0026-895X/87/060731-06$02.00/0

MOLECULAR PHARMACOLOGY, 32:731-736

Interaction of Some Estrogenic Drugs with Tubulin. Formation of Twisted Ribbon Structures

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Received April 24, 1987; Accepted September 3, 1987

SUMMARY

We studied the action of *E*-diethylstilbestrol (E-DES), erythro-hexestrol (erythro-HES), and E,E-dienestrol (E,E-DIES) on microtubule formation. The three drugs inhibit this formation from microtubular protein; the percentages of inhibition were, respectively, 15% and 45% for 1.25×10^{-5} M E-DES and E,E-DIES. With purified tubulin 6S, 7.5×10^{-6} M E,E-DIES and erythro-HES induced a 20% inhibition. In the case of E-DES, our results are in good agreement with previous ones. These drugs partially disrupt preformed microtubules. Moreover, when E,E-DIES (5×10^{-5} M) is added to tubulin, loosely organized aggregates composed of twisted ribbon structure are formed. In the case of erythro-HES, similar structures were observed but at higher concentrations. With E-DES, no organized structures are present.

Diethylstilbestrol is a synthetic estrogenic drug used in prostatic carcinoma. It has been implicated as a human carcinogen (1) and it can induce mitotic non-disjunction, aneuploidy, or polyploidy (2–5). This suggests a colchicine-like action, and several studies have proved that E-DES inhibits tubulin assembly (6–8).

As there are other synthetic estrogenic drugs with a similar structure and carcinogenic activity (9), we attempted to verify that this inhibitory effect was a specific property of E-DES. We tested the action of E,E-DIES and erythro-HES on microtubule formation. Our study shows, particularly, that E,E-DIES can induce, in tubulin preparation, the formation of loose aggregates which are composed of twisted ribbon structures reminiscent of vinblastine-induced structures.

Materials and Methods

Reagents

MES and EGTA were purchased from Sigma and GTP was purchased from Fluka.

E-DES, E,E-DIES, and erythro-HES (Fig. 1) were purchased from Sigma and dissolved in methanol from Carlo Erba. All compounds were proved pure by ^{1}H-NMR spectroscopy with a Bruker AM 200 instrument.

Preparation of Tubulin

Microtubular protein (MTP). MTP, i.e., tubulin 6S with microtubule-associated proteins was obtained as previously described (10, 11) from fresh porcine brain according to the method of Shelanski *et al.* (12) through three cycles of *in vitro* assembly (37°) and disassembly (4°).

Two buffers were used: buffer A (pH 6.5), consisting of 0.1 M MES, 1 mM EGTA, and 0.5 mM $MgCl_2$ ($6H_2O$), for disassembly and buffer B (pH 6.5), consisting of 0.1 M MES, 1 mM EGTA, 0.5 mM $MgCl_2$, ($6H_2O$), 1 mM GTP, and 8 M glycerol for assembly. Each preparation was controlled by polyacrylamide gel electrophoresis (13).

Protein concentration was determined by the method of Lowry *et al.* (14), using bovine serum albumin as a standard. The MTP solution was stored at −80°.

Tubulin 6S. Tubulin 6S was purified from MTP by using phosphocellulose chromatography (15).

Prior to use, Whatman phosphocellulose P11 was washed successively with 0.5 M NaOH, H_2O, 0.5 M HCl, H_2O and equilibrated with 0.025 M MES buffer, pH 6.7, containing 0.5 mM $MgCl_2$ ($6H_2O$), 1 mM EGTA, and 0.1 mM GTP.

The tubulin eluted from the phosphocellulose column was collected and concentrated using an Amicon concentrating cell model 8050 (PM 30 membrane). Then, tubulin was chromatographed on a Sephadex G25 column equilibrated with 0.05 M MES buffer, pH 6.7, containing 0.5 mM $MgCl_2$ ($6H_2O$), 1 mM EGTA, and 3.4 M glycerol.

