Neurochemical Research, Vol. 25, No. 1, 2000, pp. 19–25

Interactions of Bovine Brain Tubulin with Pyridostigmine Bromide and *N,N′*-Diethyl-*m*-Toluamide*,**

Veena Prasad,[1] Rebekah Scotch,[1] Asish Ray Chaudhuri,[1] Consuelo Walss,[1] Dana B. Fathy,[1] Claudia Miller,[2] and Richard F. Ludueña[1,3]

(Accepted May 17, 1999)

Pyridostigmine bromide (PB), an inhibitor of acetylcholinesterase, has been used as a prophylactic for nerve gas poisoning. *N,N′*-diethyl-*m*-toluamide (DEET) is the active ingredient in most insect repellents and is thought to interact synergistically with PB. Since PB can inhibit the binding of organophosphates to tubulin and since organophosphates inhibit microtubule assembly, we decided to examine the effects of PB and DEET on microtubule assembly as well as their interactions with tubulin, the subunit protein of microtubules. We found that PB binds to tubulin with an apparent K_d of about 60 μM. PB also inhibits microtubule assembly in vitro, although at higher concentrations PB induces formation of tubulin aggregates of high absorbance. Like PB, DEET is a weak inhibitor of microtubule assembly and also induces formation of tubulin aggregates. Many tubulin ligands stabilize the conformation of tubulin as measured by exposure of sulfhydryl groups and hydrophobic areas and stabilization of colchicine binding. PB appears to have very little effect on tubulin conformation, and DEET appears to have no effect. Neither compound interferes with colchicine binding to tubulin. Our results raise the possibility that PB and DEET may exert some of their effects in vivo by interfering with microtubule assembly or function, although high intracellular levels of these compounds would be required.

KEY WORDS: Pyridostigmine bromide; pyridostigmine; *N,N′*-diethyl-*m*-toluamide; microtubule; tubulin.

INTRODUCTION

Microtubules are eukaryotic cylindrical organelles involved in a wide variety of cellular processes. Their subunit protein, tubulin, is a heterodimer consisting of an α and a β subunit. One of tubulin's unusual properties is its ability to bind to a wide variety of structurally very different ligands, many of which disrupt microtubule assembly. Pyridostigmine bromide (PB) (Fig. 1) is a well-known inhibitor of acetylcholinesterase, currently used in the treatment of myasthenia gravis (1). Although PB binds reversibly to acetylcholinesterase, it is also able to inhibit the binding of irreversible inhibitors of acetylcholinesterase, such as the organophosphate nerve gases (2). For this reason, PB was given prophylactically to perhaps 250,000 military personnel during the Persian Gulf War (3). Since that time, some of the personnel have complained of neurological symptoms which they feel may have arisen as a side effect of receiving PB (2). Since organophosphates are known to disrupt the cytoskeleton in vivo, and to inhibit microtubule assembly (4,5), we decided to examine the effects

[1] Department of Biochemistry, University of Texas Health Science Center at San Antonio, San Antonio, Texas.
[2] Department of Family Practice, University of Texas Health Science Center at San Antonio, San Antonio, Texas.
[3] Send reprint requests to: Dr. Richard F. Ludueña, Department of Biochemistry, University of Texas Health Science Center, San Antonio, TX 78284-7760. Tel: 210-567-3732; FAX: 210-567-6595.
* Presented in part at the meeting of the American Society for Cell Biology, Washington, DC, December 1995.
** Special issue dedicated to Dr. Héctor S. Barra.

Abbreviations: DEET, *N,N′*-diethyl-*m*-toluamide: MAP2, microtubule-associated protein 2; PB, pyridostigmine bromide.

0364-3190/00/0100-0019$18.00/0 © 2000 Plenum Publishing Corporation

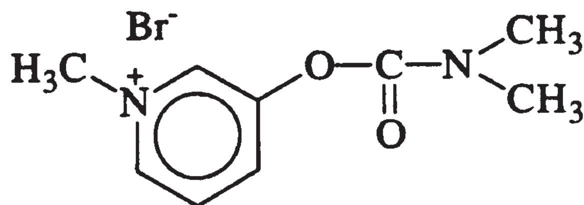

Fig. 1. Structure of pyridostigmine bromide (PB).

of PB on microtubule assembly and to study its interaction with tubulin. In addition, we included in this study the insect repellent *N,N′*-diethyl-*m*-toluamide (DEET) (Fig. 2), a compound speculated to have aggravated the toxicity of PB, perhaps by aiding its absorption into cells (3,5). DEET itself can cause neurotoxicity (7–11) and can be absorbed through the skin, so the possibility of interaction with PB is not remote (12,13). We found that PB and DEET both inhibit microtubule assembly in vitro, that they both interact with tubulin, although not as strongly as do other ligands, and that they have no apparent effects on the conformation of tubulin.

