oxidized surficial layer of the fine-grained ooze.

Chondrites is a facies breaker because it occurs in situations where there is a gradual redox boundary within burrowing reach of the sea floor and in sediment that is rich in organic (nutritious) material; this situation underlies many sorts of sea floors. The burrow has a distinctive and elaborate morphology; it does not reflect the sort of generalized pattern that might be expected in a trace-making animal with broad environmental tolerances. The occurrence of *Chondrites* is related to chemically reducing conditions deep within the sediment and is only indirectly dependent on sea-floor conditions.

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References and Notes

1. A. Seilacher, in *Approaches to Paleoecology* (Wiley, New York, 1964), pp. 296–316; *Mar. Geol.* **5**, 413 (1967); J. D. Howard, in *The Study of Trace Fossils* (Springer, New York, 1975), pp. 131–146; D. C. Rhoads, in *ibid.* pp. 147–160.
2. S. Simpson, *Q. J. Geol. Soc. London* **112**, 475 (1957); A. Seilacher and D. Meischner, *Geol. Rundsch.* **54**, 596 (1964); R. G. Osgood, *Palaeontogr. Am.* **6**, 281 (1970); M. L. Shourd and H. L. Levin, *J. Paleontol.* **50**, 260 (1976).
3. W. Häntzschel, in *Treatise on Invertebrate Paleontology* (Univ. of Kansas Press, Lawrence, 1975), part W, Suppl. 1, pp. 1–269.
4. A. A. Ekdale and W. H. Berger, *Palaeogeogr. Palaeoclimatol. Palaeoecol.* **23**, 263 (1978); A. Wetzel, *Meteor Forschungsergeb. Ergeb.* **34**, 1 (1981).
5. D. D. Swinbanks and Y. Shirayama, in preparation.
6. K. Brenner and A. Seilacher, *Neues Jahrb. Geol. Palaeontol. Abh.* **157**, 11 (1978); E. G. Kauffman, *ibid.*, p. 18.
7. A. A. Ekdale, *Soc. Econ. Paleontol. Mineral. Spec. Pub. 35*, (1984).
8. M. A. Arthur and S. O. Schlanger, *Am. Assoc. Petrol. Geol. Bull.* **63**, 870 (1979).
9. R. G. Bromley, *Geol. Soc. London Q. J.* **123**, 157 (1967); W. J. Kennedy, *Br. Mus. Nat. Hist. Geol. Bull.* **15**, 127 (1967).
10. A. A. Ekdale and R. G. Bromley, *Bull. Geol. Soc. Den.* **31**, 107 (1983).
11. D. Rhoads and J. Morse, *Lethaia* **4**, 413 (1971).
12. A. A. Ekdale, *Geol. J.* (special issue) **9**, 163 (1977).
13. We thank J. Aagaard for photographic assistance. Supported in part by NSF grant EAR 8305869 to A.A.E.

26 January 1984; accepted 20 March 1984

Inhibition of Plant Microtubule Polymerization in vitro by the Phosphoric Amide Herbicide Amiprophos-Methyl

Abstract. *The phosphoric amide herbicide amiprophos-methyl (APM) produced a concentration-dependent inhibition of taxol-induced rose microtubule polymerization in vitro. Parallel studies on taxol-induced assembly of bovine brain microtubules showed no effect of APM at a concentration ten times that required to give complete inhibition of rose microtubule assembly. The data indicate that (i) APM is a specific and potent antimicrotubule drug and (ii) APM directly poisons microtubule dynamics in plant cells, rather than indirectly depolymerizing microtubules through a previously proposed mechanism involving deregulation of intracellular calcium levels.*