Tubulin concentration was determined spectrophotometrically at 280 nm using an extinction coefficient of 1.2 ml cm^{-1} mg^{-1} (16).

The purity was checked with tubulin (2.0 ± 0.1 mg/ml) diluted with an equal volume of a solution containing 1% sodium dodecyl sulfate, 2% β-mercaptoethanol, 70% glycerol, and 0.1 mg/ml bromophenol and boiled for 3 min. The sample was applied to dodecyl sulfate-polyacrylamide slab gels (13). Sodium dodecyl sulfate (from Sigma) contained 25% (w/w) myristyl sulfate and 5% cetyl sulfate (17). The tubulin solution was then stored at −80°.

Assembly Study

Microtubular protein (MTP). Microtubule formation was monitored by continuous recording of turbidimetry (18, 19) at 340 nm using

This work was supported by INSERM Grants 851003 and 851007.

ABBREVIATIONS: E-DES, *E*-diethylstilbestrol; E,E-DIES, *E,E*-dienestrol; erythro-HES, erythro-hexestrol; MES, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis (2-aminoethyl ether)tetraacetic acid; MTP, microtubular protein.

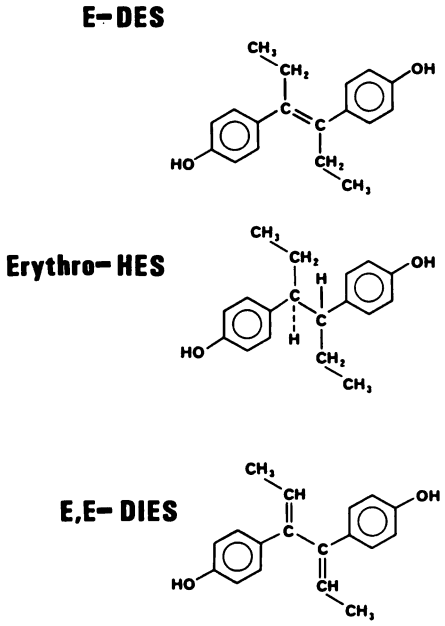

Fig. 1. Chemical structures of E-DES, erythro-HES, and E,E-DIES.

a Uvikon LS Printer 48 Spectrophotometer with a thermostated 1-cm light path cell. Assembly was initiated by a jump of temperature from 4° to 37°.

The MTP concentration was 0.8 mg/ml. The plateau value of the optical density allowed the determination of the degree of assembly.

For the measurements of the inhibitory effects, 5 μl of estrogen solution in methanol, mixed with 250 μl of MTP in buffer A, were then added to 250 μl of assembly buffer (buffer A, 1 mM GTP).

At the steady state level of assembly, samples were chilled at 4°. Half an hour later, a second assembly was started by a jump of temperature from 4° to 37°.

In all cases, controls were measured with 5 μl of methanol alone.

The degree of assembly in the presence of estrogen was expressed as a percentage of the control value. Each experiment was performed in triplicate.

Tubulin 6S. The tubulin 6S assembly was carried out as described for MTP except that the buffer differed: 0.05 M MES, 10 mM $MgCl_2$ ($6H_2O$), 1 mM EGTA, 1 mM GTP, and 3.4 M glycerol, pH 6.7. The tubulin concentration was 10^{-5} M.

Disruption of Preformed Microtubules

Microtubules were obtained from MTP or tubulin 6S as previously described. When the plateau value of optical density was reached, 5 or 3 μl of estrogenic drug in methanol solution (5×10^{-5} M or 10^{-4} M) were added. The variations in optical density were monitored. These variations were corrected from those induced by the same volume of pure methanol.

Electron Microscopy

Electron microscopy was performed using a Philips EM 400 T microscope (Service Commun de Microscopie Electronique, Faculté des Sciences St. Jérome, Marseille, France). Samples were taken directly in the UV spectrophotometer cell and were negatively stained with 1.5% uranyl acetate on carbon-coated grids.