EXPERIMENTAL PROCEDURE

Materials. Microtubules were prepared by cycling from bovine brain cerebra according to the procedure of Fellous et al. (14). Tubulin was purified from these microtubules by chromatography on phosphocellulose; the microtubule-associated proteins tau and MAP2 were also purified from the microtubules (14). Pyridostigmine bromide and DEET were from Sigma Chemical Co. (St. Louis, MO). Other materials were obtained or prepared as previously described.

Fluorescence Measurements. For direct measurement of the interaction of PB with tubulin, tubulin was incubated with PB and the quenching of the fluorescence of the tryptophan residues of tubulin was measured at 336 nm; excitation was at 296 nm. Prior to measuring the interaction between PB and tubulin, we did emission and excitation spectra of PB, finding that it does not absorb at either 296 or 336 nm and, when excited at 296 nm, does not emit at 336 nm. In contrast, tubulin's maximum fluorescence is at 336 nm when excited

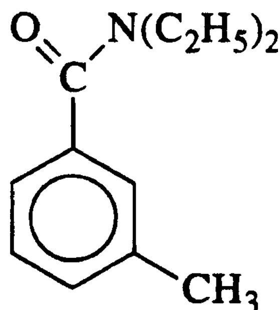

Fig. 2. Structure of N,N¢-diethyl-m-toluamide (DEET).

at 296 nm. The observed fluorescence data were fitted using a nonlinear curve fitting program MINSQ version 3.2 (Scientific Software, Salt Lake City, UT) (15), using a one-site binding equation as follows:

$$F = F_m D/(K_d + D), \text{ where}$$

F is the total fluorescence value at a given drug concentration, D is the drug concentration, F_m is the maximum fluorescence and K_d is the apparent dissociation constant.

To measure exposure of hydrophobic areas, tubulin was incubated at 37°C; then, at periodic intervals, aliquots were removed, made 10 µM in BisANS, and the fluorescence was determined as previously described (16). Excitation and emission were at 385 nm and 490 nm, respectively.

Electron Microscopy. Samples were mixed 1:1 with 1% glutaraldehyde; a 10 µl droplet of the mixture was placed for 60 seconds on a carbon-coated 200-mesh copper grid that had been treated with nitrocellulose in 1% amyl acetate (1:1). The grid was washed with 3 drops of water and then with one drop of cytochrome *c* (1 mg/ml in water). The grid was then negatively stained with 1% uranyl acetate. The grids were examined under a Jeol 100CX electron microscope at an accelerating voltage of 60 kV.

Other Methods. Aliquots of tubulin were incubated with tau at 37°C; microtubule assembly was monitored by turbidimetry at 350 nm (14). Tubulin was reacted with iodo[^{14}C]acetamide and the extent of alkylation was determined by filtration of the samples (17). Measurement of [^{3}H]colchicine binding to tubulin was carried out by the DEAE-filter method of Borisy (18). Protein concentrations were determined by the method of Lowry et al. (19) as modified by Schacterle and Pollock (20) using bovine serum albumin as a standard.

RESULTS

Binding of PB to Tubulin. We developed a fluorometric assay for the binding of PB to tubulin (Fig. 3). The binding data were consistent with a single class of

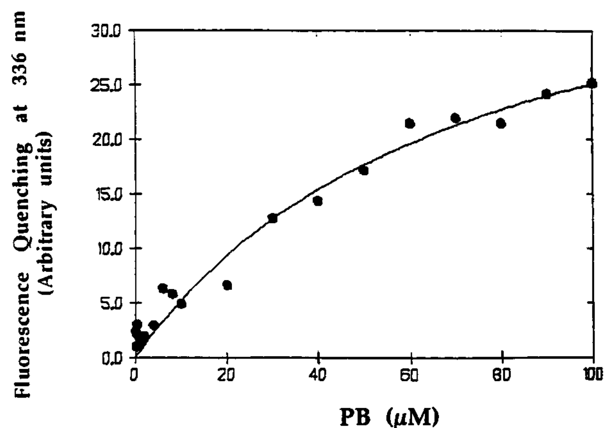

Fig. 3. Binding of PB to tubulin. Aliquots of tubulin (1 µM) were incubated with 0–110 µM PB at 37°C for 30 minutes and the fluorescence quenching of the tubulin was measured at 336 nm. Excitation was at 296 nm.

site. Two experiments yielded apparent K_d values of 40 and 80 μM, consistent with an average K_d of 60 μM. In contrast to PB, DEET did not fluoresce nor did it affect tubulin fluorescence so no fluorescence binding assay could be developed.

