Proc. Nat. Acad. Sci. USA
Vol. 69, No. 11, pp. 3146–3150, November 1972

Enhancement of RNA Polymerase Activity by a Factor Released by Auxin from Plasma Membrane*

(soybean/transcription factor/chromatin)

JAMES W. HARDIN†, JOE H. CHERRY†, D. JAMES MORRÉ‡, AND CAROLE A. LEMBI‡

† Department of Horticulture and ‡ Department of Botany and Plant Pathology, Purdue University, Lafayette, Indiana 47907

Communicated by Kenneth V. Thimann, August 7, 1972

ABSTRACT **Using recently developed techniques for solubilization of RNA polymerase from soybean chromatin and isolation of plasma membrane fractions from plants we can show the presence of a transcriptional factor specifically released from the membranes by auxin, 2,4-dichlorophenoxyacetic acid. The nonauxin, 3,5-dichlorophenoxyacetic acid, does not release the factor, but subsequent exposure of the membranes to auxin results in its release. Factor activity could not be demonstrated in fractions devoid of plasma membranes. The presence of a regulatory factor for RNA polymerase associated with plant plasma membrane and specifically released by auxin provides a mechanism whereby both rapid growth responses and delayed nuclear changes could be derived from a common auxin receptor site associated with plasma membrane.**

Stimulation of cell enlargement by auxin in soybean hypocotyls is accompanied by increases in chromatin-bound RNA polymerase activity (1). We previously isolated a factor that stimulates chromatin-bound RNA polymerase from control tissue but not from auxin-treated tissue (2). Such a factor might interact with auxin, with subsequent transfer of either an auxin–factor complex or a modified factor across the nuclear membrane. Transcription factors have also been described by Matthysse and Phillips (3) and Venis (4).

The plasma membrane is a primary site of hormone activity (5–9). We reasoned that these lines of evidence were compatible with interaction of auxin with specific receptor sites on the plasma membrane to release or modify a factor that controls transcription. In this study, plasma membrane fractions isolated from soybean hypocotyls were treated with physiological amounts of auxin (0.1 μM) to release a factor that enhances the activity of purified soybean hypocotyl RNA polymerase. The stimulatory activity is not released by the nonauxin, 3,5-dichlorophenoxyacetic acid, but subsequent treatment of the same fraction with an auxin, indoleacetic acid or 2,4-dichlorophenoxyacetic acid, mediates the release. The findings provide an interesting new possibility for regulation of nucleic acid synthesis by auxin.

MATERIALS AND METHODS

Plant Material. Soybean seeds (*Glycine max* L. var. Wayne) were soaked in water overnight and sown in moist Vermiculite. Seeds were germinated in darkness at 29° in a constant humidity chamber. After 4 days of germination hypocotyls were excised and used for either chromatin or plasma membrane isolations.

Chromatin Isolation. Chromatin was isolated by a procedure modified from that of Huang and Bonner (10). Details of the isolation, assay, activity, and storage in liquid nitrogen of chromatin are published elsewhere (1, 2, 11).

RNA Polymerase Solubilization and Assay of Activity. RNA polymerase was solubilized from isolated chromatin preparations and assayed as described (11) by the Whatman DE81 disc technique of Blatti *et al.* (12). α-Amanitin was added immediately before addition of the enzyme.