Results

Microtubular Protein (MTP)

The three drugs tested showed an inhibitory effect on MTP assembly, independently of incubation time (Table 1).

E-DES and erythro-HES. In the case of E-DES, our results were in good agreement with previous results (6–8). Table 1 shows that erythro-HES exerted a similar inhibitory effect.

In the case of drug doses inducing partial inhibition, the microtubules were less numerous and shorter but exhibited normal ultrastructure, as revealed by electron microscopy (not shown). This was confirmed by the total cold disassembly of these microtubules.

For higher doses, such as 7.5×10^{-5} M E-DES and erythro-HES, assembly was completely inhibited. Electron micrographs showed that, in the case of erythro-HES, some organized structures appeared (Fig. 2, A and B), whereas in the case of E-DES, only clumped aggregates of protein were found (not shown).

Preformed microtubules were only partially disassembled, even with high doses of E-DES (5×10^{-5} M): 30% as calculated from the decrease of optical density. In contrast, erythro-HES seemed to have no effect.

E,E-DIES. When the doses of E,E-DIES are increased from 0.5×10^{-5} M to 7.5×10^{-5} M, the plateau value of optical density, paradoxically, first decreases, then increases (Table 1).

From 0.5×10^{-5} M to 2.5×10^{-5} M E,E-DIES, the percentages of inhibition increased (Table 1), and the electron micrographs showed cold-sensitive microtubules that were less numerous and shorter, similar to those obtained in the presence of low doses of E-DES or erythro-HES (not shown).

When the E,E-DIES concentrations were higher ($>2.5 \times 10^{-5}$ M), the percentages of inhibition decreased (Table 1), and the electron micrographs indicated the presence of few microtubules and the appearance of twisted ribbon structures. At 7.5×10^{-5} M, the microtubules were no longer observed. Twisted ribbon structures seemed to be formed of six protofilaments (Fig. 2C). These curly protofilaments appear to be cold stable as proved by turbidimetric measurements and electronic microscopy observations.

E,E-DIES (5×10^{-5} M) induced no apparent disassembly of preformed microtubules, as determined by optical density measurement, but electron micrographs showed that the number of microtubules decreased and that aggregates were formed (not shown).

Tubulin 6S

E-DES, erythro-HES, and E,E-DIES inhibited the assembly of tubulin 6S, independently of incubation time.

E-DES. E-DES induced a dose-dependent inhibition of tubulin 6S assembly (Fig. 3) but at lower concentrations than MTP. In addition, the rate of assembly was sharply decreased. Electron micrographs showed shorter and less numerous mi-

TABLE 1

Action of E-DES, erythro-HES, and E,E-DIES on MTP (0.8 mg/ml) assembly

Percentages of inhibition (mean from three experiments ± standard deviation) were calculated from the variation of optical density of the solution.

Concentration (10^{-5} M)	E-DES	Erythro-HES	E,E-DIES
		%	
0.50	NS*	NS	13.0 ± 1.5
1.25	15.0 ± 1.5	NS	45.0 ± 3.0
2.50	26.0 ± 2.0	20.0 ± 2.0	65.0 ± 6.0
3.75	51.0 ± 3.5	47.0 ± 3.0	57.0 ± 4.0
5.00	90.0 ± 4.5	78.0 ± 4.5	53.0 ± 3.0
7.50	95.0 ± 5.0	94.0 ± 5.0	39.0 ± 2.0

* NS, not significant.

