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QUALITATIVE STUDY OF THE INTERACTION MECHANISM OF ESTROGENIC DRUGS WITH TUBULIN

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Abstract—Synthetic estrogenic drugs (*E*-diethylstilbestrol, erythro-hexestrol and *E,E*-dienestrol) inhibit tubulin assembly and erythro-hexestrol and *E,E*-dienestrol lead to the formation of twisted ribbon structures. For the inhibitory effect on tubulin assembly, estrogenic drugs seem to interact directly with tubulin 6S on site(s) analogous to the colchicine-site, but independent of the GTP- and vinblastine-sites. This binding does not involve tubulin tryptophanyl residues or sulfhydryl groups. The influence of temperature, calcium and magnesium on the formation of twisted ribbon structures induced by the binding of estrogenic drugs to microtubular protein and tubulin has also been studied. This formation is strongly magnesium-dependent whereas preformed twisted ribbon structures are calcium- and chilling-insensitive.

Several studies have dealt with the action of estrogenic drugs on the tubulin–microtubule system [1, 2]. It has been showed that some estrogenic drugs such as *E*-diethylstilbestrol (E-DES§), *E,E*-dienestrol (E,E-DIES) or erythro-hexestrol (erythro-HES) (Fig. 1) induce mitotic arrest and aneuploidy in Chinese hamster cells *in vitro* [2]. Consequently, Tucker and Barrett [1] suggested that E-DES may lead to neoplastic transformation by means of depolymerization of microtubules, thus inducing non disjunction and aneuploidy.

These findings led to several *in vitro* studies of the action of these drugs on microtubule formation [3–8]. In a previous paper [7], we confirmed this inhibitory effect. Furthermore we proved that, in tubulin preparation, E,E-DIES and erythro-HES induced the formation of loose aggregates composed of twisted ribbon structures. In contrast, E-DES did not exhibit this effect.

In the present paper, we attempted to study the formation of these twisted ribbon structures in more detail, particularly the influence of chemico-physical factors. We characterized the tubulin–estrogen interaction in the presence of Mg^{2+} and Ca^{2+} ions at various temperatures. Then, as concerns the estrogen binding site on tubulin, we demonstrated that tubulin tryptophan and sulfhydryl residues were not involved in this binding. Finally, we compared this site with the colchicine and vinblastine binding sites on tubulin.

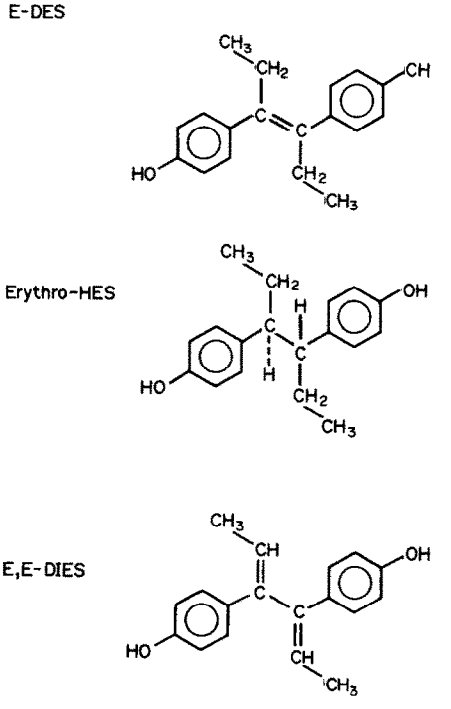

Fig. 1. Chemical structures of E-DES, erythro-HES and E,E-DIES.

MATERIALS AND METHODS

Reagents

MES and EGTA were purchased from Sigma. GTP and DTNB were from Fluka. E-DES, E,E-DIES and erythro-HES were purchased from Sigma and dissolved in methanol from Carlo Erba or in dimethylsulfoxide (DMSO) from Merck. E-DES, E,E-DIES and erythro-HES concentrations were measured spectrophotometrically. The extinction coefficient, ε, was determined by dissolving estrogenic drugs in methanol: $\varepsilon_{240} = 16{,}000 \pm 674\ M^{-1}\ cm^{-1}$

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§ Abbreviations: E-DES, *E*-diethylstilbestrol; E,E-DIES, *E,E*-dienestrol; MES, 2-*N* morpholinoethane sulfonic acid; EGTA, ethylene glycol bis (β-amino ethyl ether) *N,N'* tetracetic acid; DTNB, 5,5'-dithio (2 nitrobenzoate).

