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THE EFFECT OF ESTRAMUSTINE DERIVATIVES ON MICROTUBULE ASSEMBLY *IN VITRO* DEPENDS ON THE CHARGE OF THE SUBSTITUENT

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Abstract—Estramustine, and derivatives of estramustine with a charged substituent at position 17 on the estrogen moiety, have been investigated for their effects on bovine brain microtubules *in vitro*. The negatively charged estramustine phosphate has been found previously to be a microtubule-associated protein (MAP)-dependent microtubule inhibitor [Wallin M, Deinum J and Fridén B, *FEBS Lett* **179**: 289–293, 1985]. In the present study the binding of estramustine phosphate to MAP2 and tau was investigated. Both these MAPs were found to have two to three binding sites for estramustine phosphate which is compatible with the reported number of basic amino acid repeats of these MAPs, considered to be the ultimate tubulin binding domains. The K_d for the binding of estramustine phosphate to MAP2 was estimated to be 20 μM at 4°, and for the binding of tau, 200 μM. The rate of dissociation was very low ($T_{1/2} > 2$ hr), which indicates that the binding of estramustine phosphate may stabilize the protein–drug complex by changing the protein conformation. Two new negatively charged estramustine derivatives, estramustine sulphate and estramustine glucuronide, were found to be similar MAP-dependent microtubule inhibitors. The concentration for 50% inhibition of assembly was 100 μM for the sulphate derivative, the same as found previously for estramustine phosphate, and 250 μM for the more bulky estramustine glucuronide. A positively charged derivative, estramustine sarcosinate, did not inhibit microtubule assembly or alter the composition of the coassembled MAPs. The morphology of the microtubules was, however, affected. The uncharged estramustine bound to both tubulin and MAPs, but no effects were seen on microtubule assembly, the composition of coassembled MAPs or the microtubule morphology. Our results suggest that only negatively charged estramustine derivatives have a MAP-dependent microtubule inhibitory effect. The two new negatively charged derivatives could therefore be valuable tools in the study of tubulin-MAP interactions. The results also confirm that these interactions between tubulin and MAPs are mainly electrostatic.

The microtubule-associated proteins (MAPs§) are highly diverse proteins [1] attached externally to the tubulin structure. Two main categories are usually distinguished: one of high molecular mass proteins around 300 kDa, MAP1 and MAP2; and one of smaller proteins around 60 kDa, called tau. These proteins bind to tubulin and stimulate the assembly of microtubules. Both MAP2 and tau bind to the acidic C-terminals of tubulin [2]. Moreover, a cationic tubulin-binding domain common to the C-terminals of MAP2 and tau has been identified recently [3–5], with a high degree of homology. This tubulin-binding domain of both MAP2 and tau was found to consist of three repetitive sequences of 18 amino acids [5] and to assemble with tubulin.

Estramustine phosphate (Fig. 1) has been found previously to inhibit microtubule assembly *in vitro* by binding to the microtubule-associated proteins [6–8]. Since classical anti-microtubule drugs, such as colchicine and vinblastine, all bind to tubulin (for a review see Ref. 9), the main component of microtubules, estramustine phosphate must belong to a new group of microtubule inhibitors.

The existence of a common tubulin-binding sequence in MAP2 and tau corresponds with our previous results which show comparable inhibition by estramustine phosphate (50% inhibition of assembly at around 100 μM) of microtubules reconstituted from tubulin with either MAP2, tau or the tubulin binding fragment of chymotrypsin-digested MAP2 [8]. The binding of estramustine phosphate was therefore localized to the tubulin-binding part of the MAPs. It is of interest to relate the number of tubulin-binding sites with the number of binding sites for estramustine phosphate. Preliminary results have shown that only a limited number of estramustine phosphate molecules bind to each MAP [8]. Therefore, a more thorough binding study of estramustine phosphate was performed.

Estramustine phosphate differs from estramustine by only the phosphate group at position 17 of the estrogen moiety (Fig. 1). However, in the literature the two molecules have been regarded as identical with respect to interaction with the microtubule system. This is probably not correct since these two compounds have highly different polarities. Estramustine phosphate is 1000 times more soluble

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§ Abbreviations: MAPs, microtubule-associated proteins; Pipes, 1,4-piperazinediethanesulphonic acid; EDTA, ethylenedinitrilo tetraacetic acid; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; DMSO, dimethyl sulphoxide; MES, 4-morpholine ethane sulphonic acid.

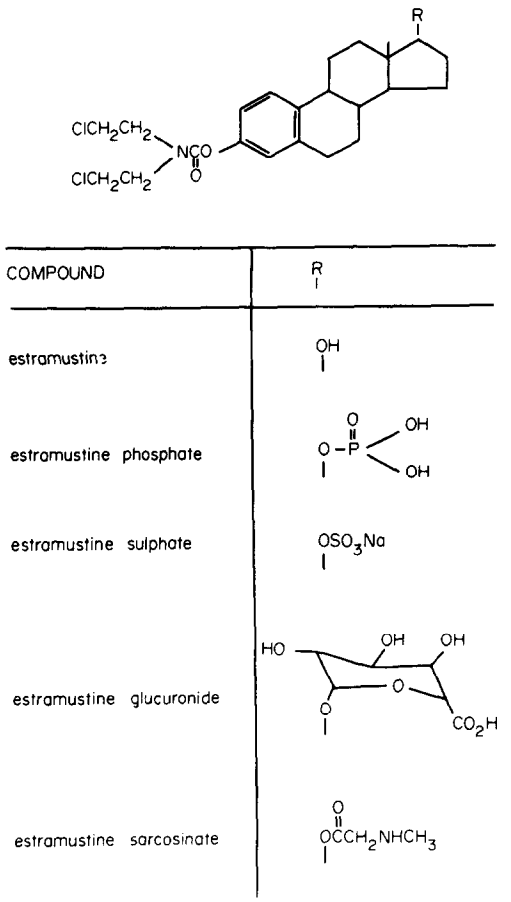

Fig. 1. Structure formula of estramustine and polar derivatives of estramustine.

in water than estramustine [10]. The binding of estramustine to microtubule proteins was therefore investigated further, as well as its effect on microtubule assembly.