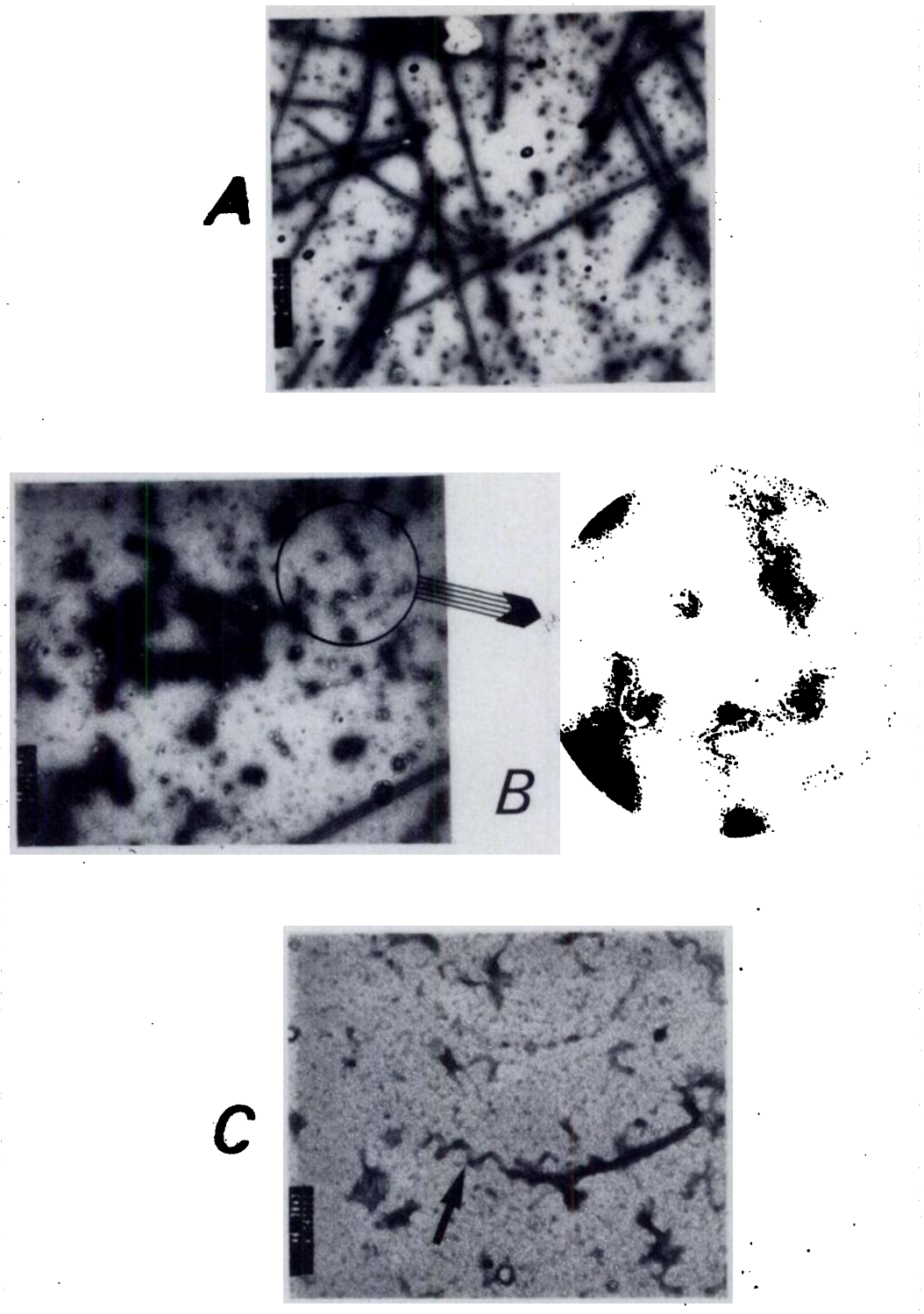

Fig. 2. Electron micrographs. A. MTP alone (0.8 mg/ml) (× 13,000). B. MTP with 7.5×10^{-5} M erythro-HES (× 36,000). C. MTP with 7.5×10^{-5} M E,E-DIES (× 60,000).

crotubules, and, with sufficient doses, no organized structure remained as in the case of MTP (not shown).

