10^{-8}.
C: binding data from Ray (19).
D: binding data from Batt, Wilkins and Venis (2).
E: pC_{50} values from Ray (19).

*No competition

Table III. Effects of Enzymes and Inhibitors on Binding of NAA by Microsomes and Solubilized Binding Activity.
Data on NAA binding by intact microsomes are taken from the work of Dohrmann and Ray (7). Solubilized material prepared by the acetone precipitation method was mixed with an equal volume of 50 mM HEPES/NaOH, pH 7.5 containing the indicated enzyme or inhibitor. Incubations were terminated by chilling the sample in an ice bath and duplicate samples were immediately assayed on a column of Sephadex G-25 pre-equilibrated with 1 x 10^{-7} M [^{14}C]NAA. Dithioerythritol (DTE) was 3 mM; pCMB, 50 µM; Pronase (predigested 15 min at 25 C), 18.5 mg·ml^{-1}; trypsin, 1.0 mg·ml^{-1}; phospholipase C (reaction mix included 1 mM $CaCl_2$), 1.5 mg·ml^{-1}; DNase, 0.5 mg·ml^{-1}; RNase, 0.1 mg·ml^{-1}. Values of percentage of inhibition are calculated relative to the control at 0 C, which bound 5350 dpm/0.25 ml sample (or 67 pmoles·mg protein^{-1}).

	Treatment	Percentage of Inhibition: Intact microsomes	Solubilized fraction
A.	0 C, control	(0)	(0)
	+ DTE (120 min)	84	5
B.	50 C (20 min)	--	5
	50 C (60 min)	96	36
C.	25 C (60 min)	17	0
	+ trypsin	--	71
	+ Pronase	55	60
	+ phospholipase C	61	0
	+ DNase	--	3
	+ RNase	--	3
	+ pCMB	--	90

Table IV. NAA-Binding Site Sulfhydryl Requirement. Acetone-buffer-solubilized protein was treated with the indicated substances in resuspension buffer (without sucrose) in a final volume of 1.0 ml. pCMB was 50 µM; unlabeled NAA was 10 µM; 2-mercaptoethanol was 14 mM.

	Treatment	dpm [^{14}C]NAA bound	% inhibition
A.	0°, control	1762	0
	pCMB	1461	17
	pCMB + NAA	1773	0
B.	25°, 5 min control	1802	0
	pCMB	440	75
	pCMB + NAA	1148	35
C.	25°, 15 min control	1809	0
	pCMB	132	92
	pCMB + NAA	787	45
	pCMB + 2-mercaptoethanol	1169	34

Table V. Inhibition of Binding by a Supernatant Factor.
A high speed supernatant was prepared as described in the Methods, and it was added in the indicated concentration as a percentage of the total volume of binding buffer. Thus 100% supernatant factor would be the supernatant from one gram f. wt. tissue per two ml of buffer. All assays were conducted at pH 5.5 with 2 x 10^{-7} M [^{14}C]NAA, using the acetone-buffer-solubilized binding protein.

	Percentage of supernatant	Net dpm bound	Percentage of control
1.	0	4033	100
2.	1	3193	79
3.	10	1522	38
4.	40	264	6
5.	60	0	0

Moreover, pCMB strongly inhibits the binding. pCMB forms covalent bonds with sulfhydryl groups and not to disulfides (8). However, it is possible that the results of Ray (7, 18) are affected by the lipid, or micellar environment of the binding protein under their conditions. If this were true, then it might imply that the sulfhydryl requirement in our preparation is not involved in the over-all conformation of the protein, but simply at the binding site. Therefore, we have tested the active site sulfhydryl dependence of binding by the standard means of supplying the inhibitor (pCMB) in the presence and absence of ligand (NAA). The results (Table IV) demonstrate a substantial protection of the binding activity when the binding protein is incubated in the presence of ligand. Therefore, the binding protein has a sulfhydryl group at the binding site, or protects such a group when it is in its binding conformation. Venis has obtained similar results with the intact microsomes of corn (22).

Supernatant Factor Inhibition of Binding. Ray *et al.* (18, 19) have shown that high speed supernatants from corn contain a heat-stable, dialyzable factor which inhibits the binding of [^{14}C]-NAA to microsomal membranes. Our experiments (Table V) show that the binding of [^{14}C]NAA by solubilized receptors is also inhibited by such a boiled, filtered supernatant preparation. In contrast to the results obtained earlier, however, we find that binding can be completely abolished by the addition of sufficient supernatant (Table V). Our supernatant was prepared from a PMSF-treated homogenate for this experiment, but similar supernatants from untreated homogenates inhibit the binding to the same degree (not shown).

DISCUSSION

Since several different preparations of auxin-binding substances from corn (1, 2, 5–8, 18, 19, 21) and from other plants (10, 11, 15) have been reported in the literature, it has become desirable to determine their relationship to each other. Most of these binding activities have been derived from microsomal fractions, although auxin-binding activities have also been isolated from the cytosol (10, 15). In this paper, we have established that when proper protection from proteolysis is used, only one chromatographically distinct auxin-binding "site" of high affinity can be isolated from

corn microsomes. The affinity of this protein is similar to the high affinity site reported by Venis (21) in his solubilized preparation. The mol wt of the protein is 80,000, the same as that reported by us previously for the same activity solubilized by Triton X-100, and both preparations become similarly aggregated at low ionic strength. We believe that this auxin-binding protein is probably involved in the action of auxin. First, it shows binding affinities for various synthetic auxins which roughly parallel their activities for coleoptile lengthening *in vivo*. Second, studies of the unsolubilized material in different plant organs indicate a good correlation between presence of the binding sites and sensitivity of the given organ to stimulation of lengthening by auxin (19). Moreover, a soluble heat-stable factor appears to regulate the specific affinity of the binding sites for auxins both in the intact microsomes, and in the solubilized protein. The nature of this soluble factor will be the subject of another aper.

The data of Dohrmann and Ray (7) indicated that the detergent-solubilized binding activity was only partly sensitive to pronase, but was completely inactivated by pronase preceded by incubation with phospholipase C. However, the activity of the acetone-extracted preparation is fully sensitve to trypsin without preincubation with phospholipase C. The preparation is less sensitive to pronase than to trypsin. Taken together these results indicate that removal of the phospholipid from the protein probably exposes new sites for proteolytic cleavage.

Our results shown in Tables III and IV demonstrate that the binding protein contains a free sulfhydryl group which is required for binding. This result substantiates the results of Venis (21), but is surprising in view of the contrary finding of Ray *et al.* (7, 18) that NAA binding is inhibited by DTE, and that this inhibition is reversed by the addition of oxidation agents. However, in a crude microsomal preparation such as that of Ray *et al.*, there may be secondary effects of the application of exogenous agents (*i.e.* reducing agents), such as the activation of proteolytic enzymes or allosteric effects. Moreover, it has been shown that small amounts of DTE or of the related compound DTT catalyze the formation of H_2O_2 under suitable conditions (13, 20). It has been shown that H_2O_2 is a potent inhibitor of auxin action and that its effect can persist many hr *in vivo* (14). Therefore, it is premature to think that this difference between our results has basic significance.

We have demonstrated previously that the auxin-binding protein of corn coleoptiles and primary leaves is not an ATPase (5). We do not yet know how the binding of auxin to the receptor causes the cell elongation of the shoot. Our preliminary studies indicate that the binding protein is present not only in the ER, as reported by Ray (17), but also to a lesser extent in certain membranes of greater density such as the plasmalemma. Further work will be needed to confirm this result and to determine the interaction of this receptor protein with other enzyme systems involved in the auxin response.

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