The phosphate group of estramustine phosphate is negatively charged at pH 6.8, the pH used in our *in vitro* assay. This suggests that the anionic phosphate group is most probably involved in the binding of estramustine phosphate to the cationic tubulin-binding domains of MAPs. In order to determine if the charge of the substituent is of importance for the effect on microtubules, three other derivatives of estramustine were studied (Fig. 1). Two negatively charged estramustine derivatives, estramustine sulphate and estramustine glucuronide, and one positively charged derivative, estramustine sarcosinate, were used. Their effects were compared with the negatively charged estramustine phosphate and with the uncharged estramustine.

The effect of the compounds was found to be charge-dependent. Only the negatively charged estramustine derivatives inhibited the assembly of microtubules in an MAP-dependent way. Our results therefore provide further support for the finding that the interaction between tubulin and MAPs is mainly ionic.

MATERIALS AND METHODS

Purification of microtubule proteins. Microtubule proteins were prepared from bovine brain in the absence of glycerol by two cycles of assembly–disassembly [8]. Tubulin was separated from MAPs by phosphocellulose chromatography in the presence of 0.5 mM $MgSO_4$ [11].

Microtubule-associated protein 2 and tau were purified by heat treatment of purified microtubule proteins, followed by gel filtration [12].

Assembly. Microtubule proteins were assembled in 0.1 M Pipes, 0.5 mM $MgSO_4$ and 1 mM GTP. In the study of estramustine sarcosinate 0.1 M MES, 0.5 $MgSO_4$, 1 mM GTP and 0.01% Tween 20 were used, since estramustine sarcosinate precipitates in Pipes buffer. There was no obvious difference in the characteristics of microtubule assembly between the two systems. The assembly was started by addition of concentrated microtubule proteins at 4° to a preheated buffer at 37°, or by addition of the buffer–microtubule protein solution to the measuring cell at 37°. Inhibitors or an equivalent amount of solvent were either added from a stock solution to the buffer at 37° or preincubated with the proteins at 4°. Assembly was followed by turbidity [8], and in some cases also by viscosity [13].

Chemicals. Estramustine, estradiol-3-[*N,N*-bis-(2-chloroethyl)carbamate]; estramustine phosphate, estradiol-3-[*N,N*-bis-(2-chloroethyl)carbamate] 17β-phosphate; estramustine 17β-sulphate; estramustine 17β-D-glucopyranosiduronic acid (estramustine glucuronide); estramustine 17-*N*-methylglycinate (estramustine sarcosinate) and the radiolabelled estramustine and estramustine phosphate (2,4,6,7-^{3}H) isotopes were all synthesized and kindly given by Pharmacia LEO Therapeutics AB. Stock solutions of estramustine phosphate and estramustine sarcosinate were made in distilled water, estramustine sulphate and glucuronide derivatives in 99% ethanol and estramustine in DMSO. Taxol was a gift from Dr M. Sufness at the National Institutes of Health (Bethesda, MD, U.S.A.) and was added from a 3 mM stock solution in DMSO. The final concentration of ethanol or DMSO in the microtubule assay did not exceed 1%, unless otherwise indicated. In all experiments with estramustine phosphate, the solution contained 1 mM EDTA added initially or at steady state of assembly to avoid precipitation of an insoluble Mg^{2+}–estramustine phosphate complex [7]. This addition of EDTA to microtubule proteins isolated in the presence of Mg^{2+} had no obvious effect on the assembly characteristics.

Binding assays. Estramustine phosphate and estramustine were incubated for 10 min at 4° with either tubulin, MAP2 or tau. Thereafter, quantitation of bound and free drugs was carried out after gel filtration on a Biogel P-6DG column (1.6 × 10 cm), by measuring the protein concentration at 280 nm and the drug content by scintillation in each fraction [6].

SDS–PAGE. SDS–PAGE was performed according to the method of Laemmli [14] on a linear (5–12%) polyacrylamide gradient, as described previously [8].

Protein concentrations. Microtubule protein concentrations were determined according to Lowry *et al.* [15], using bovine serum albumin as a standard. The concentrations of the separated microtubule proteins were determined by measuring the absorbance at 280 nm using the extinction coefficients for tubulin (1.2 mL/mg cm; M_r = 110,000 daltons; [16]), tau (2.8 mL/mg cm; M_r = 60,000 daltons; [17]) and MAP2 (3.2 mL/mg cm; M_r = 280,000 daltons; [12]).

When required the proteins were concentrated by pressure dialysis with an Amicon ultrafiltration cell and a PM30 membrane at 4°.

Electron microscopy. Negatively stained microtubule specimens for electron microscopy were prepared as described in [8].

Statistical methods. The experimental data on binding of estramustine phosphate to purified MAP2 and tau were used in parameter determination by non-weighted non-linear regression analysis, using the Marquardt algorithm.