Preformed microtubules were greatly disassembled with 5×10^{-5} M E-DES: a 75% decrease in absorbance was observed (Fig. 3).

Erythro-HES and E,E-DIES. *Microtubule assembly.* Phenomena similar to those observed in the case of MTP were observed for both erythro-HES and E,E-DIES added to tubulin 6S, but were more pronounced.

First, with erythro-HES ($\leq 2 \times 10^{-5}$ M) and E,E-DIES ($\leq 10^{-5}$ M) there was a decrease in the plateau value of optical density (Fig. 4) corresponding to an inhibition of microtubule formation

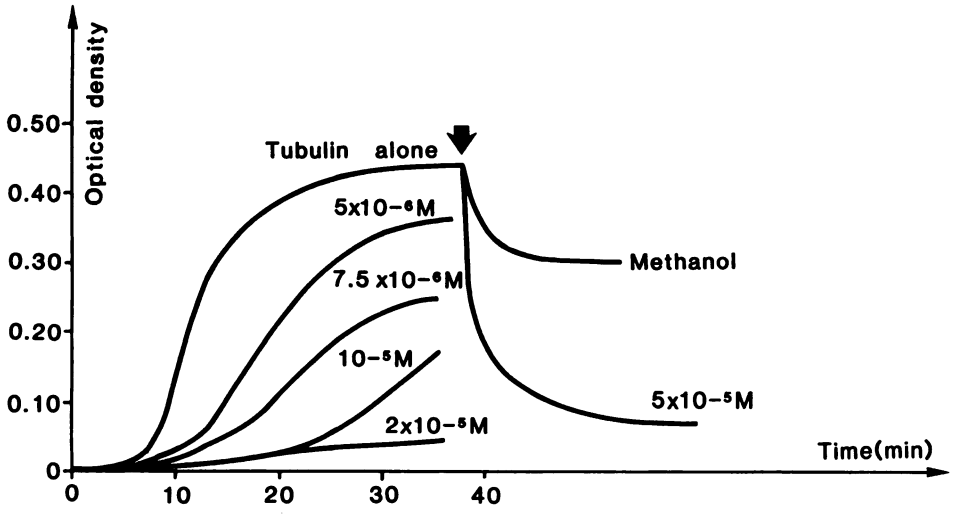

Fig. 3. Variation of optical density versus time with E-DES, on tubulin (10^{-5} M) assembly and on preformed microtubules. ▾ corresponds to the addition of 5×10^{-5} M E-DES.