Table 1. Determination of sulfhydryl concentration of tubulin alone and with E,E-DIES (mean from three experiments ± SD)

Tubulin 6S concentration (μM)	E,E-DIES concentration (μM)	Number of SH
1.4	0	19.85 ± 0.05
1.4	50	20.10 ± 0.05
0.5	0	20.25 ± 0.05
0.5	50	21.05 ± 0.05
0.5	100	20.30 ± 0.05

for E-DES, $\varepsilon_{228} = 36{,}000 \pm 898\ M^{-1}\ cm^{-1}$ for E,E-DIES and $\varepsilon_{262} = 4360 \pm 345\ M^{-1}\ cm^{-1}$ for erythro-HES. [^{3}H]Vinblastine sulfate (sp. act. 17 Ci/mmol), ring [*methoxy*-^{3}H]colchicine (sp. act. 2.5 Ci/mmol) were purchased from Amersham International.

Preparation of tubulin

Microtubular protein (MTP) (tubulin and microtubule associated proteins) and tubulin 6S were obtained as previously described [7] from fresh pig brains using the method of Shelanski *et al.* [9] through three cycles of *in vitro* assembly (37°) and disassembly (4°). Tubulin 6S was purified from MTP according to the method of Weingarten *et al.* [10].

Tubulin concentration was determined spectrophotometrically at 280 nm using an extinction coefficient of 1.2 mL/cm/mg [11].

Assembly studies

Microtubular protein. MTP assembly was monitored by recording turbidity at 340 nm with a Beckman DU-70 spectrophotometer equipped with a thermostated 1 cm light path cell. Since the absorbance at 340 nm is proportional to the weight concentration of long microtubules, it can be used to quantitate plateau microtubule assembly. However, the turbidity is not quantitative for sheets and other protein aggregates [12].

The MTP concentration was 0.8 mg/mL. The assembly buffer was 0.1 M MES, 1 mM EGTA, 0.5 mM $MgCl_2$ ($6H_2O$), and GTP at various concentrations, pH 6.5. Assembly was initiated by a jump of temperature from 4° to 37°.

Thirty minutes after the beginning of assembly, samples were taken directly from the UV spectrophotometer cuvette and negatively stained with 1.5% uranyl acetate on carbon-coated grids. Electron micrographs were performed using a Philips EM 400 T microscope (Service Commun de Microscopie Electronique, Faculté des Sciences St-Jérôme, Marseille, France). Each experiment was performed in triplicate.

MTP assembly at various GTP concentrations. Estrogens were dissolved in methanol and the final assembly solution contained 1% solvent. The GTP concentration varied from 2 to 20 μM. For each GTP concentration, a control was carried out without drug. For these experiments, MTP was chromatographed on a Sephadex G25 column to rid the protein of GTP before assembly.

MTP assembly with dimethylsulfoxide. Estrogens were dissolved in DMSO and the final assembly solution contained 1% solvent. GTP (1 mM) was added in the assembly buffer. Controls were performed without drug.

Tubulin 6S. Typically, the tubulin 6S concentration was 10 μM. The assembly buffer was 50 mM MES, pH 6.7, 1 mM EGTA, 1 mM GTP and 3.4 M glycerol. The magnesium concentration varied from 1 to 10 mM. The drugs were dissolved in methanol and the final assembly solution contained 1% methanol. In all cases, controls were performed without drug.

Assembly was initiated by a jump of temperature from 4° to 37° and, 30 min later, electron micrographs were performed. Each experiment was done in triplicate.

Fluorescence

Relative fluorescence intensity of 2 mL of tubulin solution (1.1 μM) was measured at excitation and emission wavelengths of 295 and 330 nm, respectively, with slit widths of 5/5 nm. A wavelength of 330 nm corresponds to the maximum fluorescence emission by the protein. Estrogens do not absorb light at this wavelength. Then small aliquots (1–25 μL) of estrogen (final concentration: 2–60 μM) were added to the tubulin solution and the same measurements were done. Controls were performed with small aliquots of methanol without estrogen.

For the fluorescence experiments, the tubulin eluted from the phosphocellulose column was chromatographed on a Sephadex G25 column equilibrated in a phosphate/sucrose buffer [13]. Before each experiment, tubulin solutions were filtered through a Millex GV 0.22 μm unit (Millipore SA).

Correction for the inner filter effect, which is due only to light scattering by the protein solution, was performed at 295 nm using the method of Helene and Brun [14].

Determination of sulfhydryl concentration

We classically determined the free sulfhydryl groups (SH groups) using 5,5′-dithio(2-nitrobenzoate) (DTNB) [15].