RESULTS

Estramustine phosphate binds specifically to MAP2 and tau

The binding of estramustine phosphate to both MAP2 and tau, measured after 10 min of incubation with the ^{3}H-labelled compound, was found to be saturable. This incubation time was chosen in order to correlate the incorporation with the instantaneous effect of estramustine phosphate on microtubule assembly seen *in vitro*. Furthermore, preliminary results show that the binding is rapid, since 50% of the incorporation at 10 min is seen already after 1 min of incubation, and extension of the incubation to 20 min increases the binding by only 20% (B. Fridén, unpublished results). Scatchard analysis gave an approximate K_d of 20 μM of 4° for the binding of estramustine phosphate to MAP2, with 2.5 binding sites (SE = 0.2) (Fig. 2A). The same results were obtained if estramustine phosphate with a different specific activity was used. Estramustine phosphate bound to isolated tau with a K_d of approximately 200 μM, with 1.5 binding sites (SE = 0.5) (Fig. 2B). Analogous experiments with purified tubulin confirmed earlier results [6] showing that only negligible amounts, less than 0.05 mol estramustine phosphate/mol tubulin, were bound (not shown).

Estramustine phosphate dissociates slowly from MAP2

In spite of the apparent low affinity of estramustine phosphate for MAP2, the drug–protein complex could be isolated. No significant dissociation of the complex was observed during gel filtration, since no tailing was seen. In order to estimate the dissociation rate, the first isolated estramustine phosphate–MAP2 complex was incubated for a further 60 min at 4° and the remaining MAP2–estramustine phosphate complex was isolated a second time by the same gel filtration procedure. The amount of estramustine phosphate bound to MAP2 decreased herewith by only 28 ± 16%, (N = 3). The dissociation rate was thus very slow with $T_{1/2} > 2$ hr. Furthermore, when the first isolated estramustine phosphate–MAP2 complex was incubated with 100 μM unlabelled estramustine phosphate for 10 min, and rechromatographed, the amount of bound [^{3}H]-estramustine phosphate decreased by only 23 ± 10% (N = 3), further indicating a firm binding between estramustine phosphate and MAP2.

Estramustine binds to tubulin, MAP2 and tau, without any effect on microtubule assembly

Estramustine was found to bind to tubulin (0.2 ± 0.1 mol/mol, N = 2), MAP2 (2.7 ± 1.0 mol/mol, N = 4) and tau (1.0 ± 0.5 mol/mol, N = 4) at 4°. Binding was not saturated in the range measured (1–20 μM) (Fig. 3A–C). It was not possible to perform measurements at higher concentrations as estramustine precipitated.

Assembled microtubules, incubated with 20 μM estramustine for 10 min at 4° and assembled thereafter at 37°, were found to bind 7.2 ± 5.0 nmol estramustine/mg microtubule protein (N = 4). However, no effect was seen on assembly kinetics or extent of assembly when both turbidity (Fig. 4) and specific viscosity (not shown) were measured. The critical concentration for assembly, protein composition of assembled microtubules and microtubule morphology (not shown) were unaffected by 20 μM estramustine. Measurement of all these parameters showed clearly a lack of effect of estramustine on microtubule assembly *in vitro*.

Phosphocellulose-purified tubulin was incubated with 20 μM estramustine for 10 min at 4° and was assembled thereafter with taxol at 37°. A binding of 5.2 ± 0.9 (N = 2) nmol estramustine/mg tubulin was measured. This was higher than that measured when estramustine was added at steady state [2.0 ± 0.1 (N = 2) nmol estramustine/mg tubulin], showing further that estramustine does not affect microtubule assembly.

No binding competition between estramustine and estramustine phosphate

Addition of estramustine phosphate to microtubules at steady state, assembled in the presence of 20 μM estramustine, induced original, instantaneous and complete disassembly similar to that occurring in the absence of estramustine (Fig. 4). Moreover, the presence of estramustine did not reduce the incorporation of estramustine phosphate (not shown), indicating that there is no competition between the two substances.

Anionic estramustine derivatives inhibit microtubule assembly

The effect of a negatively charged substituent at position 17 of estramustine on the inhibition of microtubule assembly was studied further with two anionic derivatives. Analogous to estramustine phosphate ($pK_a = 3.6$), estramustine sulphate ($pK_a < -1$) and estramustine glucuronide ($pK_a =$ 3.2; personal communication, Bertil Hansen, Pharmacia LEO Therapeutics AB) were deprotonated at the pH of the assay, pH 6.8. Since neither the sulphate or the glucuronide derivatives precipitated in the presence of Mg^{2+}, no addition of EDTA was needed in the assembly assay. Both derivatives inhibited the assembly of microtubules with the same