Effect of PB and DEET on Microtubule Assembly. We incubated tubulin with tau and found that PB, at a concentration of 250 μM, inhibited the extent of microtubule assembly by about 70% after 30 minutes (Figure 4). Interestingly, increasing the concentration of PB caused large increases in turbidity, suggesting that PB was inducing aggregate formation. Similar results were obtained using MAP2, instead of tau, to stimulate polymerization (not shown). Higher concentrations of PB appeared to enhance polymer formation. At concentrations of 100–200 μM, PB would not block microtubule assembly as measured by turbidimetry, but the assembling microtubules contained mostly microtubules and some ring-like structures. At a PB concentration of 500 μM, there were no microtubules visible and only amorphous structures were seen (Fig. 5). No differences in the morphology of assembled structures were seen when MAP2 was used instead of tau (not shown).

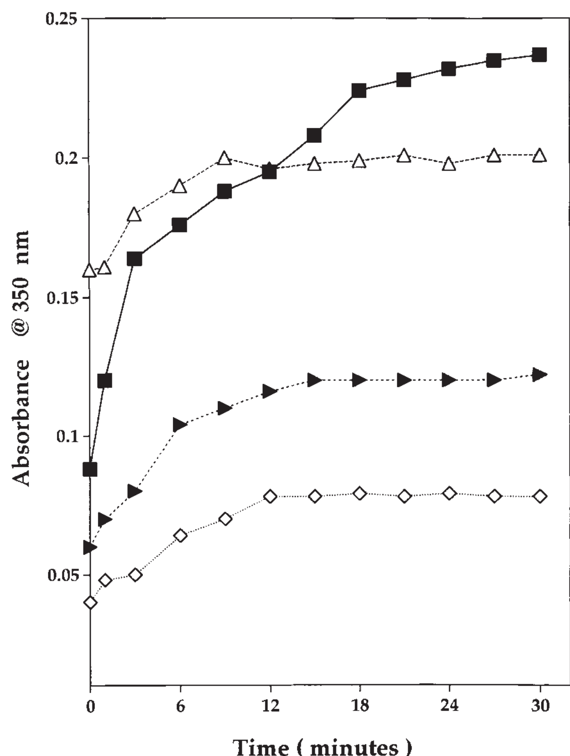

Fig. 4. Effect of PB on microtubule assembly. Aliquots of tubulin (1.0 mg/ml) were incubated at 37°C with tau (0.15 mg/ml) in the absence or presence of a series of concentrations of PB. Microtubule assembly was monitored by turbidimetry at 350 nm. Concentrations of PB were as follows: 0 μM (■); 250 μM (◇); 500 μM (▶); 1 mM (Δ).

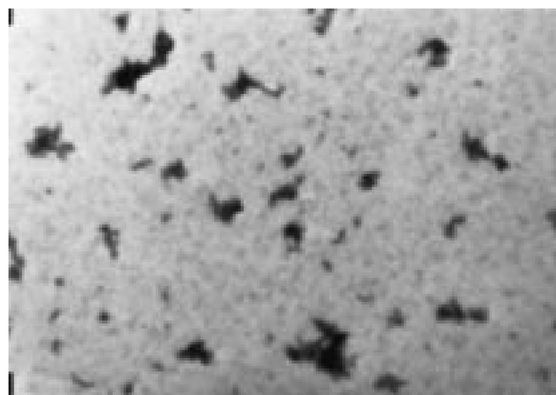

Fig. 5. Tubulin aggregates formed in the presence of PB. Tubulin (1.0 mg/ml) and tau (0.15 mg/ml) were incubated in the presence of 500 μM PB at 37°C for 30 minutes (×86,000).

DEET inhibited microtubule assembly as measured by sedimentation (Fig. 6A). Half the maximal effect was obtained at a DEET concentration between 100 and 250 μM. Interestingly, higher concentrations of DEET appeared to enhance polymer formation as measured by turbidimetry, suggesting that DEET was converting the microtubule into a polymer of higher turbidity but smaller mass (Fig. 6B). When examined by electron microscopy, 25–100 μM DEET permitted formation of microtubules (Fig. 7). Higher DEET concentrations (1–5 mM) caused formation of ribbon-like structures (Fig. 8); it is reasonable to speculate that such a polymer would have have a higher turbidity to mass ratio than would a microtubule and hence would account for the low sedimentation and high turbidity seen at these concentrations of DEET in Fig. 6A and 6B.