A volume of 0.1 mL tubulin solution (14 μM) was added to 0.9 mL of phosphate buffer 0.05 M, pH 7.5, containing DTNB (1 mM) and urea (7 M). Sulfhydryl concentration was determined from the absorbance of the solution at 412 nm using an extinction coefficient of $13.6\ mM^{-1}\ cm^{-1}$. We also measured the amount of sulfhydryl groups in the presence of various estrogen concentrations.

Binding assay of [^{3}H]*vinblastine and* [^{3}H]*colchicine to tubulin in the presence of estrogen*

The binding of vinblastine (VLB) and colchicine in the presence of E-DES or E,E-DIES was performed using the DEAE filter paper method [16]. Two DEAE 81 disks (Whatman, diameter 2.5 cm) were washed with the cold buffer D: 10 mM NaH_2PO_4, 10 mM $MgCl_2$, 0.1 mM GTP, pH 6.8.

The estrogen solution (10 μL, methanol solution, final concentration 80–100 μM) was mixed with 30 μL tubulin (final concentration 2.6–5 μM) and buffer D. Various concentrations of labelled vinblastine

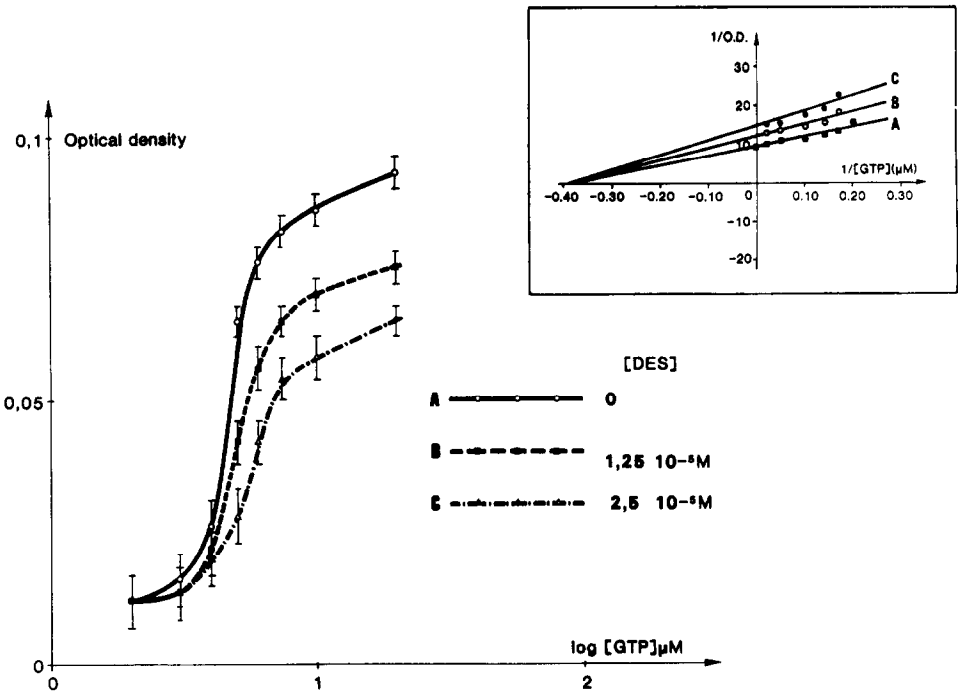

Fig. 2. Effect of GTP on MTP assembly in presence (12.5 and 25 µM) or absence of E-DES. The optical density (O.D.) values after 30 min of MTP assembly was plotted versus the logarithm of GTP concentration. The inset showed the double-reciprocal plot representation of these results.