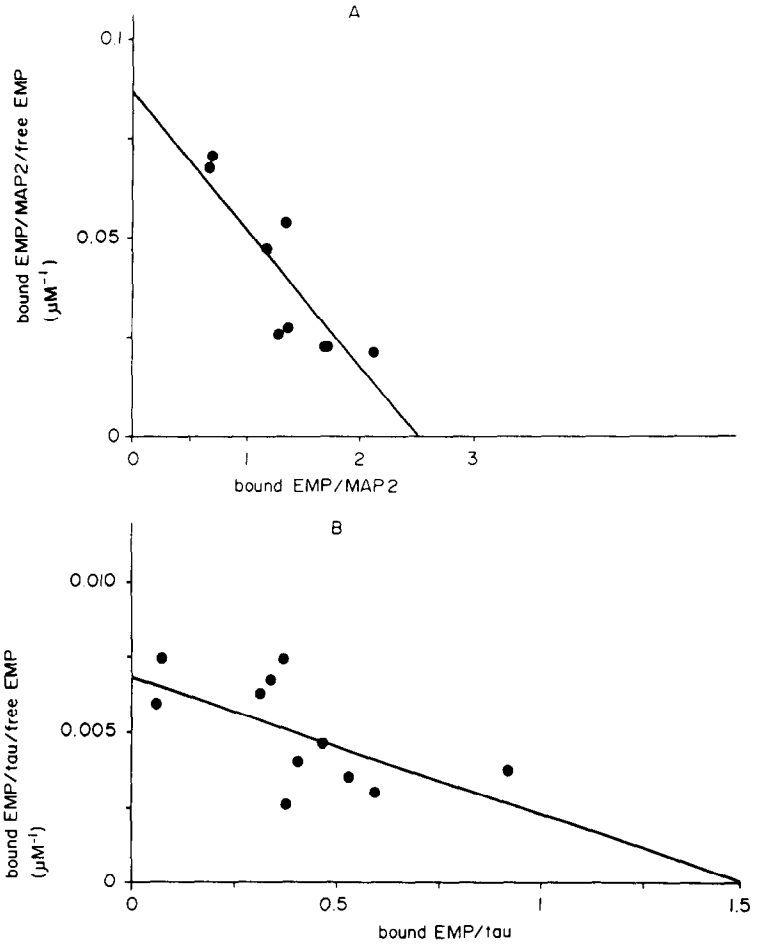

Fig. 2. Binding of estramustine phosphate to purified microtubule-associated protein 2 and tau. Microtubule-associated proteins were incubated with different concentrations of estramustine phosphate and a fixed amount of [^{3}H]estramustine phosphate at 4° for 10 min in 0.1 M Pipes, 0.5 mM $MgSO_4$, 1 mM EDTA, pH 6.8. Free and protein-bound drug were separated on a Biogel column (1.6 × 10 cm). Binding was determined by measuring the protein content at 280 nm and the amount of [^{3}H]estramustine phosphate in each fraction, and was analysed further by a Scatchard plot. The lines were drawn by regression analysis. (A) MAP2 (0.023 mg/mL) was incubated with different concentrations (10–100 μM) of estramustine phosphate and with a constant amount of [^{3}H]estramustine phosphate. (B) Tau proteins (0.023 mg/mL) were incubated with different concentrations (10–250 μM) of estramustine phosphate and a constant amount of [^{3}H]estramustine phosphate.

characteristics as found for estramustine phosphate (Fig. 5A and B). In contrast to estramustine phosphate, disassembly induced by these two derivatives was lower when the drugs were added at steady state, than when they were present initially. The inhibition of microtubule assembly could be reversed by taxol, indicating a MAP-dependent effect. This was confirmed by SDS–PAGE (Fig. 6), since microtubules pelleted in the presence of 20 μM taxol and either of the two drugs showed a reduction in MAP content, dependent on the concentration of the estramustine derivative. The concentration required for 50% inhibition of assembly of 2 mg/mL microtubule protein was approximately 100 and 250 μM for estramustine sulphate (Fig. 7) and estramustine glucuronide, respectively. We were not able to determine any binding data for these derivatives, since they are not yet available as radiolabelled compounds.

Anionic estramustine derivatives increase the critical concentration for microtubule assembly

The critical concentration for microtubule assembly was investigated in the presence of 100 μM estramustine sulphate and 250 μM estramustine glucuronide (Fig. 8). The critical concentration for assembly was found to increase from approximately 0.4 [7] to approximately 1 mg/mL in the presence of 250 μM estramustine glucuronide, and 2 mg/mL in the presence of 100 μM estramustine sulphate.