The phosphoric amide herbicides have been considered antimicrotubule drugs on the basis of the observed disappearance of microtubules (*1*) and the disruption of microtubule-dependent processes (*2*) in vivo after the drugs are applied to cells of either higher or lower plants. That these drugs interact directly with the microtubule system in plant cells has not been shown. In fact, the best characterized member of this class of herbicides, amiprophos-methyl (APM), affects cellular processes other than the microtubule system. For example, APM at concentrations greater than 5 μM inhibits the uptake of $^{45}Ca^{2+}$ by mitochondria and microsomes isolated from corn and squash (*3*), and at 10 μM inhibits $^{45}Ca^{2+}$ uptake, photosynthesis, and respiration in the unicellular alga *Chlamydomonas* (*4*). However, APM does not inhibit the assembly of mammalian brain microtubules in vitro, depolymerize preformed brain microtubules, or prevent the initiation of brain microtubule assembly on algal microtubule organizing centers in vitro (*1*). These observations, along with the knowledge that brain microtubules are depolymerized in vitro by calcium and that microtubules in animal cells depolymerize after ionophore-mediated calcium influx (*5*), have led to the proposal that APM acts only indirectly on microtubules in plant cells by deregulation of cellular calcium stores with subsequent microtubule depolymerization (*3*, *4*, *6*).

To determine whether APM interferes directly with plant microtubule assembly, we examined the effects of this herbicide on the polymerization of plant tubulin into microtubules in vitro. We showed earlier (*7–9*) that tubulin isolated from cultured cells of rose (*Rosa* sp.) assembles into microtubules after being warmed in the presence of either glycerol or taxol (*10*). Because the taxol-mediated assembly of rose tubulin was so efficient and easily controlled (*7*, *9*) we used this system in the present study of APM.

Rose tubulin was isolated by DEAE–Sephadex A50 chromatography as described (*7*), with minor modifications (*8*), and was more than 85 percent pure as judged by quantitative densitometry of Coomassie blue–stained sodium dodecyl sulfate slab gels (*9*). The kinetics of taxol-mediated rose microtubule assembly at 24°C was monitored turbidimetrically by measuring absorbancy at 400 nm (*11*). Rose tubulin at 10 μM (*12*) assembled rapidly in the presence of a saturating concentration of taxol (40 μM) (*9*), with the kinetics showing a short lag phase followed by a rapid increase in absorbancy and maximum absorbancy reached within 1 hour (Fig. 1). Assembly of tubulin in the presence of micromolar levels of APM produced concentration-dependent increases in the lag time and decreases in both the rate and extent of turbidity development. Maximum inhibition of turbidity development was obtained at nearly 1:1 molar ratios of APM to tubulin. The kinetics of turbidity change with 10 μM APM contained early negative absorbancy values followed by a small amount of recovery to produce a net change in absorbancy of zero by 25 minutes. The early decrease in absorbancy probably results from the dissociation of small aggregates of tubulin that were not sedimented during the tubulin isolation procedure (*8*, *9*). When DEAE-purified (*8*, *13*) bovine brain tubulin (10 μM)

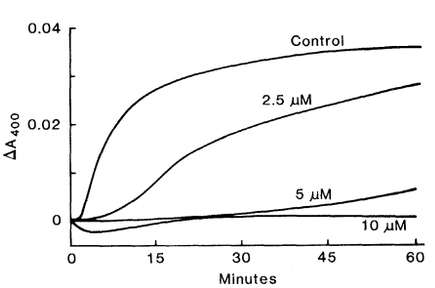

Fig. 1. Effect of APM on the kinetics of taxol-induced microtubule assembly. Samples of tubulin (10 μM) in sucrose isolation buffer (SIB) (*8*) at 0°C were mixed rapidly with SIB at 24°C containing saturating taxol (40 μM) and a given amount of APM (2.5, 5, or 10 μM). Assembly reaction mixtures were monitored continuously at 24°C in 400-μl quartz cuvettes (light path, 1 cm) in a recording spectrophotometer (Beckman Acta III), and turbidity was recorded as the change in absorbancy at 400 nm (ΔA_{400}). All assembly mixtures contained 1.5 percent DMSO, including the control, which contained no APM.

was assembled in the presence of saturating taxol (27 μ*M*) (*10*) and 100 μ*M* APM, no difference was observed in the pattern of turbidity development from those of control assemblies without APM.