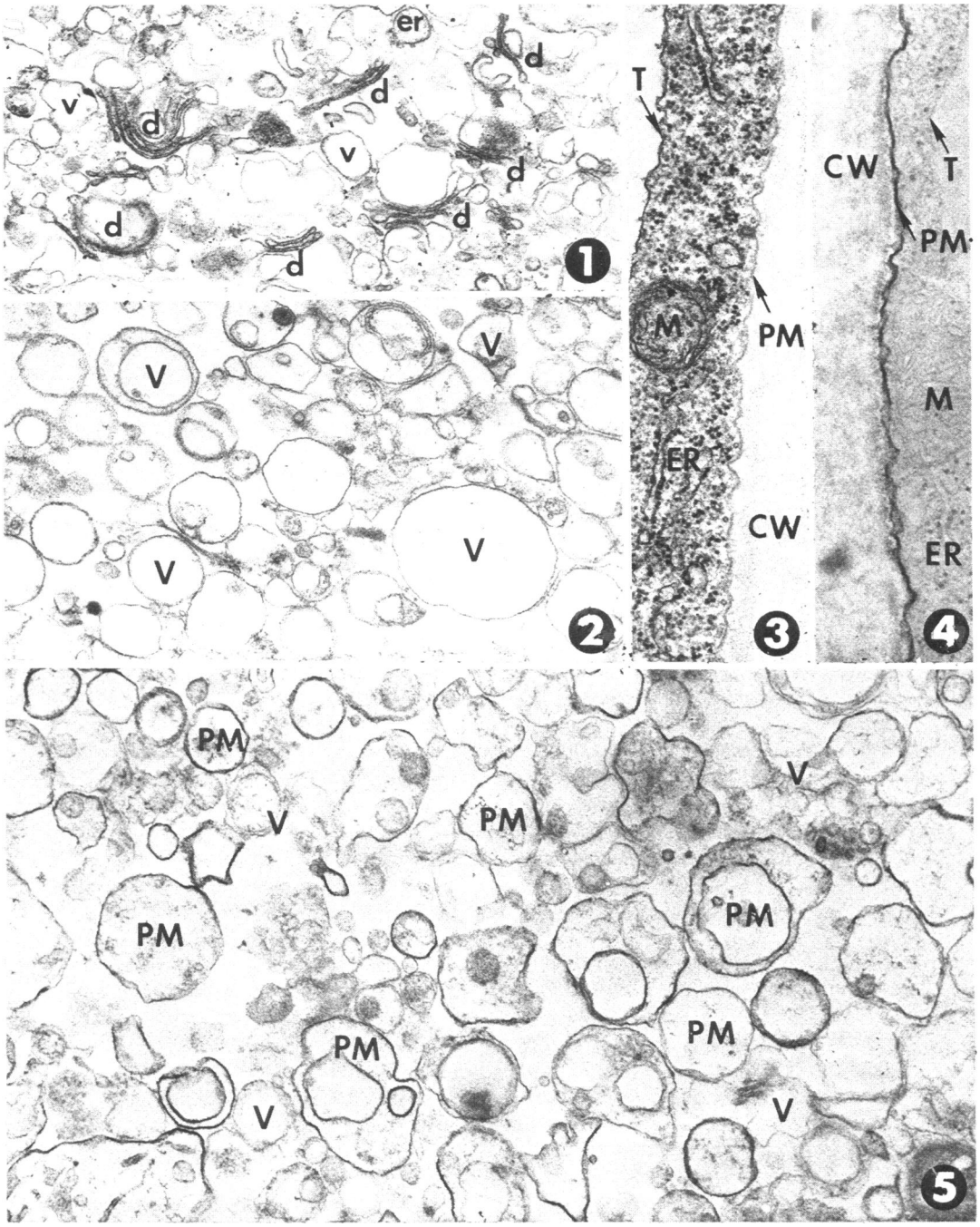
Membrane Isolations. Plasma membrane and other cell fractions were isolated by the procedure of Lembi *et al.* (8) modified as follows. About 25 g of hypocotyl tissue were homogenized in 40 ml of a freshly prepared medium consisting of 0.1 M K_2HPO_4, 1.0 mM dithiothreitol, and 20 mM EDTA in centrifuged (100,000 × *g*, 90 min) coconut milk (pH 6.5) (coconut milk medium) and containing 0.5 M sucrose. Homogenates were prepared with a Polytron 20ST (Kinematica, Lucerne, Switzerland) homogenizer (13) and centrifuged in 50-ml tubes for 12 min at 20,000 × *g* (Sorvall RC2-B, HB-4 rotor) to remove cell walls, nuclei, plastids, mitochondria, microbodies and large membrane fragments. The supernatant containing microsomes and most of the cytoplasmic membranes (dictyosomes, plasma membrane fragments, etc.) was then layered onto a step gradient containing equal volumes of 0.65, 0.8, 1.0, 1.2, and 1.3 M sucrose in coconut milk medium having sucrose equivalent densities of 0.8, 1.0, 1.2, 1.4, and 1.5 M, respectively. The gradient was centrifuged for 90 min at 100,000 × *g*. Membrane fractions were recovered from the various interfaces, resuspended in homogenization medium, and centrifuged for 45 min at 100,000 × *g*. Membrane fractions were designated as follows: *A* = medium/0.8 M sucrose equivalent interface; *B* = 0.8/1.0 M sucrose equivalent interface; *C* = 1.0/1.2 M sucrose equivalent interface; and *D* = 1.2/1.4 M sucrose equivalent interface.