Effects of PB and DEET on Tubulin Conformation. We used three different assays to measure the effect of PB on tubulin conformation. In one, we examined the effect of PB on the time-dependent exposure of hydrophobic areas on the surface of the tubulin molecule (Fig. 9). The results showed no effect up to 250 μM PB. Also, PB did not affect the inhibition of exposure by vinblastine. In another assay, we examined the effect of PB on the exposure of sulfhydryl groups on tubulin; the degree of exposure was assayed by the extent of interaction with the alkylating agent iodo[^{14}C]acetamide. We found that PB had a very small inhibitory effect on the exposure of sulfhydryl groups on the tubulin molecule. PB, at 25 μM, inhibited alkylation by 6%. The effect, although small, was only marginally significant ($P < .05$). In contrast, at

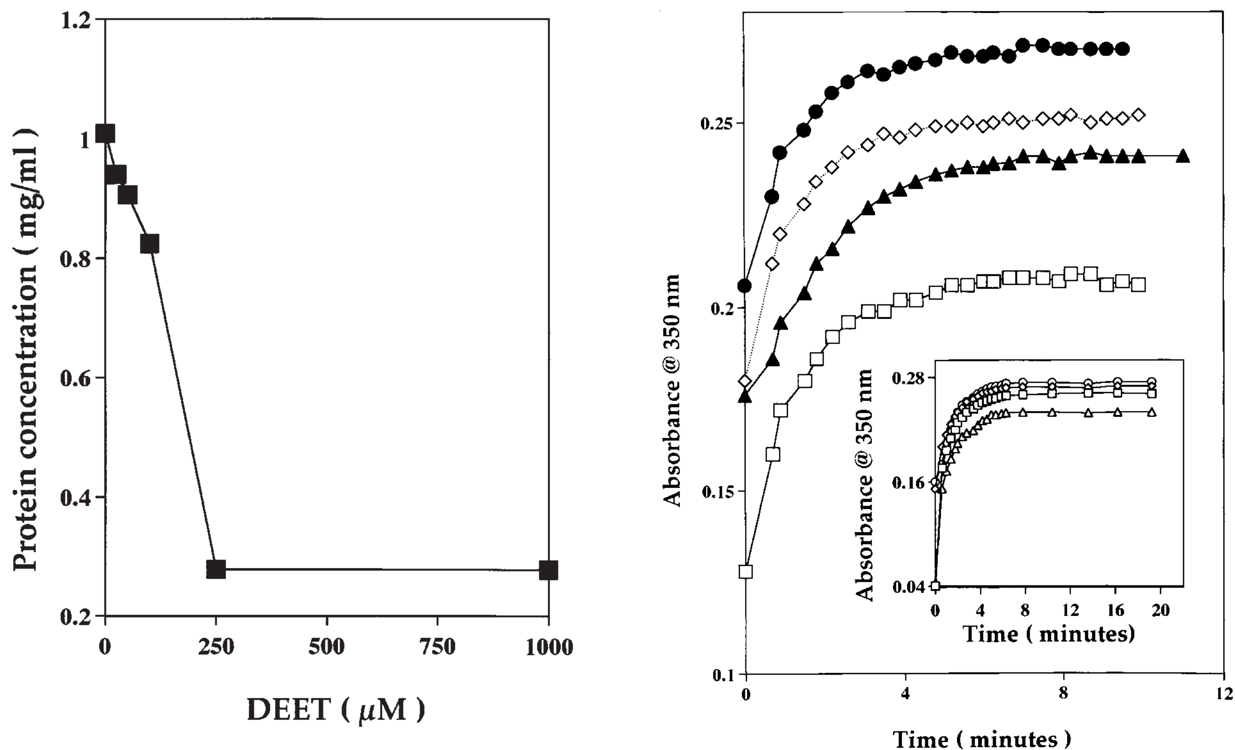

Fig. 6. Effect of DEET on microtubule assembly. Aliquots of tubulin (1.0 mg/ml) were incubated at 37°C for 40 minutes with tau (0.15 mg/ml) in the absence or presence of the indicated concentrations of DEET. **A**. The polymerized samples were spun in the Airfuge at 16,000 rpm at 37°C and the amount of protein pelleted was determined. The figure shows the concentration of the polymer as a function of the concentration of DEET. **B.** Microtubule assembly was monitored by turbidimetry at 350 nm. *Inset:* Concentrations of DEET were as follows: 0 μM (□), 25 μM (◇), 50 μM (O), and 100 μM (Δ). *Main figure:* Concentrations of DEET were as follows: 0 μM (□), 250 μM (◇), 1 mM (•), and 5 mM (▲).