The appearance of polymeric structures formed in the presence or absence of APM was studied by negative-stain electron microscopy. In the absence of APM, numerous short microtubules were formed with 40 μ*M* taxol (Fig. 2A); but with increased concentrations of APM, more amorphous debris and fewer and shorter microtubules were seen (Fig. 2B). At 10 μ*M* APM, no ordered polymeric structures were observed. These observations, along with the turbidity data above, suggest that APM inhibits the nucleation phase of microtubule polymerization.

Quantitation of the APM-induced inhibition of taxol-mediated polymerization was made by sedimentation assays (*14*). After 1 hour of assembly in the presence or absence of APM, rose tubulin or brain tubulin polymer was sedimented by centrifugation in a Beckman Airfuge at 48,000*g* for 1 hour at 23°C. Pellets were suspended in sucrose isolation buffer (SIB), and protein was determined by the dye-binding method (Bio-Rad) (*8, 15*). At micromolar levels of APM a concentration-dependent decrease in the yield of rose tubulin polymer was produced, with maximum inhibition of polymerization at approximately equimolar concentrations of APM and tubulin (Table 1). At a tenfold higher concentration of APM (100 μ*M*), no decrease was found in the yield of brain microtubules.

Because taxol is such a potent microtubule polymerization-promoting agent (*9, 10*), the induction of rose microtubule polymerization in vitro by taxol is much stronger than would be expected under more physiological assembly conditions. Since micromolar concentrations of APM inhibit taxol-induced rose tubulin assembly, it is likely that much lower levels of APM would be effective in disruption of microtubules in vivo. Hertel *et al.* (*16*) reported, however, that APM induces similar levels of Ca^{2+} efflux from isolated plant and animal mitochondria. If deregulation of Ca^{2+} stores in vivo is solely responsible for the depolymerization of microtubules in plant cells, then microtubules in animal cells and plant cells could also be expected to depolymerize after treatment with similar concentrations of APM.

To test this possibility, we incubated cultured mammalian cells (PTK_2) with APM (0.1, 1.0, 10, or 100 μ*M*) in Ca^{2+}-free phosphate-buffered saline, *p*H 7.4 and 1 percent dimethyl sulfoxide (DMSO) at 37°C for 1 hour. Cells were fixed and processed for indirect immunofluorescence staining with rabbit antibodies to bovine brain tubulin and fluorescein-labeled goat immunoglobulin G (IgG) directed against rabbit IgG (*17*). Mitotic spindles were unaffected by APM except that at 100 μ*M* some, but not all, of the spindles were disrupted (data not shown). At all concentrations of APM tested, cytoskeletal microtubules remained intact. A control treatment of cells with 1 μ*M* Colcemid in 1 percent DMSO showed complete microtubule depolymerization. After treatment of endosperm cells from ovules of the African blood lily (*Hemanthus*) with 0.1 μ*M* APM in a solution containing 0.5*M* citrate, *p*H 5.1, 4 percent glucose, and 1 percent DMSO for 20 minutes at 22°C, indirect immunogold staining showed the complete disappearance of cytoskeletal microtubules and depolymerization of all spindle microtubules, except the proximal ends of microtubules in kinetochore bundles (*18*). This approximately 1000-fold greater sensitivity of plant microtubules to APM in vivo suggests that the observed disappearance of microtubules in plant cells at relatively low concentrations of APM is due to direct poisoning of microtubule dynamics rather than to indirect Ca^{2+}-induced depolymerization.

We showed recently (*9*) that the requirement of rose tubulin assembly in

Table 1. Effect of APM on polymer yield.

APM concentration (μ*M*)	Amount of sedimented polymer (μg)	Percent of control
	Rose tubulin	
0	71	100
2.5	55	77
5.0	21	30
10.0	4	6
	Brain tubulin	
0	84	100
100.0	84	100

