Biochemical Pharmacology, Vol. 35, No. 3, pp. 371–375, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

IN VITRO MECHANISM STUDY OF MICROTUBULE ASSEMBLY INHIBITION BY *CIS*-DICHLORODIAMMINE-PLATINUM(II)

V. PEYROT,* C. BRIAND,*† R. MOMBURG and J. C. SARI‡

*Laboratory of Pharmaceutical Physics, Faculty of Pharmacy, 27, Bd Jean Moulin, 13385 Marseille Cédex 5, France, and ‡Institute of Technology Luminy, Case 909, 13288 Marseille Cédex 9, France

(*Received* 31 *December* 1984; *accepted* 12 *June* 1985)

Abstract—The inhibitions of microtubule protein (MTP) and tubulin 6S polymerizations by *cis*-dichlorodiammine-platinum(II) (CDDP) have been investigated by turbidity measurements and electron microscopy. For 2.5×10^{-4} M CDDP after 40 min contact time at 27°, the inhibition was 60% for MTP (1.2 mg/ml) and nearly 90% for tubulin 6S (1.2 mg/ml). Microtubules were not present after a 1 hr contact time at 27° with 2.5×10^{-4} M CDDP. Free sulfhydryl group determinations with 5,5′-dithio-bis-(2-nitrobenzoate) (DTNB) showed that 20.10 (±0.05) sulfhydryl groups were found per tubulin dimer. In the presence of excess CDDP, this number was reduced to 17.74 (±0.05) after a 1 hr contact time at 27°. By using CDDP–tubulin dialysis assays, the CDDP–tubulin complex formation was found to be an irreversible reaction through a covalent binding at the sulfhydryl group sites. By the DEAE filter paper method, CDDP was shown to slightly decrease vinca-alkaloid and colchicine bindings to tubulin likely by inducing a conformational change of the protein.

Cis-dichlorodiammine-platinum(II) (CDDP) is a widely used anticancer drug, its antitumor activity is closely correlated with its interaction with DNA [1].

However, our initial study [2] showed that CDDP was a potent *in vitro* inhibitor of microtubule polymerization. This inhibition was dependent of the contact time between drug and protein and was related to temperature, although this reaction is still possible at 4°.

The percent inhibition of MTP polymerization was 40% after 1 hr contact time at 37° with CDDP at equimolar concentrations and 80% after 2 hr.

This inhibition may be assumed to contribute to neurotropic properties of *cis*-platinum [3, 4].

The aim of the present study was to verify precisely the mechanism of action of CDDP by studying its effects on the microtubule protein (MTP) and on tubulin 6S (MTP without associated proteins named MAPs). Furthermore, we have also studied the influence of CDDP on vinca-alkaloids and colchicine bindings to tubulin. Indeed, these two classes of drugs are the most studied microtubule inhibitors. Colchicine and vinca-alkaloids have played key roles as tools in the investigation of drugs action on microtubule function.

MATERIALS AND METHODS

Reagents

2-(*N*-Morpholino)ethane sulfonic acid (MES) and ethylene glycol bis(2-aminoethyl-ether)tetraacetic acid (EGTA) were purchased from Sigma (U.K.) and guanosine 5′-triphosphate (GTP), 5,5′-dithio-bis-(2-nitrobenzoate) (DTNB) from Fluka (F.R.G.).

Vinblastine, vincristine and vindesine were gifts from Eli Lilly Laboratories.

† To whom correspondence should be addressed.

[^{3}H]Vinblastine sulfate (specific activity 11.4 Ci/mmol) and [^{3}H]vincristine sulfate (specific activity 7.7 Ci/mmol) were purchased from Amersham International (U.K.)

(Ring *C* methoxyl-H^3)colchicine was also from Amersham International (specific activity 2.5 Ci/mmol). All other reagents were Merck's analytical grade.

Cis-dichlorodiammine-platinum(II) (CDDP) of the highest purity was a gift from Roger Bellon Laboratories and other platinum compounds were a gift from Johnson Matthey Research Center.

Tubulin purification

Microtubule protein (MTP) was obtained from fresh porcine brain by the method of Shelanski *et al.* [5] by two consecutive cycles of polymerization/depolymerization. Before each experiment, a third cycle was performed in 4 M glycerol buffer. Additional purification of tubulin was carried out on a phosphocellulose column according to Weingarten *et al.* [6]. Prior to use, Whatman phosphocellulose P11 was washed with 0.5 M NaOH, H_2O, 0.5 M HCl and H_2O successively and equilibrated with 0.05 M MES buffer, pH 6.7, containing 0.5 mM $MgCl_2$, 1 mM EGTA and 0.1 mM GTP.