250 μM concentration, PB had no effect on alkylation. Overall, therefore, we could conclude that PB has little or no effect on alkylation. Finally, PB had a small effect on the rate of decay of [^{3}H]colchicine binding to tubulin (Fig. 10). To do this experiment, tubulin was incubated with [^{3}H]colchicine at 37°C with 250 μM PB. At periodic intervals, aliquots were removed to see how much [^{3}H]colchicine was bound. After 90 minutes of incubation, the amount of [^{3}H]colchicine bound to tubulin was 0.37 ± .04 mole/mol in the presence and 0.30 ± .005 in the absence of PB; this is a small but

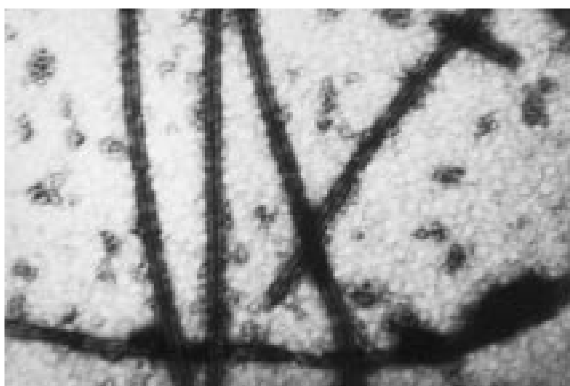

Fig. 7. Microtubules formed in the presence of DEET. Tubulin (1.0 mg/ml) and tau (0.15 mg/ml) were incubated at 37°C for 30 minutes in the presence of 100 μM DEET (×86,000).

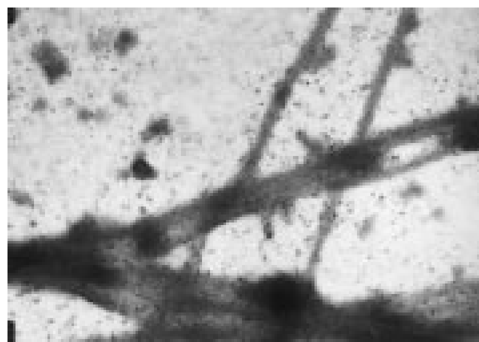

Fig. 8. Ribbon-like structures formed in the presence of DEET. Tubulin (1.0 mg/ml) and tau (0.15 mg/ml) were incubated at 37°C for 30 minutes in the presence of 500 μM DEET (×65,000).

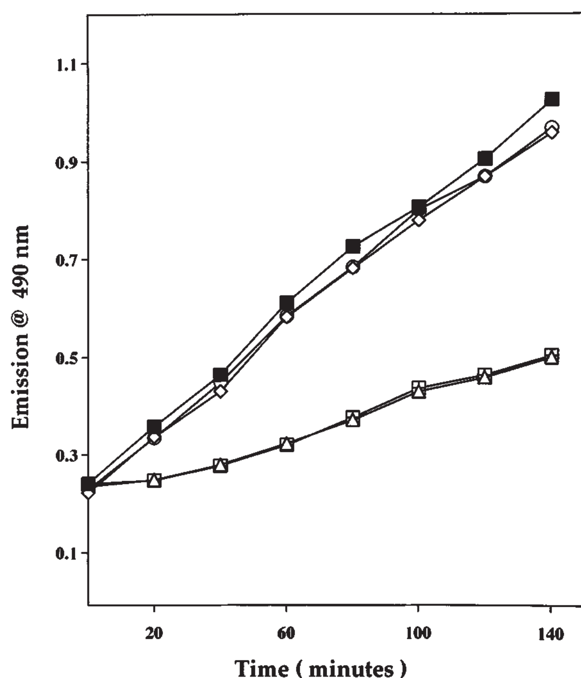

Fig. 9. Effect of PB on the exposure of hydrophobic areas on tubulin. Samples of tubulin (0.2 mg/ml) were incubated at 37°C in the presence of either PB, vinblastine or both PB and vinblastine. At the indicated times, 1-ml aliquots were removed and made 10 µM in BisANS and the fluorescence was determined. Concentrations of drugs were as follows: 0 µM (■), 25 µM PB (O), 250 µM PB (◇), 20 µM vinblastine (Δ), 20 µM vinblastine and 250 µM PB (⊞).

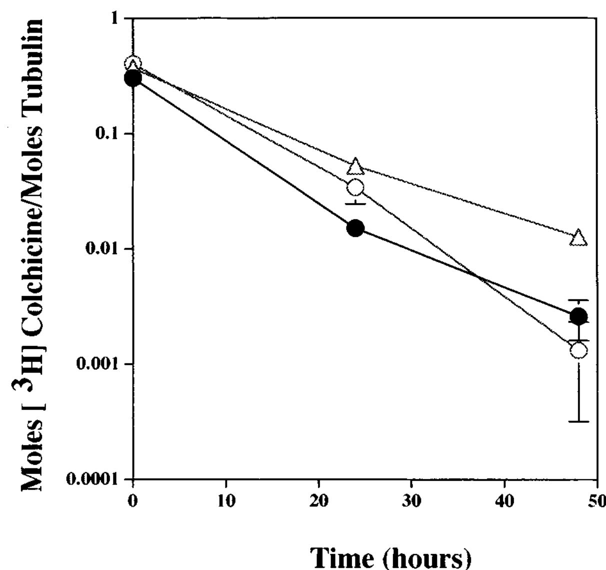

Fig. 10. Effect of PB and DEET on the decay of colchicine binding. Triplicate samples of tubulin (0.3 mg/ml) were pre-incubated for 90 minutes with [^{3}H]colchicine (10 µM) in the absence (O) or presence of either PB (250 µM) (Δ) or DEET (1 mM) (•) for up to 48 hours. After 90 minutes, the first aliquots were removed for the zero-time measurement. At the indicated times, subsequent aliquots were removed and the amount of bound label determined. Standard deviations are shown.