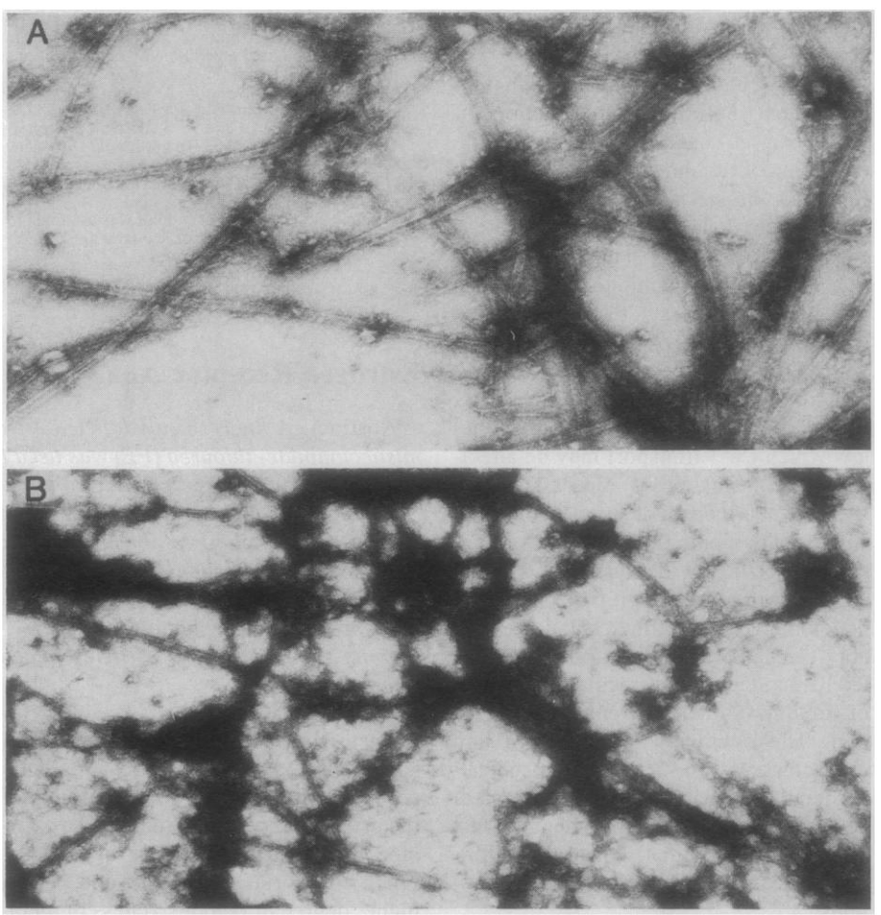

Fig. 2. Electron micrographs of negatively stained polymers formed in the absence (A) or presence (B) of 2.5 μ*M* APM. A 5-μl sample from each reaction mixture described in Fig. 1 was taken after 60 minutes of assembly, placed on Formvar- and carbon-coated copper grids, and stained with 2 percent uranyl acetate for 1 minute. Grids were drained with filter paper and air dried, and polymers were photographed at ×8000 with an electron microscope (Zeiss 9S-2) at 60 kV. Both micrographs are ×34,600.

vitro for taxol is not significantly different from that of brain tubulin assembly (*10*), and we proposed that the taxol binding sites on these diverse tubulins have been conserved over evolution. Because APM did not inhibit taxol-induced brain tubulin assembly and because taxol sites appear to be conserved, it is unlikely that APM inhibits rose tubulin assembly by competing with taxol for binding to tubulin. Our findings that (i) APM inhibited polymerization at least at the nucleation step, and (ii) maximum inhibition of taxol-induced polymerization occurred at nearly 1:1 molar ratios of APM to tubulin are analogous to the effects of colchicine and podophyllotoxin on taxol-induced brain tubulin polymerization (*19*). These data suggest that APM interferes with rose tubulin polymerization by a mechanism similar to that proposed for the inhibition of brain tubulin assembly by colchicine and podophyllotoxin (*19*). However, the APM binding site appears to be distinct from the low-affinity colchicine binding site on rose tubulin (*8*), since concentrations of colchicine more than 100-fold higher are required for the inhibition of taxol-induced rose tubulin assembly (*9*).