Tubulin purity was checked by polyacrylamide gradient gel electrophoresis [7]. Glycerol (3.4 M) was added to the eluted tubulin fraction and this solution was concentrated using an Amicon concentrating Cell Model 8050. The resulting tubulin solution (5–8 mg/ml) containing 0.1 mM GTP was then stored at −80°.

Protein concentration measurements

In the absence of free nucleotides, tubulin concentrations were determined spectrophotometrically

using an extinction coefficient of 1.2 ml/cm mg at 280 nm [8]. When nucleotides were present, tubulin concentration was determined by the method of Lowry *et al.* [9] using bovine serum albumin as standard.

Polymerization measurements with CDDP

It is known that platinum complexes interact with nucleotides [10] and especially with guanosine nucleotides [1]. In order to avoid nucleotide interference during the contact time of CDDP and tubulin, it was necessary to chromatograph the protein solution through Sephadex G 25 previously equilibrated with buffer A: (0.05 M MES, pH 6.7, 0.5 mM $MgCl_2$, 1 mM EGTA and 3.4 M glycerol).

The *in vitro* polymerization of tubulin was monitored by turbidimetry at 340 nm on a Uvikon L.S. Printer 48 spectrometer with a thermostated 1 cm light path cell at 37°. The polymerization was studied in a buffer solution consisting of 0.05 M MES, pH 6.7, 10 mM $MgCl_2$, 1 mM EGTA, 1 mM GTP and 3.4 M glycerol [11]. Assembly was initiated by transferring sample from ice to the prewarmed (37°) cuvette holder. MTP assembly was followed at 37° by turbidimetric method at 340 nm, as previously described [2].

Electron microscopy

Electron microscopy was performed with a Philips EM 400 T microscope. Samples were taken 30 min after the addition of reassembly buffer and were negatively stained with 1% uranyl acetate on carbon-coated grids.

Determination of tubulin sulfhydryl concentrations

The determination of tubulin-free sulfhydryl groups was performed according to Ellman [12]. Tubulin solution (0.1 ml) was added to 0.9 ml of 0.05 M phosphate buffer, pH 7.5 containing urea (final concentration 6.4 M) and 5,5′-dithio-bis(2-nitrobenzoate) (DTNB) (final concentration 1 mM). Sulfhydryl concentration was calculated from the absorbance at 412 nm, using ε of 13.6/mM cm.

Vinca-alkaloids and colchicine binding assays

Vinca-alkaloids and colchicine bindings were determined by a modified DEAE filter paper method [13]. Two DE-81 paper discs (Whatman, 2.5-cm diam.) were washed with ice-cold assay buffer by mild suction. The assay buffer contained 10 mM phosphate buffer pH 6.8, 10 mM $MgCl_2$ and 0.1 mM GTP. Tritiated colchicine or vinca-alkaloids at various concentrations and either tubulin alone or tubulin + CDDP (tubulin: 10^{-5} M, CDDP: 2×10^{-4} M, contact time: 3 hr at 4°) were incubated at 37° for 2 hr for the colchicine binding assay and 30 min for the vinca-alkaloids binding assay. After the incubation periods, samples (100 μl) were applied to the discs. After 5 min, the filters were rinsed three times with 3 ml of ice cold buffer by mild suction. The radioactivity of the discs was determined in 10 ml of Bray's solution. In all cases, controls were run in the absence of tubulin and their radioactivity values were subtracted from experimental results. Measurements were duplicated and agreed to within 10%.

Dialysis assays

A mixture of *cis*-platinum–tubulin (10:1) was dialyzed against 600 ml of cold buffer A (4°). The dialysis was stopped after 6 hr although the equilibrium was reached after 3 hr.

Platinum concentrations were followed in and out the dialysis tubings by flameless atomic absorption using a Philips Pye Unicam SP9 spectrophotometer. A three-stage temperature program was used for each determination: a 20 sec dry stage at 115°; a 15 sec ash stage at 950° and a 3 sec atomizer stage at 2800°.