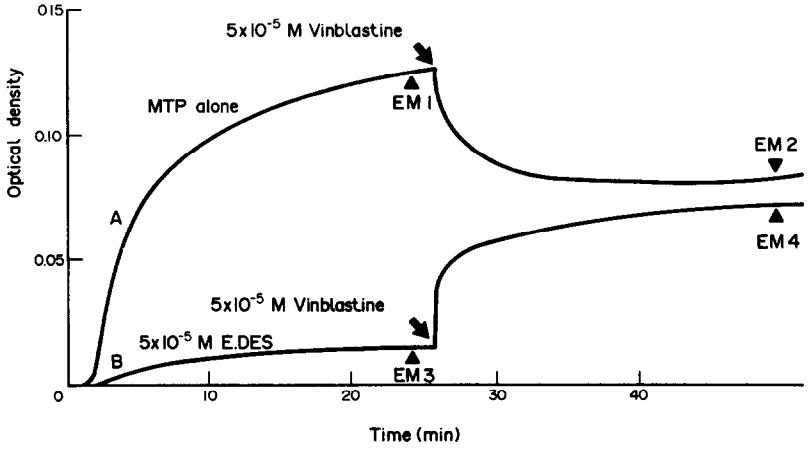

Fig. 3. Action of vinblastine after MTP assembly in presence of estrogen: turbidimetric assay. We used estrogen doses (50 µM) which completely inhibited the MTP assembly (curve B). Twenty minutes later, 50 µM vinblastine were added. The variation of optical density versus time was recorded at 340 nm and 37°. A control was performed without estrogen (curve A).

(final concentration 8–60 µM) or colchicine (final concentration 4–12 µM) were then added. The final volume was 120 µL.

After a contact time of 30 min for vinblastine and 2 hr for colchicine at 37°, aliquots were directly applied on the disks. These disks were washed three times with 3 mL of cold buffer D by mild suction. The radioactivity of the filter papers was determined in 10 mL of Biofluor solution using a Beckman LS 1701 Counter.

In all cases, a control was performed without the estrogen solution in order to determine the amount