Earlier studies (*20*) have shown that the potent antimicrotubule action of APM can be used to investigate the regulation of tubulin synthesis in the alga *Chlamydomonas*. When *Chlamydomonas* cells are deflagellated and then treated with 2.5 μM APM, flagellar regeneration is completely inhibited (*4*). Weeks and his co-workers (*20*) demonstrated that the induction of tubulin synthesis, which normally accompanies flagellar regeneration, is inhibited by 3 μM APM. Indeed, when used at concentrations below which Ca^{2+} transport may be affected (≤ 5 μM) (*3*), APM appears to be a specific and sensitive probe for such studies. The finding that tubulin synthesis is specifically depressed in animal cells after their treatment with drugs that depolymerize microtubules and presumably increase the tubulin pool size has led to a model of autoregulatory control of tubulin synthesis in eukaryotic cells (*21*). The continued identification and characterization of drugs that specifically depolymerize microtubules in plant cells should facilitate experiments to test whether the proposed autoregulatory model is also applicable to plant systems.

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References and Notes

1. D. G. Robinson and W. Herzog, *Cytobiologie* **15**, 463 (1977); H. Quader, I. Wagenbreth, D. G. Robinson, *ibid.* **18**, 39 (1978); M. E. Sterns and D. C. Brown, *J. Ultrastruct. Res.* **77**, 366 (1981).
2. S. Sumida and M. Ueda, *Plant Cell Physiol.* **17**, 1351 (1976); O. Kiermayer and C. Fedtke, *Protoplasma* **92**, 163 (1977); H. U. Koop and O. Kiermayer, *ibid.* **102**, 295 (1980); C. Fedtke, *Biochemistry and Physiology of Herbicide Action* (Springer-Verlag, Berlin, 1982), pp. 123–141; C. Fedtke, in *Biochemical Responses Induced by Herbicides*, D. E. Moreland, J. B. St. John, F. D. Hess, Eds. (American Chemical Society, Washington D.C., 1982), p. 231; S. Sumida and R. Yoshida, in *ibid.*, p. 251.
3. C. Hertel, H. Quader, D. G. Robinson, D. Marmé, *Planta* **149**, 336 (1980).
4. H. Quader and P. Filner, *Eur. J. Cell Biol.* **21**, 301 (1980).
5. R. C. Weisenberg, *Science* **177**, 1104 (1972); A. C. Rosenfeld, R. V. Zackroff, R. C. Weisenberg, *FEBS Lett.* **65**, 144 (1976); M. Schliwa, *J. Cell Biol.* **70**, 527 (1976).
6. D. G. Robinson and H. Quader, in *The Cytoskeleton in Plant Growth and Development*, C. W. Lloyd, Ed. (Academic Press, London, 1982), p. 109.
7. L. C. Morejohn and D. E. Fosket, *Nature (London)* **297**, 426 (1982).
8. L. C. Morejohn *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1440 (1984).
9. L. C. Morejohn and D. E. Fosket, *J. Cell Biol.*, in press. Maximum rates and extents of rose microtubule polymerization in vitro were obtained at approximately 2:1 molar ratios of taxol to tubulin. Thus, 10 μM tubulin assembly is saturated at taxol concentrations greater than 20 μM.
10. Taxol is a taxane alkaloid from the western yew (*Taxus brevifolia*) that binds to tubulin in the form of microtubules and promotes the polymerization and stability of microtubules of plant and animal cells both in vivo and in vitro. Maximum levels of polymerization of brain microtubules in vitro are obtained at equimolar concentrations of taxol and tubulin [P. B. Schiff, J. Fant, S. B. Horwitz, *Nature (London)* **277**, 665 (1979); P. B. Schiff and S. B. Horwitz, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1561 (1980); A. S. Bajer, C. Cypher, J. Molé-Bajer, H. M. Howard, *ibid.* **79**, 6569 (1982)].
11. F. Gaskin, C. R. Cantor, M. L. Shelanski, *J. Mol. Biol.* **80**, 737 (1974).
12. The molecular weight of tubulin was assumed to be 100,000 on the basis of sequencing data [E. Krauhs, M. Little, T. Kempf, R. Hofer-Warbinek, W. Ade, H. Ponstingl, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4156 (1981); H. Ponstingl, E. Krauhs, M. Little, T. Kempf, *ibid.*, p. 2757; P. Valenzuela, M. Quiroga, J. Zaldivar, W. J. Rutter, M. W. Kirschner, D. W. Cleveland, *Nature (London)* **289**, 650 (1981)].
13. J. C. Lee, R. P. Frigon, S. N. Timasheff, *J. Biol. Chem.* **248**, 7253 (1973).
14. K. A. Johnson and G. G. Borisy, in *Molecules and Cell Movement*, S. Inoué and R. E. Stephens, Eds. (Raven, New York, 1975), p. 119.
15. Bio-Rad Laboratories, Richmond, Calif.; M. M. Bradford, *Anal. Biochem.* **72**, 248 (1976).
16. C. Hertel, H. Quader, D. G. Robinson, I. Roos, E. Carafoli, D. Marmé, *FEBS Lett.* **127**, 37 (1981).
17. Experiment performed by D. Pepper, Department of Developmental and Cell Biology, University of California, Irvine.
18. J. Molé-Bajer, personal communication.
19. N. Kumar, *J. Biol. Chem.* **256**, 10435 (1981); P. B. Schiff and S. B. Horwitz, *Biochemistry* **20**, 3247 (1981).
20. P. S. Collis and D. P. Weeks, *Science* **202**, 440 (1978); S. A. Minami, P. S. Collis, E. S. Young, D. P. Weeks, *Cell* **24**, 89 (1981).
21. A. Ben-Ze'ev, S. R. Farmer, S. Penman, *Cell* **17**, 319 (1979); D. W. Cleveland, M. A. Lopata, P. Sherline, M. W. Kirschner, *ibid.* **25**, 537 (1981); D. W. Cleveland and J. C. Havercroft, *J. Cell Biol.* **97**, 919 (1983); D. W. Cleveland, M. F. Pittenger, M. A. Lopata, *J. Submicrosc. Cytol.* **15**, 353 (1983); D. W. Cleveland, M. F. Pittenger, J. R. Feramisco, *Nature (London)* **305**, 738 (1983).
22. Taxol was provided by M. Suffness, Natural Products Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. APM (*O*-methyl-*O*-(4-methyl-6-nitrophenyl)-*N*-isopropyl-phosphorothioamidate) was obtained from Chemagro, Mobay Chemical Corporation, Kansas City, Mo. We thank D. Pepper for performing the indirect immunofluorescence experiments, J. Molé-Bajer for sharing her unpublished results, C. D. Silflow and P. A. Lefebvre for their helpful comments on the manuscript and to G. Erickson for preparation of the typescript. Supported by a grant from Monsanto and by NSF grants 7722398 and PCM 8216035.
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20 January 1984; accepted 6 March 1984