Estramustine sarcosinate alters the morphology of assembled microtubule proteins

Microtubule assembly in the presence of the

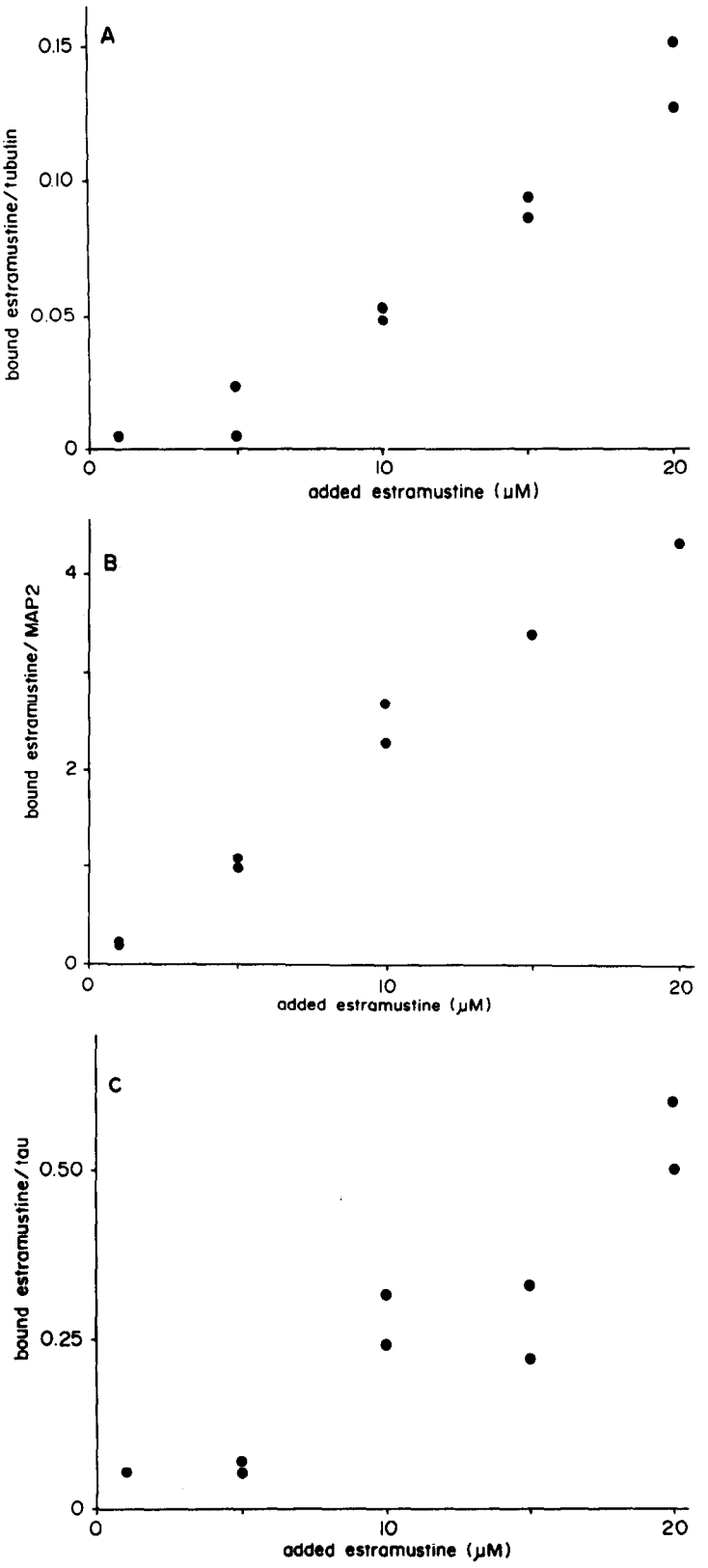

Fig. 3. Binding of estramustine to purified microtubule proteins. Tubulin (A), MAP2 (B), and tau (C) (1.6, 0.023 and 0.023 mg/mL, respectively) were incubated with different concentrations of estramustine (1–20 μM) and a constant amount of [^{3}H]estramustine under the conditions described in the legend to Fig. 2. The amount of estramustine incorporated was measured as described in the legend to Fig. 2. The amount of bound estramustine differed slightly between different protein preparations. Binding data from a single protein batch is therefore shown in this figure.

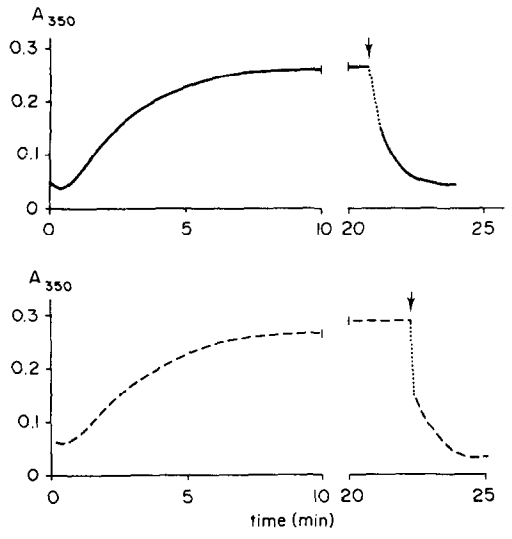

Fig. 4. Microtubule assembly in the presence of estramustine. Microtubule proteins (2 mg/mL) were assembled in 0.1 M Pipes, 0.5 mM $MgSO_4$, 1 mM EDTA and 1 mM GTP at pH 6.8 and 37°, in the absence (——) or presence (– – –) of 20 μM estramustine. At the arrow, 250 μM estramustine phosphate was added.

estramustine amino acid derivative, estramustine sarcosinate, was also investigated. This derivative is in contrast with the other estramustine derivatives by being cationic, with a pK_a of 7.5 (personal communication, Bertil Hansen, Pharmacia LEO Therapeutics AB). Estramustine sarcosinate precipitated in Pipes buffer but was soluble in 0.1 M MES in the presence of 0.01% Tween 20. Microtubules assembled in the presence of 100 μM estramustine sarcosinate showed increased turbidity measured at 350 nm (Fig. 9) SDS–PAGE showed the microtubule protein composition of the polymers formed to be normal (Fig. 10A). However, electron microscopy revealed the presence of aberrant forms (Fig. 10C) which might scatter light more than normal microtubules. In evaluating whether there was any competition between estramustine sarcosinate and estramustine phosphate, 1 mM EDTA had to be added before addition of estramustine phosphate to avoid a precipitation of estramustine phosphate–Mg^{2+} complexes. The subsequent addition of 400 μM estramustine phosphate induced a rapid and complete disassembly, suggesting that there is no competition between the two drugs.