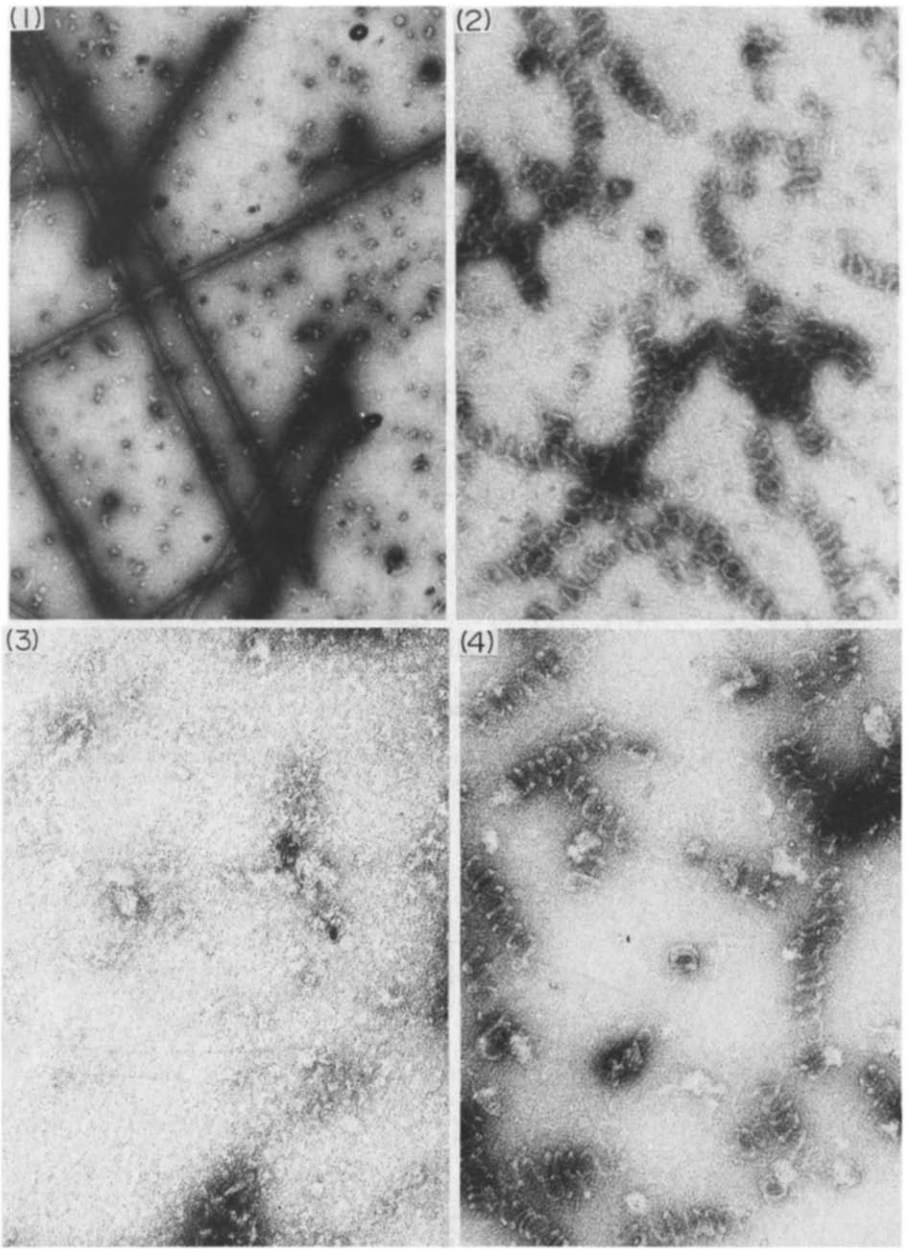

Fig. 4. Action of vinblastine after MTP assembly in presence of estrogen: electron micrographs (EM). (1) MTP alone (×26,000). (2) Addition of 50 μM vinblastine on preformed microtubules (×72,000). (3) MTP with 50 μM E-DES (×72,000). (4) Addition of 50 μM vinblastine after MTP assembly with 50 μM E-DES (×72,000).

of labelled product bound to tubulin. Each experiment was done in triplicate.

Theoretical percentage of colchicine binding to tubulin was determined assuming a binding constant value of $10^7 M^{-1}$ [17].

RESULTS

The three drugs showed no fluorescence in the range of protein emission when excited at 295 nm, even in the presence of tubulin. The tubulin fluorescence emission spectrum was not modified by these drugs. Moreover, no significant quenching of the protein intrinsic fluorescence was observed after addition of E-DES, E,E-DIES or erythro-HES aliquots.

Based on the sulfhydryl concentrations reported in Table 1, it can be seen that E,E-DIES did not block SH groups. Similarly, the two other estrogenic drugs did not modify the sulfhydryl concentration of

Table 2. Effect of E-DES on colchicine binding to tubulin (2.6 μM)

Colchicine concentration (μM)	E-DES concentration (μM)	Radioactivity (dpm)	Colchicine bound (%)
4	0	5580 ± 85*	92
	80	4003 ± 237	65
	100	3359 ± 64	55
8	0	6285 ± 173	96
	80	5029 ± 250	77
	100	4280 ± 374	65
12	0	7426 ± 208	99
	80	6162 ± 314	83
	100	6356 ± 255	85

The percentage of bound colchicine is evaluated assuming a binding constant value of $10^7\ M^{-1}$ [17].
* Mean dpm from three experiments ± SD.

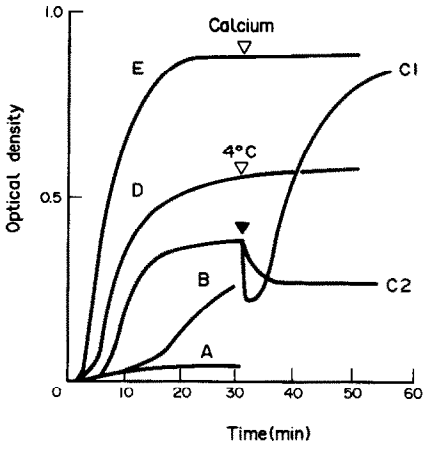

Fig. 5. Effect of erythro-HES and E,E-DIES on tubulin 6S (10 μM) assembly and preformed microtubules and effect of calcium and cold temperature on twisted ribbon structures. The tubulin 6S assembly was carried out as described previously ($MgCl_2$ 10 mM). The two lower curves (A and B) represent the inhibitory effects of 20 μM erythro-HES and 7.5 μM E,E-DIES, respectively. The three upper curves (C_1, D and E) represent the formation of twisted ribbon structures. This formation was obtained by addition of 20 μM E,E-DIES (curve D) or 50 μM E,E-DIES (curve E) before the polymerization. Curve C_1 represents the formation of twisted ribbon structures from preformed microtubules. When the plateau value of optical density was reached, 3 μL of estrogenic drug in methanol solution were added. The arrow corresponds to the addition of 100 μM erythro-HES in methanol solution (curve C_1). Control with methanol alone is shown on curve C_2. The effect of calcium on twisted ribbon structures is shown on curve E. Calcium (3 mM) was added on preformed twisted ribbon structures obtained with 50 μM E,E-DIES. The effect of cold temperature is reported on curve D. The twisted ribbon structures obtained with 20 μM E,E-DIES were chilled 30 min at 4°.

tubulin (not shown). The number of SH groups found in this study, about 20, was in good agreement with the values of literature [18].

In Fig. 2, we plotted the optical density plateau value versus the logarithm of GTP concentration for several MTP assembly experiments with and without various E-DES concentrations. Similar results were obtained with E,E-DIES and erythro-HES (not shown). On the right hand of the figure we represented the same results using a double-reciprocal plot.