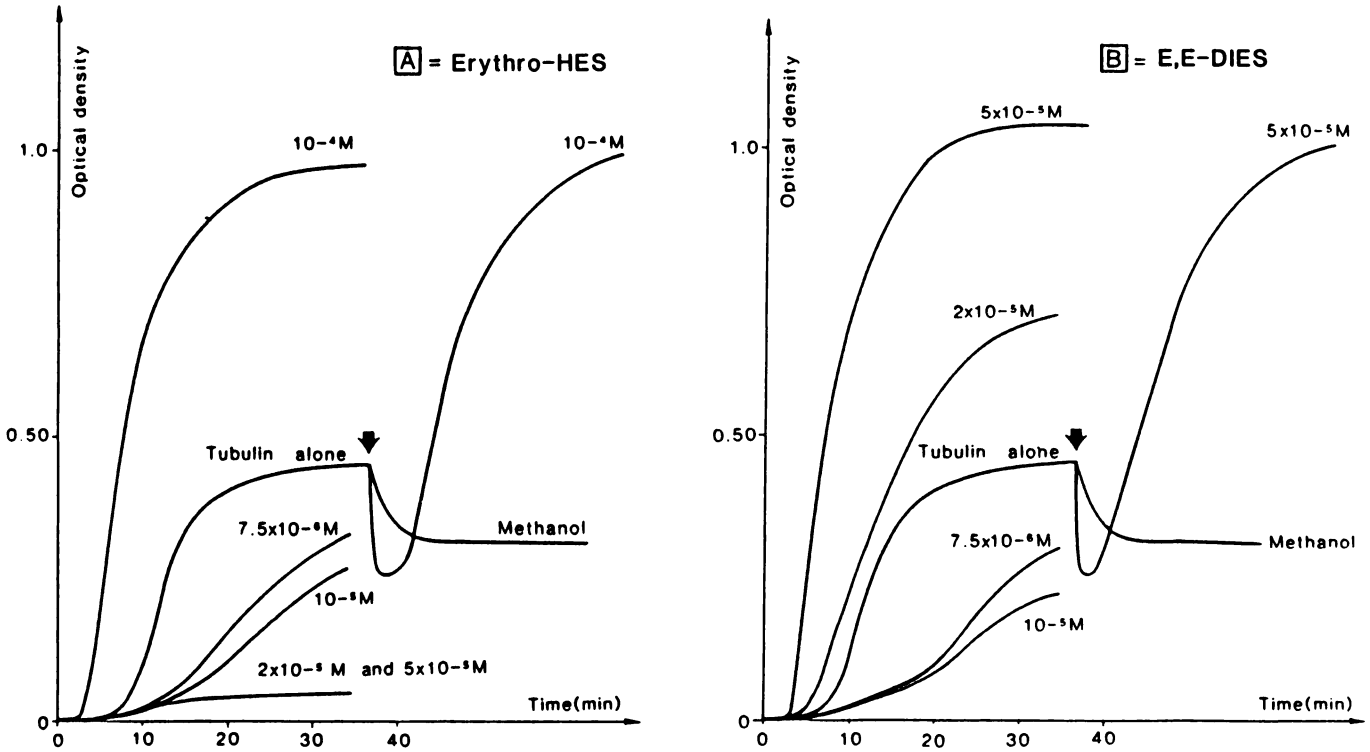

Fig. 4. Variation of optical density versus time with erythro-HES (A) and E,E-DIES (B) on tubulin (10^{-5} M) assembly and on preformed microtubules. The *arrows* correspond to the addition of 10^{-4} M erythro-HES (A) and 5×10^{-5} M E,E-DIES (B).

as proved by electron micrographs which showed a decrease in the number and the length of microtubules (not shown).

Then, with sufficient doses of drug (E,E-DIES, 2×10^{-5} M), the plateau value of optical density increased beyond that obtained with tubulin alone (Fig. 4). This increase in turbidity was due to the formation of aggregate structures (Fig. 5B). When the doses were further increased (erythro-HES 10^{-4} M, E,E-DIES $\geq 5 \times 10^{-5}$ M), the aggregates were replaced by twisted ribbon structures (Fig. 5, C and D).

Disassembly of preformed microtubules. The action of erythro-HES (10^{-4} M) and E,E-DIES (5×10^{-5} M) on preformed microtubules first induced a decrease in optical density and then an increase (Fig. 4). The decrease of optical density corresponds to a disassembly of microtubules, whereas the following increase traduces the appearance of structures similar to that in Fig. 5D, as proved by electron micrographs (Fig. 5E). Under the same conditions, E-DES induces only a disassembly of preformed microtubules.

Discussion

E-DES. In the case of E-DES, our findings are in good agreement with previous results (6–8). Tubulin 6S seems to be more sensitive to the effect of E-DES, as proved both by comparison of results in Table 1 with those in Fig. 3 and by the total disruption of microtubules formed from tubulin 6S.