Electron Microscopy. Fractions were prepared for electron microscopy as described (14) except that fixation was for 1–2 hr in glutaraldehyde, rather than overnight, to enhance specificity of staining (14–16) for plasma membrane.

Membrane Characterization. Distributions of mitochondria, plasma membranes, and dictyosomes on the sucrose–coconut milk gradients were estimated by assay of succinate:2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride oxidoreductase (EC 1.3.99.1) (18), glucan synthetase (EC 2.4.1.11) (9), and latent IDPase (EC 3.6.1.6) (19, §). Sterols were determined by the colorimetric procedure of Jorgensen and Dam (20). Lipids were extracted by the procedure of Folch *et al.* (21) and determined gravimetrically.

* Journal paper no. 4799 of the Purdue Agricultural Experiment Station.

Membrane Incubations. Membrane fractions were suspended in a Dounce homogenizer in TGMED buffer that contained either 0.1 μM 2,4-dichlorophenoxyacetic acid, 0.1 μM indoleacetic acid, or 0.1 μM 3,5-dichlorophenoxyacetic acid.



FIGS. 1–5. (*1*) Portion of fraction *A* from the sucrose–coconut milk gradient. This preparation was section-stained with lead citrate (17) and shows numerous dictyosomes (*d*), occasional fragments of endoplasmic reticulum (*er*), and unidentified vesicles (*v*) some of which may respresent swollen Golgi apparatus cisternae. ×35,000.

(*2*) Portion of fraction *C* from the sucrose-coconut milk gradient that was section-stained with lead citrate. Numerous vesicles (*V*) characterize the fraction. ×35,000.

(*3*) Electron micrograph of a portion of a soybean hypocotyl cell stained with lead citrate (17). All endomembranes and organelles are intensely stained, and the cell wall (*CW*) is lightly stained. The vacuole membrane or tonoplast (*T*) is more heavily stained than the plasma membrane (*PM*). *M* = mitochondrion; *ER* = endoplasmic reticulum. Glutaraldehyde–osmium tetroxide fixation. ×35,000.

(*4*) As in Fig. 3 except treated with the phosphotungstic acid–chromic acid procedure (14). Except for occasional regions of the cell wall (*CW*) and portions of dictyosomes (not illustrated), the plasma membrane (*PM*) is the only cell component that is darkly stained. Tonoplast (*T*), mitochondria (*M*), endoplasmic reticulum (*ER*), proplastids, microbodies, etc. are unstained. ×35,000.

(*5*) Portion of fraction C from the sucrose–coconut milk gradient that was treated by phosphotungstic acid–chromic acid procedure. Plasma membrane (*PM*) is easily detected as vesicles with intensely-staining membranes. Other vesicles (*V*) and occasional mitochondria are only faintly stained. ×35,000.

TABLE 1. *Distribution of "marker" enzymatic activities and cell constituents among fractions from sucrose–coconut milk gradients*

Fraction	Specific activity*			Amount†		
	Succinate:INT oxidoreductase†	Glucan synthetase		Latent IDPase	Phytosterols (mg/100 mg lipid)	Total lipid (% of lipid + protein)
		1.5 μM UDPG	1 mM UDPG			
A	0.05 ± 0.005	0.100	99	7.5	3.8 ± 1.0	47 ± 4
B	0.01 ± 0.02	0.072	189	5.0	8.5 ± 3.2	45 ± 5
C	0.38 ± 0.04	0.031	234	1.0	11.3 ± 0.7	44 ± 3
D	0.76 ± 0.03	0.013	136	0	4.1 ± 1.0	34 ± 4
D_m‡	4.34	—	—	—	—	—

* Units of specific activity are μmol 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazoliun chloride (INT) reduced/hr per mg of protein for succinic-INT reductase, nmol [^{14}C]glucose incorporated into alkali-insoluble glucan from [^{14}C]UDP-glucose per hr, per mg of protein for glucan synthetase, and μmol inorganic phosphate released/hr per mg of protein for latent IDPase.