marginally significant (P < .05) increase, which could reflect PB's ability to stabilize the decay of colchicine binding (Fig. 10). It thus appears likely that PB had little or no effect on colchicine binding. As tubulin decay progressed, the half-time of decay was found to be about 9.8 hours in the presence and 7.0 hours in the absence of PB. Thus, PB appeared to exert a small protective effect to slow down the decay of colchicine binding.

The same assays were performed on the interaction of DEET with tubulin. Here, DEET, at concentrations of 1–5 mM, appeared to enhance the exposure of hydrophobic areas (Fig. 11) both in the presence and absence of vinblastine. However, DEET had no significant effect on exposure of sulfhydryl groups; alkylation with iodo[^{14}C]acetamide showed an incorporation of 4.08 ± .17 mol ^{14}C/mol tubulin in the absence of DEET, 4.30 ± .17 in the presence of 100 µM DEET, and 4.20 ± .18 moles in the presence of 1 mM DEET; these are not significant differences. DEET also did not affect the decay of [^{3}H]colchicine binding (Fig. 10). Incubation of tubulin with for 90 minutes at 37°C with 1 mM DEET caused a small enhancement of colchicine binding (0.40 ± 0.022 mol/mol compared to 0.30 ± .005, P < .01). The half-time of decay in the presence of DEET was 5.8 hours.

DISCUSSION

PB appears to bind to tubulin with a K_d of approximately 60 µM. This is substantially weaker than the binding of drugs such as colchicine, podophyllotoxin, vinblastine and maytansine, whose K_d's are all in the range of 0.1–0.9 µM (21). Although it does not bind as strongly as do these other drugs, PB nevertheless causes a large inhibition of microtubule assembly in vitro. It binds to tubulin at what appears to be a single site and appears to have very little effect on the conformation of tubulin except to cause a slight stabilization against spontaneous decay. DEET can also cause a very weak inhibition of microtubule assembly. The two drugs do not inhibit colchicine binding, suggesting that they bind at a site or sites distinct from the colchicine site. This is consistent with their chemical structures, which bear little resemblance to that of colchicine. Furthermore, PB has does not influence vinblastine's inhibition of tubulin

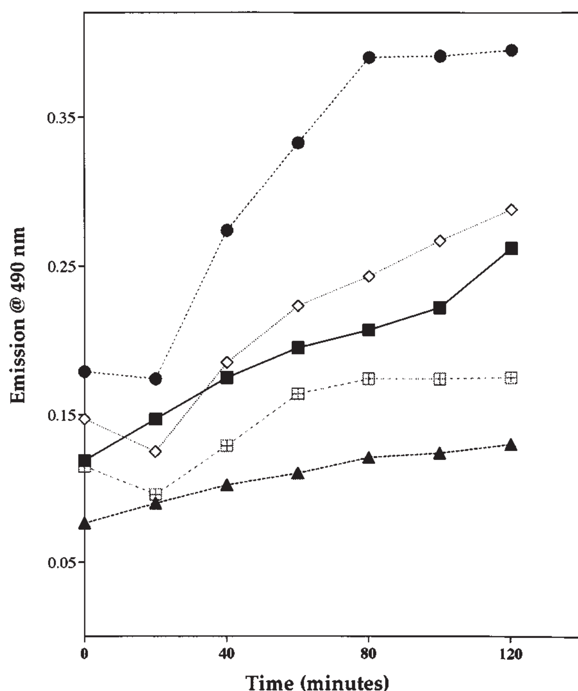

Fig. 11. Effect of DEET on the exposure of hydrophobic areas on tubulin. Samples of tubulin (0.2 mg/ml) were incubated at 37°C in the presence of either DEET, vinblastine, or both. Aliquots were removed and processed as in Fig. 9. Concentrations of drugs were as follows: 0 μM (■), 1 mM DEET (◇), 5 mM DEET (•), 20 μM vinblastine (⊞), 20 μM vinblastine and 1 mM DEET (▲). In a control experiment, in the absence of tubulin, DEET had no effect on BisANS fluorescence.

decay, suggesting that PB's binding site does not overlap with that of vinblastine. DEET, however, does increase the decay in the presence of vinblastine, suggesting either that DEET's effect overcomes that of vinblastine or that they bind at the same site. At high concentrations, PB and DEET induce aggregation of tubulin into amorphous structures. This latter effect may reflect very low affinity binding to hydrophobic areas on tubulin, which could in turn cause exposure of other hydrophobic areas, leading to aggregation.