In Fig. 3, curve A presents the classical disruptive effect of vinblastine (50 μM) on preformed microtubules, i.e. a decrease in optical density. The action of vinblastine (50 μM) on a mixture of MTP and E-DES (50 μM) led to an increase in optical density (Fig. 3, curve B). Figure 4 shows the corresponding electron micrographs.

The amounts of [^{3}H]vinblastine bound to tubulin (5 μM, vinblastine from 8 to 60 μM) were not modified by the estrogenic drugs (E-DES or E,E-DIES from 5 to 100 μM) in our experimental conditions.

Table 2 shows the effect of estrogenic drugs on labelled colchicine binding to tubulin. We observed that E-DES decreased the colchicine binding to tubulin. For a given E-DES concentration, the higher the colchicine concentration, the lower the decrease in colchicine binding. Indeed, with a 4 μM colchicine concentration and 100 μM of E-DES, the percentage of bound-colchicine was 55% and reached 85% when the colchicine concentration was increased to 12 μM.

Our previous results showed the formation of twisted ribbon structures by E,E-DIES and erythro-HES with tubulin at 37° [7]. The kinetics of assembly are shown in Fig. 5; E,E-DIES was the most potent on a comparative molar basis.

Calcium and chilling had no effect on preformed twisted ribbon structures. Indeed, adding 3 mM $CaCl_2$ or a half an hour chilling at 4° had no disruptive effect on these structures, as proved by electron microscopy and turbidimetry (Fig 5).

Table 3 shows the effect of magnesium concentration on the formation of these structures. With magnesium concentrations from 1 to 10 mM, electron micrographs of the tubulin control showed microtubules with normal ultrastructure after 30 min at 37° (Fig. 6a). At 10 mM $MgCl_2$, 75 μM E,E-DIES or erythro-HES induced the formation of twisted ribbon structures [7]. When the magnesium concentration was decreased from 10 to 5 mM, the same structures remained with E,E-DIES and some rings appeared (arrows, Fig. 6b). With erythro-HES, the electron micrographs showed similar twisted ribbon structures, some rings and microtubules (Fig. 6c). When magnesium concentration was decreased to 1 mM, some twisted ribbon structures remained with E,E-DIES but the bulk of the preparation was composed of normal and apparently open microtubules (Fig. 6d). With erythro-HES, twisted ribbon structures did not remain (not shown).

Results of experiments performed in the presence of DMSO are shown in Table 4. In the same table, we recall the results obtained without DMSO.

DISCUSSION

At an excitation wavelength of 295 nm, only

Table 3. Effects of magnesium concentration on the formation of polymeric structures induced by 75 μM E,E-DIES or erythro-HES

Magnesium concentration	1 mM	5 mM	10 mM
Tubulin 6S control	Microtubules	Microtubules	Microtubules
E,E-DIES	TRS*	TRS	TRS
	Normal & open microtubules	Rings	
Erythro-HES	Microtubules	TRS	TRS
		Microtubules	
		Rings	

* TRS, twisted ribbon structures.

tryptophanyl residues are involved in the fluorescence emission of tubulin. Thus, our results indicate that tryptophanyl residues are not implicated in the binding of estrogenic drugs to tubulin since the addition of E-DES, E,E-DIES or erythro-HES did not modify the fluorescence of the protein.

Moreover, E-DES, E,E-DIES and erythro-HES did not bind to sulfhydryl groups on tubulin, as the number of SH groups available on tubulin was not modified by the drugs. Similarly, no change in the number of SH groups was detected after incubation of tubulin with colchicine [19].

A previous study showed that the inhibitory effect of E-DES on Taxol-induced tubulin assembly could be reversed by addition of GTP [5]. Thus, we considered a possible interaction between GTP and estrogenic drugs and performed experiments to study this possible competition. Results plotted in Fig. 2 show classical curves of non competitive antagonism between the two species of ligands. It therefore seemed that the exchangeable GTP site was not involved in the binding of estrogens to tubulin.

Competition experiments with labelled vinblastine revealed that estrogenic drugs did not displace vinblastine. In addition, the vinblastine-induced formation of spirals is shown in Figs 3A and 4 (panel 2). Figure 4 (panel 3) shows the total inhibition of microtubule formation by E-DES. Figure 4 (panel 4) evidenced that the vinblastine-induced formation of twisted ribbon structures occurs even when MTP assembly is totally inhibited by estrogens. These two findings seem to indicate that there is no competition between the low and high affinity binding sites of vinblastine and estrogenic drugs in our experimental conditions.