DISCUSSION

Estramustine phosphate is a MAP-dependent

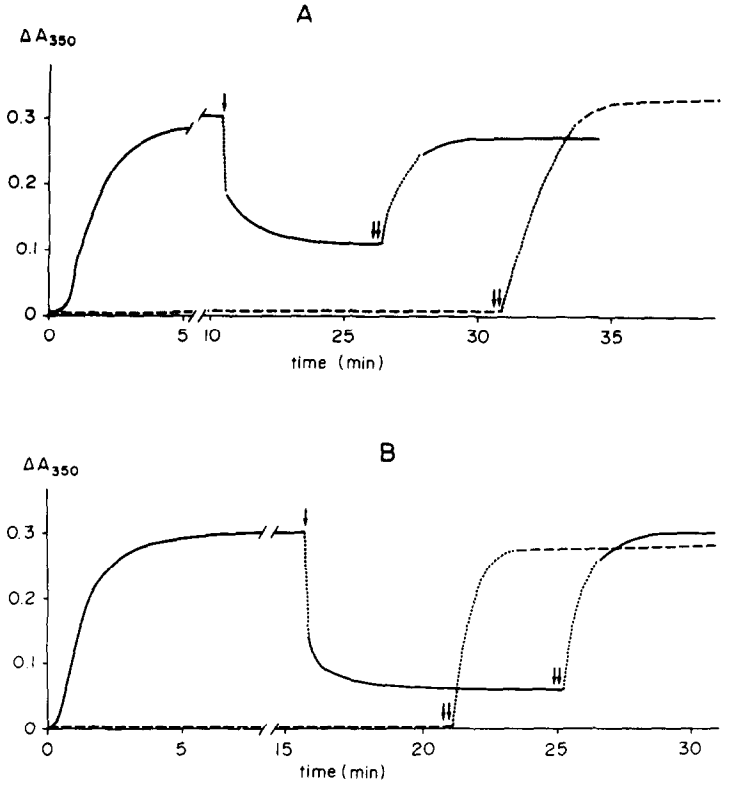

Fig. 5. Inhibition of microtubule assembly by the anionic estramustine sulphate and estramustine glucuronide. (A) Microtubule proteins (2.5 mg/mL) were assembled as described in the legend to Fig. 4 (——) and 300 μM estramustine sulphate was added at steady state (arrow). Microtubule proteins were also incubated in the presence of 300 μM estramustine sulphate (– – –) and taxol was added (double arrow). (B) Microtubule proteins were assembled as in A (——) and estramustine glucuronide (400 μM) was added at steady state (arrow). The drug was also present initially (– – –) and taxol was added (double arrow).

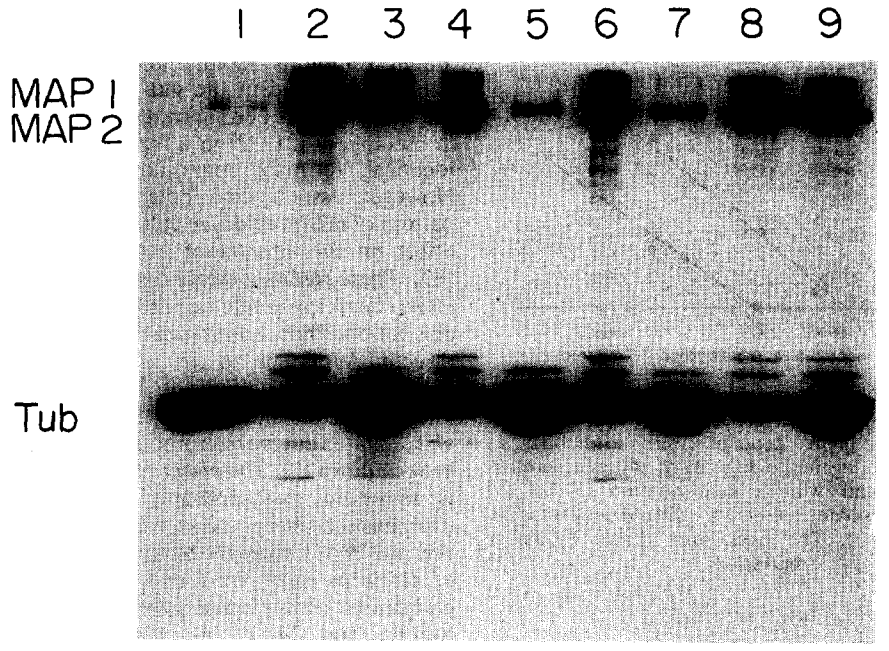

Fig. 6. Protein composition of microtubules assembled in the presence of estramustine sulphate and estramustine glucuronide. Microtubule proteins were assembled as described in the legend to Fig. 4 but in the absence of EDTA and in the presence of 20 μM taxol and 400 μM estramustine sulphate (1, pellet; 2, supernatant); 20 μM taxol and 100 μM estramustine sulphate (3, pellet; 4, supernatant); 20 μM taxol and 400 μM estramustine glucuronide (5, pellet; 6, supernatant); or 20 μM taxol and 100 μM estramustine glucuronide (7, pellet; 8, supernatant), for 20 min at 37° and then airfuged for 5 min at 100,000 *g* (9, reference). The pellets and supernatants were dissolved in sample buffer, and analysed by SDS–PAGE (5–12%).