Erythro-HES and E,E-DIES. Low doses of erythro-HES induce an inhibition of assembly similar to that obtained with

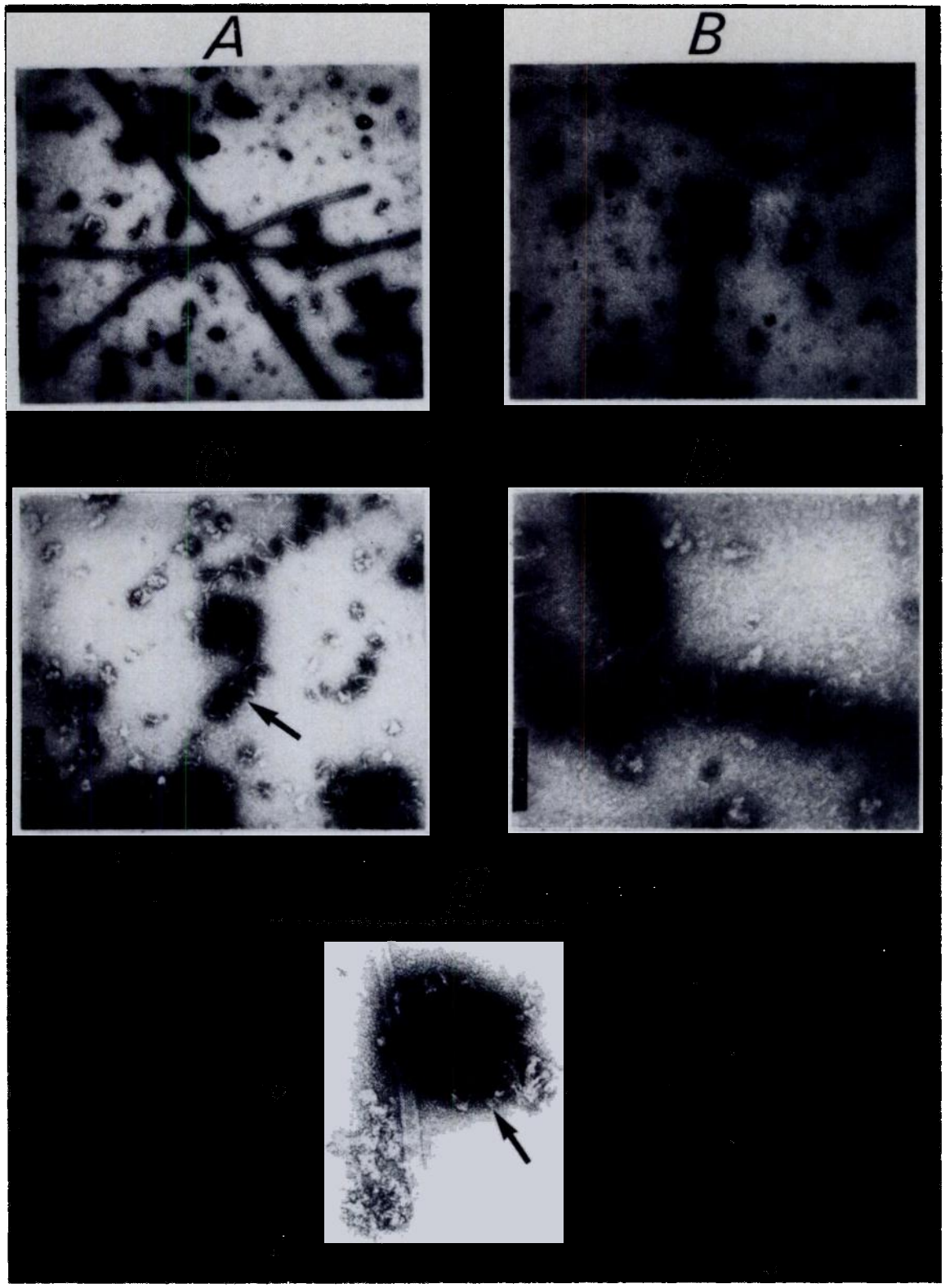

Fig. 5. Electron micrographs. A. Tubulin alone (10^{-5} M) (× 36,000). B. Tubulin with 2×10^{-5} M E,E-DIES (× 46,000). C and D. Tubulin with 6×10^{-5} M E,E-DIES (× 46,000 and × 100,000). E. Action of 10^{-4} M HES on preformed microtubules from tubulin 6S.