In contrast, our results showed that E-DES hindered the binding of colchicine on pure tubulin. These findings confirm those of Sharp obtained with MTP, i.e. tubulin and microtubule associated proteins [4]. This suggests a direct binding of estrogen to tubulin, which is in good agreement with the results of Lin *et al.* [20] obtained with several compounds structurally similar to E-DES.

Magnesium enhances the formation of twisted ribbon structures by E,E-DIES and erythro-HES (Table 3). The effects may be due to an action on estrogen molecules and/or an effect on tubulin. The first hypothesis is unlikely because the formation of a complex between Mg^{2+} ions and estrogen molecules seems difficult: E,E-DIES is a conjugated molecule and is therefore unable to fold itself to complex Mg^{2+}. The second hypothesis is more convincing because it has been reported that magnesium appears to play a role in the polymorphism of tubulin polymers. Mg^{2+} ions seem to reduce the net charge of the protein and thus they favor tubulin–tubulin interactions [21]. Moreover, Haskins *et al.* [22] showed that vincristine-induced spiral formation is stimulated by high magnesium concentration. Also, in this case, the stimulation by Mg^{2+} ions results from cationic effects [22]. Thus, one may postulate that an analogous effect is involved in our case.

The twisted ribbon structures obtained with E,E-DIES and erythro-HES are cold- and calcium-insensitive. Inversely, the abnormal filamentous formations obtained with colchicine are classically cold- and calcium-sensitive [23–26]. The insensitivity to cold of estrogen-induced twisted ribbon structures is in contradiction with previous results obtained by Sakakibara *et al.* [8]. The discrepancy may arise from a different composition of the various buffers, namely the presence of glycerol and that of DMSO, the influence of which will be discussed below. In this regard, Saltarelli and Pantaloni [26] have shown for colchicine that glycerol stabilizes the formation of colchicine-induced structures, whereas Andreu *et al.* [25] have obtained cold labile structures in a different buffer. However, only a high percentage of glycerol can induce a complete insensitivity of ribbon structures to cold: thus as concerns colchicine, a 50% (v/v) concentration of glycerol is required [26]. This well-known stabilizing effect of glycerol on proteins is, in our case, likely to be responsible for the lower sensitivity of estrogen twisted ribbon structures to cold. In our experimental conditions, the concentration of glycerol was 25% (v/v) inferring that estrogen twisted ribbon structures are more stable to low temperatures than are colchicine structures.

The previous results, as compared to those obtained in competition experiments (see above), seem to indicate that estrogenic drugs inhibit the assembly of tubulin by acting on a site analogous to the colchicine site. This hypothesis is consistent with previous works showing that estrogenic drugs had a mechanism of mitotic arrest similar to that of colchicine [2, 27, 28].