RESULTS AND DISCUSSION

Effect of CDDP on tubulin 6S

No effect has been noted with nonhydrolyzed CDDP. Therefore, hydrolyzed CDDP, which is present in intracellular fluid [1], was used in all experiments. CDDP hydrolysis was checked by conductimetric measurements [10].

Figures 1 and 2 represent the action of CDDP on MTP and on tubulin under the same conditions of temperature (27°) and of contact time (40 min). For 2.5×10^{-4} M CDDP, the percent inhibition is 60% with MTP (1.2 mg/ml) and 90% for tubulin (1.2 mg/ml). Similar effect can be seen with *trans*-dichlorodiammine-platinum; for 2.5×10^{-4} M, the percent inhibition is 100% for tubulin (1.2 mg/ml). These data show a direct interaction between CDDP or *trans*-dichlorodiammine-platinum and tubulin. The greater sensitivity of tubulin assembly over that of MTP can be adequately explained by the higher critical protein concentration in the former system. Thus, inactivation of equal amounts of tubulin in pure tubulin and MTP solutions will lead to greater inhibition of assembly of pure tubulin.

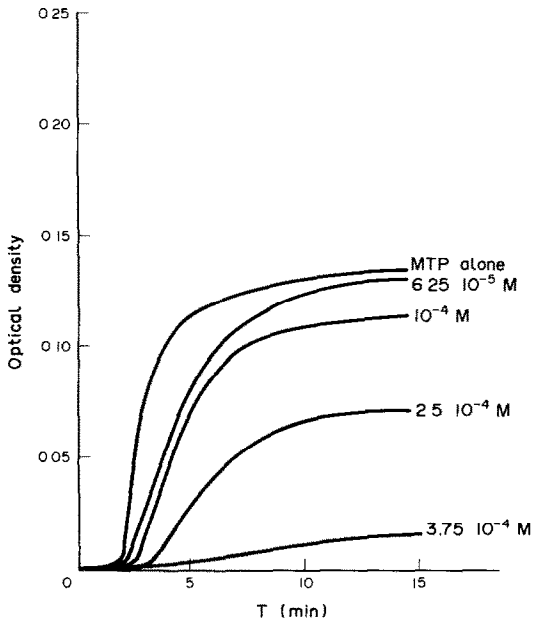

Fig. 1. Variation of optical density vs time during MTP (1.2 mg/ml) polymerization with CDDP, 40 min contact time, 27°.

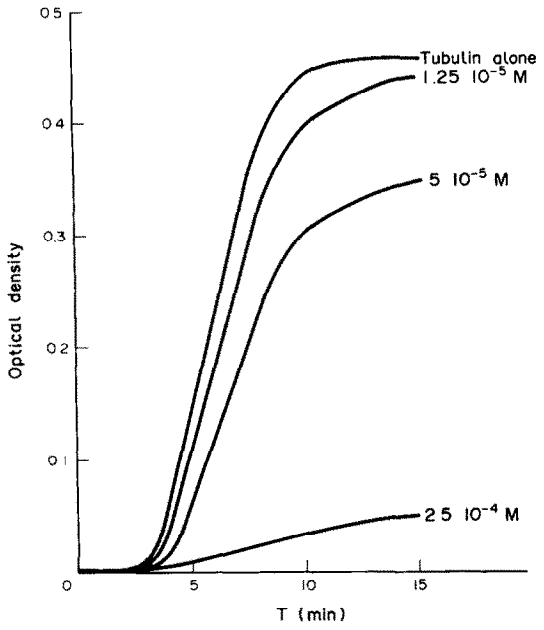

Fig. 2. Variation of optical density vs time during tubulin (1.2 mg/ml) polymerization with CDDP, 40 min contact time, 27°.

Figure 3 represents electron micrographs of tubulin polymerization in the absence and presence of CDDP. Microtubules cannot be observed with 2.5×10^{-4} M CDDP after a 1 hr contact time at 27°.

The polymerization of microtubules, from their subunits tubulin, is inhibited by organic sulfhydryl blocking agents [14]. Moreover, it is known that platinum(II), a soft acid, is more likely to form a stable bond with sulfur. Consequently, a possible CDDP binding to tubulin thiol residues was also studied [15].

Approximately 20 sulfhydryl groups are present per tubulin dimer [16]. Some free sulfhydryl groups are directly involved in the polymerization process [14]. In the present study, the number of sulfhydryl groups was found to be 20.10 (±0.05) per tubulin dimer.