E-DES, with both MTP and tubulin 6S. E,E-DIES seems to be more active (see Table 1).

When the doses of erythro-HES and E,E-DIES are increased, aggregate structures appear. When the drug/tubulin 6S molar ratio is ≥5, the drugs induce the formation of twisted ribbon structures. Similar formations occur when the drugs disrupt the preformed microtubules.

The twisted ribbon structures seem to be protofilaments (Figs. 2 and 5) and appear only when the drug/tubulin 6S molar ratio is higher than 5, indicating that each molecule of tubulin

in the aggregate is probably bound to the drug. These observations evoke the ones described in the case of the vinblastine-tubulin interaction. Therefore, the hypothesis of Luduena *et al.* (20) concerning vinblastine-tubulin interactions seems appropriate in the case of E,E-DIES-tubulin interactions. Let us recall that Luduena *et al.* (20) think that "at low vinblastine concentrations, microtubule assembly is inhibited, but the ratio of liganded to unliganded tubulin is too low to permit aggregation of tubulin-vinblastine complexes. At high vinblastine concentrations, the ratio of liganded to unliganded tubulin is high enough to permit aggregation of these complexes." Thus, the organized formations that we observed may be due to sequences of tubulin-drug liganded molecules bound one to another but at angles incompatible with those normally found in a microtubule. The twisted ribbon structures observed in the case of E,E-DIES are compatible with a still tight and relatively specific interaction of poisoned tubulin molecules. In the case of erythro-HES, the twisted ribbon structures are obtained with higher doses than for E,E-DIES; this can be imputed to a looser tubulin-erythro-HES bond.

The action of the three drugs on tubulin may be correlated with their chemical structures (Fig. 1). Indeed, in the case of E-DES, which cannot induce the formation of twisted ribbon structures, it must be emphasized that the partial conjugation of aromatic cycles through the ethylene bond leads to a more rigid structure than in erythro-HES and E,E-DIES.

Metzler (21) asserted that *in vivo* toxicologic and oncogenic effects of estrogens require metabolic activation. Tsutsui *et al.* (22) showed that unscheduled DNA synthesis is induced by DES only after addition of an exogenous metabolic activator. Moreover, Li and Li (23) concluded that "one is compelled to consider a role for reactive intermediates formed during the oxidative metabolism of estrogenic compounds in these processes." Thus, it would not be too cautious to correlate directly our *in vitro* findings with the *in vivo* toxicity of these drugs.

However, the three drugs studied inhibit *in vitro* tubulin assembly that might be involved in their *in vivo* carcinogenic activity. Indeed, in the case of E-DES, it has been shown that this *in vitro* inhibitory effect on microtubule formation may be observed *ex vivo* since E-DES arrested mitotic spindle growth in hamster embryo cells (24). Tucker and Barrett (24) suggest that E-DES may produce neoplastic transformation by means of depolymerization of mitotic microtubules, thus inducing nondisjunction and aneuploidy.

Acknowledgments

We wish to thank Prof. J. M. Andreu for helpful and constructive criticism of the manuscript. We are grateful to Mrs. M. Vidalin and Mr. H. Bouteille for their skillful assistance. We thank Mr. M. Passerel and Mr. P. de Nantes for recording the electron micrographs.

Added in Proof

After submission of this manuscript we became aware of a study by Y. Sato *et al.* [*J. Biochem.* **101**:1247–1252 (1987)]. Using microtubular protein, they found, in agreement with our study, that *E,E*-dienestrol and erythro-hexestrol inhibited *in vitro* microtubule assembly and formed twisted ribbon structures.

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