† Averages of four different preparations ± SD.

‡ A fraction enriched in mitochondria obtained by differential centrifugation from fraction *D* of the sucrose–coconut milk gradient.

Suspensions were incubated at 37° for 15 min and then centrifuged at 100,000 × *g* for 1 hr (Spinco 50 rotor with 2-ml adaptors). The supernatants were examined directly for stimulatory activity in the RNA polymerase assay (2–10 μg protein per assay). The pellets were either discarded or suspended in appropriate buffers and again subjected to the above procedure.

Alternatively, the membrane fractions (10–25 μg of protein per assay) were suspended in appropriate buffers, incubated at 37° for 15 min, and assayed directly. Membrane suspensions were monitored for endogenous RNA polymerase activity.

Protein was determined by the method of Lowry *et al.* (22). All chemicals were of reagent grade. [^{3}H]UTP was obtained from Schwarz/Mann, Orangeburg, New York; high purity 2,4-dichlorophenoxyacetic acid was a gift of Dow Chemical Co., Midland, Mich.; and α-amanitin was purchased from Henley and Co., New York, N.Y.

RESULTS

Membrane fractions assayed for stimulatory activity in the RNA polymerase assay were characterized as shown in Tables 1 and 2 and Figs. 1–5. Fractions *A* and *B* contained a "latent" IDPase of high specific activity and a glucan synthetase with a high affinity for UDP-glucose. These activities have been assumed as characteristics of dictyosome fractions in other studies (13, 19, §). Electron microscopy of fractions *A* and *B* revealed numerous dictyosomes as expected (Fig. 1), since plant dictyosomes sediment at an average density of about 1.125 (13, 23). The greatest concentration of plasma membrane was in fraction *C*, a relatively homogeneous pellet of vesicles (Fig. 2). To identify these vesicles as plasma membrane-derived, we used the stain of Roland *et al.* (14, 15). This procedure specifically stains plasma membranes in intact tissues prepared for electron microscopy (Figs. 3 and 4) as well as in isolated cell fractions (Fig. 5). Morphometric analysis showed the *C* fraction to contain 55–75% plasma membrane (Table 2). A glucan synthetase of high specific activity and low affinity for UDP-glucose was also concentrated in this fraction (Table 1). This activity characterizes plasma-membrane fractions from onion stem (9, §) and other tissues (W. J. VanDerWoude, T. K. Hodges, and R. Leonard, unpublished) and appears to be a marker enzyme for the plant plasma membrane. By these same criteria, fraction *B* contained both dictyosomes and plasma membranes. Fraction *D* contained principally plasma membranes, intact mitochondria, and mitochondrial fragments (Table 2).

TABLE 2. *Distribution of morphologically recognizable cell components among cell fractions from sucrose–coconut milk gradients as determined by the quantitative morphometric procedure outlined by Lembi et al. (8)*

Cell component	*A*	*B*	*C*	*D*
Plasma membranes*	13 ± 2	33 ± 6	65 ± 11	32 ± 11
Dictyosomes	45 ± 3	31 ± 12	3 ± 3	0
Mitochondria	1 ± 1	1 ± 0	10 ± 7	60 ± 6
Endoplasmic reticulum	1 ± 1	1 ± 0	Trace	0
Microbodies	0	0	0	0
Nuclei	0	0	0	0
Tonoplast, plastid fragments, and unidentified membranes	40 ± 5	34 ± 6	22 ± 4	8 ± 4

Averages of four different preparations ± SD.

* Identified on the basis of phosphotungstic acid–chromic acid staining. All other cell components were determined from sections stained with lead citrate (17).