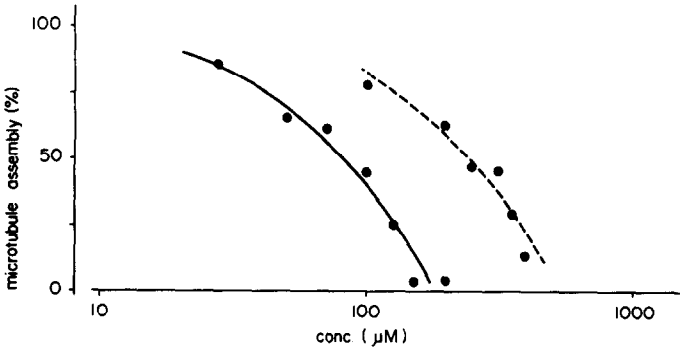

Fig. 7. The concentration-dependent inhibition of estramustine sulphate and estramustine glucuronide. Microtubule proteins (2.5 mg/mL) were assembled as described in the legend to Fig. 4 in the presence of different concentrations of estramustine sulphate (——) or estramustine glucuronide (– – –). The extent of assembly was calculated as a percentage of the reference assembly and plotted against the drug concentration of the estramustine derivatives.

inhibitor of microtubule assembly [6–8]. Here we found that two to three molecules of estramustine phosphate bound to each MAP2 with an apparent K_d of 20 μM at 4°. The number of estramustine phosphate molecules bound to tau was comparable, between one and two drug molecules to each tau, but with a lower K_d, approximately 200 μM. We have reported previously that estramustine phosphate inhibits the assembly of tubulin via an interaction with the tubulin-binding part of MAP2 [8]. The number of bound estramustine phosphate molecules measured suggests that it might bind to the small repetitive cationic tubulin binding domains that MAP2 and tau are reported to share [4, 5]. The lower affinity of estramustine phosphate for tau may reflect that tau consists of four to five isoforms and

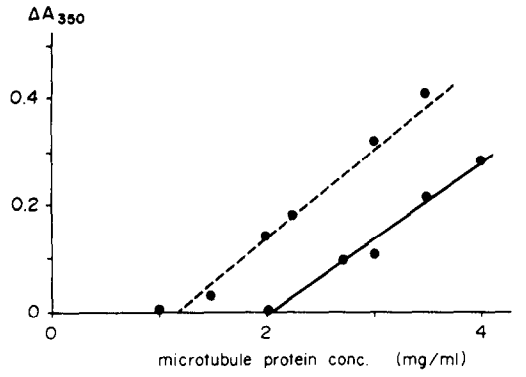

Fig. 8. Effect of estramustine sulphate and estramustine glucuronide on the critical concentration of microtubule assembly. Microtubule proteins were assembled as described in the legend to Fig. 3, at different protein concentrations and with a concentration of 200 μM estramustine sulphate (——) or 250 μM estramustine glucuronide (– – –). The lines were drawn by linear regression.

that the tubulin-binding sequences of tau are not completely homologous with those of MAP2. Tau is also known to form polymers [18] which might hide the binding sites. As shown already [6], tubulin bound only negligible amounts of estramustine phosphate, less than 0.05 mol/mol.

The estramustine phosphate–protein complex could be isolated easily by gel filtration at 4° without any obvious tailing [6], since the dissociation was low ($T_{1/2} > 2$ hr). The results suggest that the estramustine phosphate–protein complex was stabilized. Both tau [18] and MAP2 are highly flexible molecules [19] with a low degree of order, thus, binding of estramustine phosphate may induce a conformational change in MAP.

Estramustine phosphate is currently used in the treatment of prostatic carcinoma, and upon oral administration is rapidly and almost completely dephosphorylated in the gut to estramustine [20]. Estramustine has been shown to inhibit cell division in metaphase [21, 22] in a manner similar, but not identical, to many microtubule-disrupting drugs. However, estramustine induced only an abnormal pattern of microtubules in cultured cells without any effect on the amount of assembled microtubules [23]. These results indicate that estramustine has an effect on microtubule-dependent processes in the cell but does not cause disassembly of microtubules directly. We came to the same conclusion when determining the effect of estramustine on the microtubule-dependent axonal transport. Transport was inhibited but the microtubules were not obviously broken down [7]. The effect of estramustine *in vivo* is, therefore, most probably distinct from that of estramustine phosphate *in vitro*. Our data indicate that the two derivatives have different mechanisms of action *in vitro*. We found that estramustine, in contrast to estramustine phosphate, binds to both tubulin and MAPs as well as to assembled microtubules *in vitro* without any effect on microtubule assembly. We were not, however, able to determine dissociation constants for its binding to tubulin or MAPs, because the binding was unsaturable in the low solubility range (up to 20 μM). Moreover, microtubules assembled in the presence of estramustine disassembled as rapid and completely as the control upon addition of estramustine phosphate and the binding of estramustine phosphate was not suppressed by the presence of estramustine. The results indicate that the two drugs do not compete in binding to microtubule proteins.

After our report on the MAP dependency of estramustine phosphate inhibition of microtubule assembly, another group reported a similar effect of estramustine [24]. In order to reproduce this effect

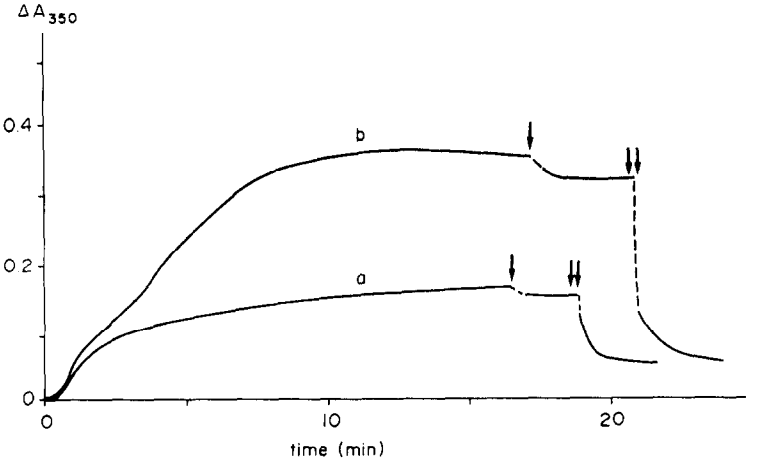

Fig. 9. Microtubule assembly in the presence of estramustine sarcosinate. Microtubule proteins (2 mg/mL) were assembled at 37° in 0.1 M MES, 0.5 mM $MgSO_4$, 1 mM GTP and 0.01% Tween 20 (a), and with 100 μM estramustine sarcosinate (b). EDTA (1 mM) was added at steady state (arrow). At the double arrow, 400 μM estramustine phosphate was added.