Furthermore, the numbers of sulfhydryl groups were 19.49 ± 0.05, 18.90 ± 0.05 and 17.74 ± 0.05 with the tubulin–CDDP ratios of 1:11, 1:22 and 1:60, respectively at 27° with a 1 hr contact time. The percent polymerization inhibition was always 100% under the same experimental conditions. Figure 4 presents the number of tubulin sulfhydryl groups in presence of CDDP vs time. It can be concluded that CDDP reacts with the tubulin thiol residues and that one blocked reactive residue is enough to inhibit the polymerization process.

A dialysis was conducted for 6 hr at 4° against buffer A following a contact time of 40 min at 27° with 10^{-5} M tubulin and 1.75×10^{-4} M CDDP to eliminate free CDDP molecules. The polymerization process could not be recovered after dialysis. As shown by platinum quantification, the CDDP–tubulin ratio of the complex molecule was nearly 1.5 and two sulfhydryls groups were blocked. Our data suggest that an irreversible reaction occurs between CDDP and tubulin through a covalent binding on the thiol residue sites.

Influence of CDDP on the colchicine and vinca-alkaloids tubulin bindings

Binding of colchicine to tubulin is a complex phenomenon which is slow and not easily reversed. In 1978, Garland [17] proposed that colchicine–tubulin interaction might be a two-step process, namely a fast reversible binding step followed by a slow ligand induced conformational change.

In this study, CDDP–tubulin complex formation was studied by using the colchicine binding activity. The influence of CDDP on the affinity and stoichiometry of colchicine binding to tubulin was investigated by Scatchard analysis (Fig. 5). The number of available colchicine sites per tubulin dimer did not change in the presence of CDDP but the affinity of colchicine to tubulin was reduced. For 2×10^{-4} M CDDP, 4.2×10^{-6} M colchicine and 10^{-5} M tubulin a 25% decrease of colchicine binding could be observed.

In the same way, a slight decrease of the apparent association constants of vinca-alkaloids (vinblastine and vincristine) to tubulin was also noted in presence of CDDP (Fig. 6).

These results suggest that the binding sites of CDDP and colchicine or vinca-alkaloids are different. A primary CDDP induced conformational change of tubulin could explain the slight modification of the colchicine and vinca-alkaloids binding. These results may contribute to the additive effect of CDDP and vinca-alkaloids on the *in vitro* MTP polymerization. Indeed, under the same experimental conditions (1 hr contact time at 37°, MTP: 0.8 mg/ml), 2.5×10^{-5} M CDDP inhibited around 40% MTP polymerization while 10^{-7} M vinblastine produced a 35% inhibition. If 10^{-7} M vinblastine was added to a MTP solution which was already treated by 2.5×10^{-5} M CDDP, a 70% polymerization inhibition was observed.

CONCLUSION

The present work presents evidence that microtubule formation is inhibited by CDDP *in vitro* at equimolecular concentrations.

The tubulin–CDDP and tubulin–*trans*-dichlorodiammine-platinum bindings may be involved essentially in the cytotoxic effect of *trans*-dichlorodiammine-platinum at high doses [18] and, *in vivo*, in the neurotoxic effect of CDDP [3, 4]. Indeed, high platinum concentrations have been found in peripheric nerves of patients who developed neuropathies [4]. The measured platinum concentrations were almost identical to those used in this study for a 50% tubulin polymerization inhibition (following a 1 hr contact time).

This tubulin binding of CDDP could also explain some effect of CDDP on the phagocytic function of mammalian cells [19]. Indeed, colchicine and vinblastine were shown to influence chemotaxis and endocytosis by altering microtubule assembly [20].

CDDP was found to bind directly to tubulin 6S via the sulfhydryl groups of the protein. One blocked CDDP thiol function is enough to stop microtubule