All the fractions contain an activity that stimulates RNA polymerase *in vitro* in proportion to the plasma-membrane content of the fraction (Table 3; see Table 2). When plasma membrane (fraction *C*) was incubated in either buffer or buffer containing 2,4-dichlorophenoxyacetic acid (Table 4), both preparations contained stimulatory activity. However, when the membranes were removed by centrifugation, the supernatant of the membranes exposed to 2,4-dichlorophenoxyacetic acid contained some of the enhancing activity while the membranes exposed to buffer alone lacked the activity (Table 4). These data indicate that a factor that stimulates RNA polymerase is associated with the plasma membrane and that this factor is released by exposure of the membranes to 2,4-dichlorophenoxyacetic acid. Plasma membranes prepared from onion stems by the techniques used for

§ Unpublished data of C. A. Lembi, W. J. VanDerWoude, D. J. Morré, J. A. Kindinger, and L. Ordin.

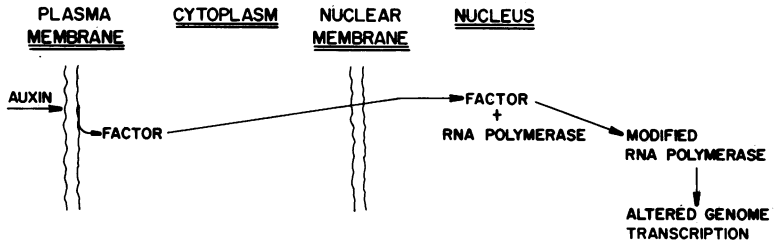


FIG. 6. Hypothesis for auxin-regulated nucleic acid biosynthesis. It is proposed that auxin interacts with a factor within the plasma membrane. The factor is then transported through the nuclear membrane into the nucleus where it regulates the activity of RNA polymerase.

soybean hypocotyls yield a fraction that enhances RNA polymerase activity of soybeans comparable to the plasma membrane fraction of soybean hypocotyl (RNA polymerase activity without and with onion stem plasma membrane was 218 and 286 pmol [^{3}H]UMP incorporated per 30 min per mg of protein, respectively).

To test the specificity of the auxin response, we used several experimental approaches. Initially, the various membrane fractions were incubated in the presence of auxin (2,4-dichlorophenoxyacetic acid or indoleacetic acid) and a nonauxin (3,5-dichlorophenoxyacetic acid). The stimulatory activity was only released in the presence of auxin and not in the presence of nonauxin (Table 5). When membranes initially incubated in the presence of 0.1 μM 3,5-dichlorophenoxyacetic acid and then removed by centrifugation were resuspended and incubated in the presence of 0.1 μM 2,4-dichlorophenoxyacetic acid, the supernatant from membranes treated with 3,5-dichlorophenoxyacetic acid contained no stimulatory activity while supernatants from the membranes treated subsequently with 2,4-dichlorophenoxyacetic acid stimulated RNA polymerase activity (Table 6). Additionally, the released factor may have some specificity for polymerase. Most of the increased RNA polymerase activity is sensitive to the inhibitor α-amanitin (Table 7), which specifically inhibits the nucleoplasmic-specific RNA polymerase in higher organisms (12).

DISCUSSION

Currently two major concepts exist as to the site of action of auxin. One of these centers on the cell wall as the site of action while the other focuses on nucleic acid metabolism. Many cell-wall loosening phenomena may be related to auxin treatment in a manner independent of nuclear activities (24). Yet an enhancement of RNA synthesis is consistently associated with sustained growth responses to the hormone (1, 25,

TABLE 3. *Effect of soybean membrane fractions on RNA polymerase activity*

	[^{3}H]UMP incorporated* (pmol/30 min per mg of protein)				
Experiment:	I	II	III	Average	Difference
RNA polymerase	200	199	280	226	—
+ fraction *A*	220	204	280	234	8
+ fraction *B*	240	196	293	243	17
+ fraction *C*	227	261	360	283	57
+ fraction *D*	346	256	267	256	30

* Experiment II was with membranes from 5-day-old hypocotyls.