Estrogen Receptor Analysis by Flow Cytometry

Abstract. *A fluorescently labeled estradiol,* N′-*fluoresceino*-N′-(17β-*estradiol hemisuccinamide) thiourea (FE) was used for measuring estrogen receptor content per cell in tumor cells. The cellular content of FE was measured quantitatively by flow cytometry. Binding of FE occurs in the nanomolar concentration range, an indication of the high affinity of the labeled estradiol. Competition of FE for binding sites is observed with estrogens, but not with progestins, androgens, or glucocorticosteroids, indicating the specificity of FE binding. In contrast to other estrogen receptor assays, this new technique requires a small sample size (about 5000 cells) and permits the assessment of heterogeneity in estrogen receptor expression among tumor cells.*

The cytoplasmic receptor for estradiol is present in a subpopulation of patients with breast cancer. The expression of the estrogen receptor in tumors is associated with a longer time interval to recurrence after primary surgery and with a higher degree of responsiveness of metastatic disease to hormonal treatment (*1*, *2*). However, there is considerable variation in the course of the clinical disease among patients with tumors expressing the estrogen receptor. The standard technique for estrogen receptor analysis may provide insufficient information to predict the course of the disease.

Standard technology involves a radioreceptor assay of whole cell extract (cytosol), without regard to normal cell contamination or heterogeneity in estrogen receptor expression among different tumor cells. Cellular heterogeneity can now be investigated in a quantitative fashion with flow cytometry, which permits objective and quantitative analysis