But there are also many similarities between the

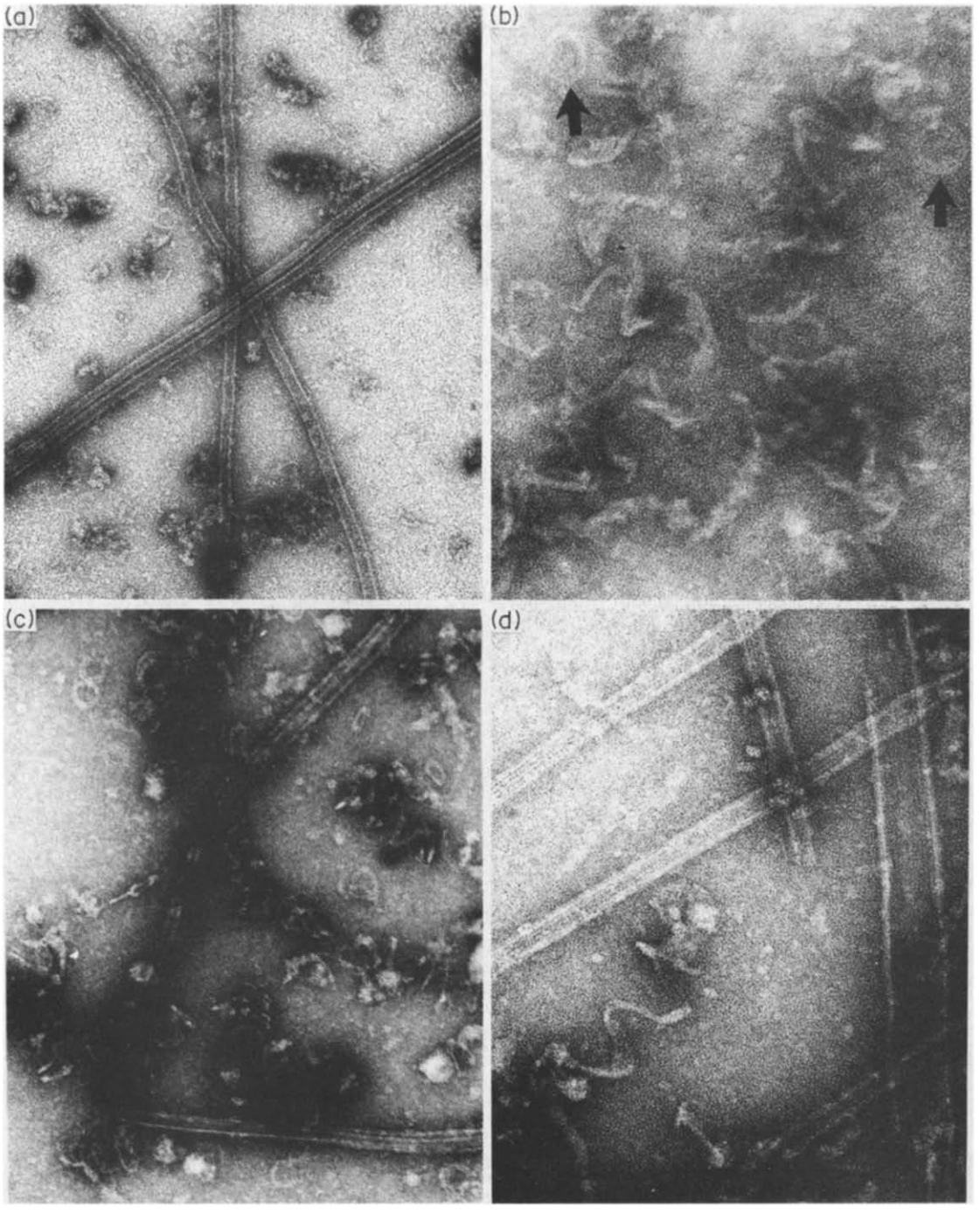

Fig. 6. Electron micrographs. (a) Tubulin alone (10 μM), 10 mM $MgCl_2$ (×72,000). (b) Tubulin with 75 μM DIES, 5 mM $MgCl_2$ (×175,000). (c) Tubulin with 75 μM HES, 5 mM $MgCl_2$ (×83,000). (d) Tubulin with 75 μM DIES, 1 mM $MgCl_2$ (×135,000).

curly structures obtained with estrogenic drugs and vinblastine, even though the site of binding is different since estrogens do not displace vinblastine. However, vinblastine-induced spirals are cold- and calcium-insensitive [29]. These similarities strengthen the hypothesis we formulated in our previous paper [7] that, as in the case of vinblastine [30], the twisted ribbon structures obtained with estrogenic drugs may be due to sequences of tubulin–drug liganded molecules bound to one another but at angles incompatible with those normally found in a microtubule. This could result from a change in the shape of the tubulin molecule upon ligand binding, which does not involve tryptophanyl or sulfhydryl

Table 4. Action of erythro-HES and E,E-DIES, dissolved in methanol or DMSO, on MTP (0.8 mg/mL) assembly

Concentration (μM)	Erythro-HES		E,E-DIES	
	Methanol	DMSO	Methanol	DMSO
	Inhibition %		Inhibition %	
25	20.0 ± 2.0	8.0 ± 1.5	65.0 ± 6.0	62.0 ± 5.0
50	78.0 ± 4.5	43.0 ± 3.0	53.0 ± 3.0	53.0 ± 3.0
75	94.0 ± 5.0	57.0 ± 4.0		

Percentages of inhibition (mean from three experiments ± SD) were calculated from the variation of optical density of the solution after 30 min at 37°C

residues or from plain steric hinderance due to the bulk of the molecule.

The action of DMSO may be the cause of the slight differences observed between the results of Sakakibara *et al.* [8] and ours. Indeed, DMSO drastically modified the inhibitory effect of erythro-HES (Table 4). We observed that the inhibition of assembly induced by erythro-HES was less pronounced when the drug was dissolved in DMSO. With E,E-DIES, the dissolution solvent (methanol or DMSO) seemed to have no influence on the inhibitory effect of the drug on MTP assembly. A further evidence of the effect of DMSO may be found in the work of Donoso *et al.* [31] who showed that DMSO hindered the formation of vincristine-induced spirals.

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