TABLE 4. *Enhancement of RNA polymerase activity by incubation of plasma membranes in 2,4-dichlorophenoxyacetic acid*

	[^{3}H]UMP incorporated (pmol/30 min per mg of protein)
RNA polymerase	72
+ PM suspended in TGMED	162
+ Repelleted PM suspended in TGMED	116
+ Supernatant after repelleted PM in TGMED	74
+ PM resuspended in TGMED + 0.1 μM 2,4-dichlorophenoxyacetic acid	236
+ Repelleted PM resuspended in TGMED + 01. μM 2,4-dichlorophenoxyacetic acid	162
+ Supernatant after PM repelleted in TGMED + 0.1 μM 2,4-dichlorophenoxyacetic acid	106
+ TGMED + 0.1 μM 2,4-dichlorophenoxyacetic acid	84

PM, plasma-membrane fraction.

TABLE 5. *Comparison of RNA polymerase activity by incubation of plasma membrane in 0.1 μM 2,4-dichlorophenoxyacetic acid, indoleacetic acid or 3,5-dichlorophenoxyacetic acid*

	[^{3}H]UMP incorporated (pmol/30 min per mg of protein)
RNA polymerase	88
+ PM–indoleacetic acid supernatant	146
+ PM–2,4-dichlorophenoxyacetic acid supernatant	138
+ PM–3,5-dichlorophenoxyacetic acid supernatant	96

TABLE 6. *Effect of sequential exposure of plasma membrane to 3,5-dichlorophenoxyacetic acid and 2,4-dichlorophenoxyacetic acid on release of RNA polymerase enhancement factor*

	[^{3}H]UMP incorporated (pmol/30 min per mg of protein)
RNA polymerase	160
+ PM–3,5-dichlorophenoxyacetic acid supernatant	151
+ PM–3,5-dichlorophenoxyacetic acid supernatant*	249

* Plasma membrane fraction (*PM*) was first exposed to 0.1 μM 3,5-dichlorophenoxyacetic acid. Then the membranes were collected by centrifugation and resuspended in 0.1 μM 2,4-dichlorophenoxyacetic acid. After centrifugation, the resultant supernatant was added to the RNA polymerase assay.

26). Our findings indicate these two concepts may have a common basis for interaction with the plasma membrane being the possible common site of auxin action. This provides a mechanism whereby the interaction of auxin with the plasma membrane could be transmitted to the nucleus by release of a receptor that could interact with a specific RNA polymerase The net result of this action would be an altered transcriptional pattern. Quantative and qualitative changes in RNA synthesis may be accounted for by the mechanisms illustrated in Fig. 6.

Multiple RNA polymerases are present in many eukaryotic organisms (12). One of these, the nucleoplasmic RNA polymerase, is specifically inhibited by the mushroom toxin, α-amanitin (12). If the RNA polymerase of soybeans, which is sensitive to α-amanitin, is functionally similar to that of other eukaryotic organisms, regulation of its activity by a hormone receptor would control the biosynthesis of mRNA. While the chemical nature of the auxin-released factor has not been determined, the factor is apparently associated with plasma membranes, requires an auxin for its release, and affects the α-amanitin-sensitive RNA polymerase.

TABLE 7. *Effect of α-amanitin on enhancenent of RNA polymerase by auxin-released factor*

	[^{3}H]UMP incorporated (pmol/30 min per mg of protein)
RNA polymerase	160
+ α-amanitin*	133
+ PM–2,4-dichlorophenoxyacetic acid supernatant	227
+ PM–2,4-dichlorophenoxyacetic acid supernatant + α-amanitin*	140
RNA polymerase	186
+ α-amanitin*	146
+ PM–indoleacetic acid supernatant	267
+ PM–indoleacetic acid supernatant + α-amanitin*	140

* Each assay contained 2 μg of α-amanitin.

We acknowledge the excellent technical assistance of R. W. Morter, S. G. Gratzer, and Catalina Montecillo. The assistance of W. J. VanDerWoude, Francis Williamson, Kerri Safranski, Wayne Yunghans, and Robert Majeska in the development of the plasma membrane isolation procedure is gratefully acknowledged. This work was supported by a Public Health Service Grant 1 R01 ES005101 to J. H. C., NSF Grant GB23183 to D. J. M., and a NSF traineeship to J.W.H.

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