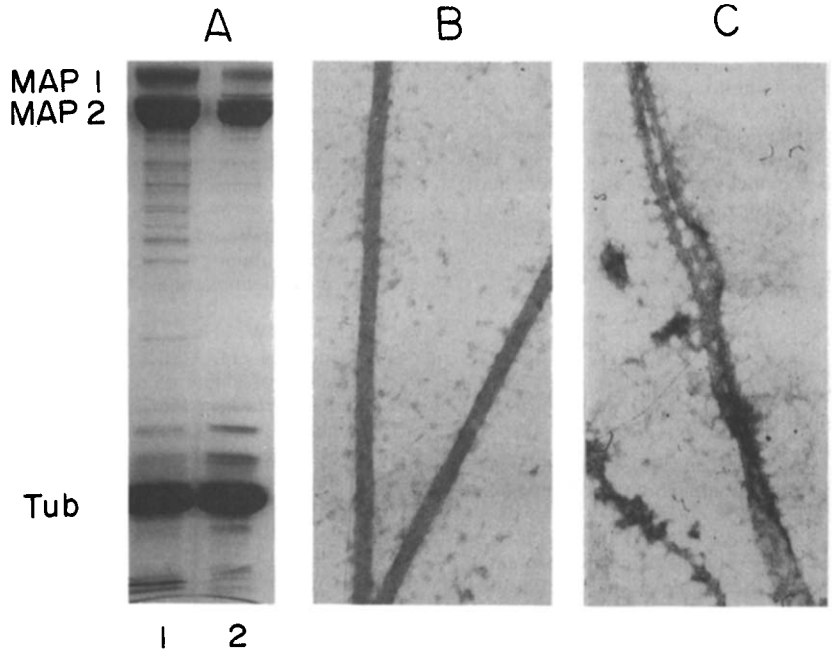

Fig. 10. Microtubules assembled in the presence of estramustine sarcosinate. (A) The microtubule proteins were assembled as described in the legend to Fig. 9, in the presence of 100 μM estramustine sarcosinate, for 20 min and pelleted at steady state. The pellets (1) and supernatants (2) were dissolved in sample buffer and the protein composition was investigated by SDS–PAGE. Negatively stained microtubules, assembled in the absence (B) or presence (C) of 100 μM estramustine sarcosinate; magnification ×90,000.

and in an attempt to increase the concentration of estramustine to the reported levels, a number of alterations to our *in vitro* system was performed. However, it was not possible to reproduce any effect of estramustine on either rat or bovine brain microtubule assembly under their conditions. The drastic use of 40% DMSO, which was necessary to increase the estramustine concentration to 100 μM, was also without effect (not shown). The reason for this discrepancy is unknown.

The effects of estramustine derivatives were found to be charge-dependent. The MAP-dependent inhibition of microtubule assembly by the charged estramustine phosphate, -sulphate and -glucuronide clearly shows the importance of an ionic group at position 17 of the estrogen moiety. Estramustine glucuronide, with the larger substituent, was less potent than estramustine phosphate and estramustine sulphate, suggesting that steric effects also are of importance. The charge of the substituent must be negative for a MAP-dependent effect, since when the positively charged estramustine sarcosinate was used the effect on microtubules was completely different. The assembly was not inhibited but the turbidity increased due to an alteration in the microtubule structure. The tubulin–MAPs interactions seemed however to be intact, since the ratio of MAPs to tubulin in the polymer was the same. The two derivatives probably bind to different sites, since estramustine phosphate could induce rapid disassembly in the presence of 100 μM estramustine sarcosinate. Our results are therefore in accordance with the previous finding that the MAPs are attached to tubulin mainly by cationic bonds. These bonds are known to be broken by an elevated ionic strength, polyanions like heparin or with negatively charged polyamino acids [25–27]. The importance of a negatively charged substituent at position 17 of the estramustine molecule for the interruption of the tubulin–MAPs association *in vitro*, supports furthermore the observation that the uncharged estramustine does not affect the tubulin–MAPs interaction. Another mechanism of action for estramustine *in vivo* is therefore probable.

Our study shows that estramustine phosphate is a valuable chemical tool for the *in vitro* study of microtubules. However, it is of limited value for *in vivo* and cellular studies because it precipitates in the presence of divalent cations and is rapidly dephosphorylated both in the body and in many cell cultures. The existence of two new drugs which do not precipitate in the presence of divalent cations and have a similar MAP-dependent mechanism of action to that of estramustine phosphate, has therefore further advantages in the study of microtubules and the role of MAPs.

Note added in proof—After submission of this manuscript, a binding study of estramustine phosphate to chick brain MAP2, has been published by Dr R. G. Burns, confirming our results (Burns RG, Stoichiometry of estramustine phosphate binding to MAP2 measured by the disassembly of chick brain MAP2: tubulin microtubules, *Cell Motility Cytoskeleton* **17**: 167–173, 1990).

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