The potential medical significance of these findings is intriguing. PB is an inhibitor of acetylcholinesterase (1). This latter activity has been explored as a possible hypothesis to explain Gulf War veterans' illnesses but has been rejected because affected veterans' symptoms should have ceased when PB was stopped if PB was the cause (3). However, our results raise another possibility, that PB can act by affecting the microtubules of cells, perhaps in the nervous system. This effect need not be limited to blocking microtubule assembly. It is conceivable that lower concentrations could inhibit microtubule dynamic behavior; in other words, without actually altering the microtubule mass in a cell, PB could in principle disrupt microtubule function. Analogous findings have already been made using taxol, vinblastine, and other drugs, namely, that, at very low concentrations, these compounds can inhibit microtubule function and dynamics in a cell without altering microtubule mass (22–24). The fact that PB binds weakly to tubulin may not be relevant. The actual intracellular concentration of PB as well as its ability to enter a cell could be influenced by other factors. One such factor is stress, which military personnel can experience to a very high degree, especially in wartime. In mice, stress has been shown to potentiate the effect of PB on acetylcholinesterase activity by 150-fold, apparently by increasing the permeability of the blood-brain barrier (25). It is therefore reasonable to hypothesize that stress could also increase PB's effect on neuronal microtubule dynamics. Other effects of PB on microtubules in vivo are possible, since it has also been observed that organophosphates can phosphorylate or degrade tubulin and microtubule-associated proteins (26,27). Nevertheless, it must be stressed that the effects of PB on microtubule assembly which were observed here required much higher concentrations than are necesary to obtain analogous effects with better-studied anti-tubulin drugs.

Another factor could be DEET, which appears to interact more weakly with tubulin than does PB. It would be hard to argue that DEET has deleterious effects on cells by binding to tubulin, since its highest known serum concentration after dermal application (3 μM) is much lower than the concentrations required to observe the effects reported here (12). However, DEET is thought to increase the permeability of cells to other factors, and it is possible that DEET could thus facilitate the entry of PB into cells (28). In addition, PB is thought to carbamylate esterases which could hydrolyze DEET; this would have the effect of increasing the intracellular concentration of DEET (29). Our results also suggest that, at millimolar concentrations, DEET, too, could have an effect on microtubules. There are thus several possible ways in which DEET and PB could act synergistically.

ACKNOWLEDGMENTS

Supported by NIH grants GM23476 and CA 26476 and Welch Foundation grant AQ-0726 to R.F.L.

REFERENCES

1. Taylor, P. 1990. Anticholinesterase agents. Pages 131–149, *in* Gilman, A. G., Rall, T. W., Nies, A. S., and Taylor, P. (eds.), Goodman and Gilman's The Pharmacological Basis of Therapeutics, 8th Edition, Pergamon Press, New York.
2. O. Y., Danon, Y. L., Berkenstadt, H., Almog, S., Mimouni-Bloch, A., Zisman, Z., Dani, S., and Atsmon, J. 1991. Survey of symptoms following intake of pyridostigmine during the Persian Gulf war. Isr. J. Med. Sci. 27:656–658.
3. Presidential Advisory Committee on Gulf War Veterans' Illnesses. 1996. Final Report. U.S. Government Printing Office, Washington, DC.
4. Abou-Donia, M. B. 1993. The cytoskeleton as a target for organophosphorus ester-induced delayed neurotoxicity (OPIDN). Chem. Biol. Interact. 87:383–393.
5. R. P. Gupta, R. P., and Abou-Donia, M. B. 1994. In vivo and in vitro effects of diisopropyl phosphorofluoridate (DFP) on the rate of hen brain tubulin polymerization, Neurochem. Res. 19:435–444.
6. Moody, R. P., Wester, R. C., Melendres, J. L., and Maibach, H. I. 1992. Dermal absorption of the phenoxy herbicide 2,4-D dimethylamine in humans: effect of DEET and anatomic site. J. Toxicol. Environ. Health 36:241–250.
7. Roland, E. H., Jan, J. E., and Rigg, J. M. 1985. Toxic encephalopathy in a child after brief exposure to insect repellents. Can. Med. Assoc. J. 132:155–156.
8. Snyder, J. W., Poe, R. O., Stubbins, J. F., and Garrettson, L. K. 1986. Acute manic psychosis following dermal application of *N,N*-diethyl-m-toluamide (DEET) in an adult. J. Toxicol. Clin. Toxicol. 24:429–439.
9. Robbins, P. J., and Cherniack, M. G. 1986. Review of the biodistribution and toxicity of the insect repellent *N,N*-diethyl-*m*-toluamide (DEET). J. Toxicol. Environ. Health 18:503–525.
10. Tenenbein, M. 1987. Severe toxic reactions and death following the ingestion of diethyltoluamide containing insect repellents, J. Am. Med. Assoc. 258:1509–1511.
11. Schaefer, C., and Peters, P. W. 1992. Intrauterine diethyltoluamide exposure and fetal outcome. Reprod. Toxicol. 6:175–176.
12. Taylor, W. G, Danielson, T. G., Spooner, R. W., and Goldsteyn, L. R. 1994. Pharmacokinetic assessment of the dermal absorption of *N,N*-diethyl-*m*-toluamide (DEET) in cattle. Drug Metab. Dispos. 22:106–112.
13. Selim, S., Hartnagel, R. E., Jr., Osmitza, T. G., Gabriel, K. L., and Shoenig, G. P. 1995. Absorption, metabolism, and excretion of *N,N*-diethyl-*m*-toluamide following dermal application to human volunteers. Fundam. Appl. Toxicol. 25:95–100.
14. Fellous, A., Francon, J., Lennon, A.-M., and Nunez, J. 1977. Microtubule assembly in vitro. Eur. J. Biochem. 78:167–174.
15. Ludueña, R. F., Roach, M. C., Prasad, V., Chaudhuri, A. R., Tomita, I., Mizuhashi, F., and Murata, K. 1995. Interaction of bovine brain tubulin with the 4(1H)-pyrizinone derivative IKP104, an antimitotic drug with a complex set of effects on the conformational stability of the tubulin molecule. Biochemistry 34:15751–15759.
16. Prasad, A. R. S., Ludueña, R. F., and Horowitz, P. M. 1986. Bis(8-anilinonaphthalene-1-sulfonate) as a probe for tubulin decay. Biochemistry 25:739–742.
17. Ludueña, R. F., and Roach, M. C. 1981. Contrasting effects of maytansine and vinblastine on the alkylation of tubulin sulfhydryls. Arch. Biochem. Biophys. 210:498–504.
18. Borisy, G. G. 1972. A rapid method for quantitative determination of microtubule protein using DEAE-cellulose filters. Anal. Biochem. 50:373–385.
19. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
20. Schacterle, G. R., and Pollock, R. L. 1973. A simplified method for the quantitative assay of small amounts of protein in biologic material. Anal. Biochem. 51:654–655.
21. Ludueña, R. F. 1979. Biochemistry of tubulin. Pages 65–116, *in* Roberts, K., and Hyams, J. S. (eds.), Microtubules, Academic Press, London.
22. Jordan, M. A., Toso, R. J., Thrower, D., and Wilson, L. 1993. Mechanism of mitotic block and inhibition of cell proliferation by taxol at low concentrations. Proc. Nat. Acad. Sci. USA 90:9552–9556.
23. Dhamodharan, M., Jordan, M. A., Thrower, D., Wilson, L., and Wadsworth, P. 1995. Vinblastine suppresses dynamics of individual microtubules in living interphase cells. Mol. Biol. Cell 6:1215–1229.
24. Jordan, M. A., Thrower, D., and Wilson, L. 1991. Mechanism of inhibition of cell proliferation by *Vinca* alkaloids. Cancer Res. 51:2212–2222.
25. Friedman, A., Kaufer, D., Shemer, J., Hendler, I., Soreq, H., and Tur-Kaspa, I. 1996. Pyridostigmine brain penetration under stress enhances neuronal excitability and induces early immediate transcriptional response. Nature Medicine 2:1382–1385.
26. Abou-Donia, M. B. 1995. Involvement of cytoskeleton proteins in the mechanisms of organophosphorus ester-induced delayed neurotoxicity. Clin. Exptl. Pharmacol. Physiol. 22:358–359.
27. Gupta, R. P., and Abou-Donia, M. B. Diisopropyl phosphofluoridate (DFP) treatment alters calcium-activated proteinase activity and cytoskeletal proteins of the sciatic nerve. Brain Res. 677:162–166.
28. Abou-Donia, M. B., Wilmarth, K. R., Abdel-Rahman, A. A., Jensen, K. F., Oehma, F. W., and Kurt, T. I. 1996. Increased neurotoxicity following concurrent exposure to pyridostigmine bromide, DEET, and chlorpyrifos. Fundam. Appl. Toxicol. 34: 201–222.
29. Abou-Donia, M. B., Wilmarth, K. R., Jensen, K. F., Oehme, F. W., and Kurt, T. L. 1996. Neurotoxicity resulting from coexposure to pyridostigmine bromide, deet, and permethrin: implications of Gulf War chemical exposures. J. Toxicol. Environ. Health 48:35–56.