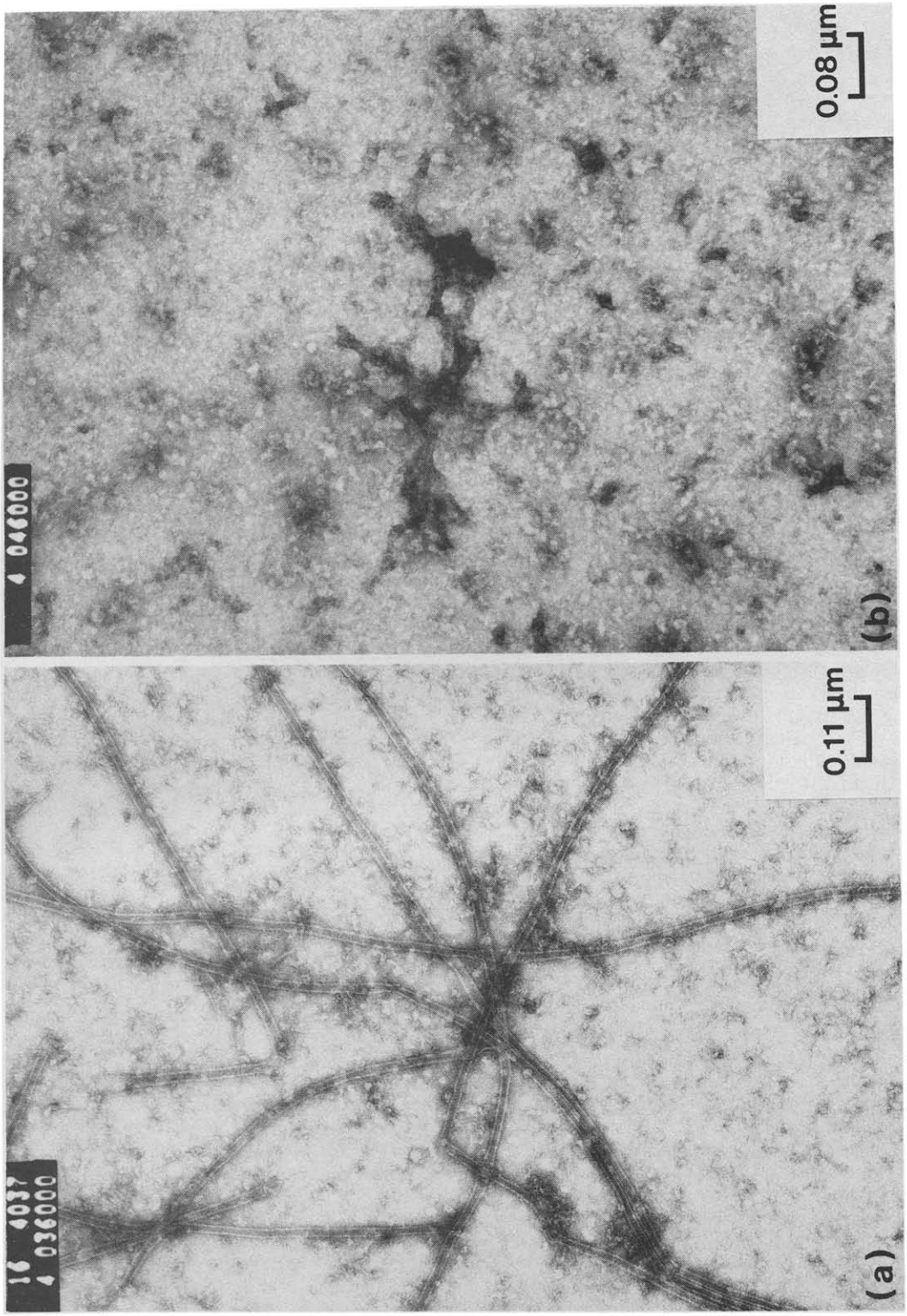

Fig. 3. Electron micrographs. a (Blank) tubulin polymerized after a 1 hr contact time at 27° (×36,000). b: Tubulin with 2.5×10^{-4} M CDDP after a 1 hr contact time at 27° (×46,000).

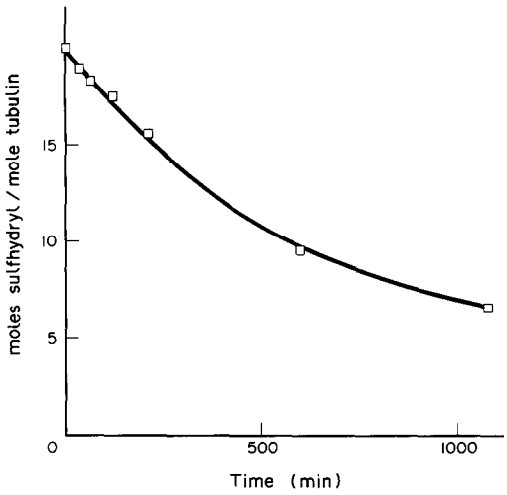

Fig. 4. The reaction of tubulin sulfhydryls with CDDP tubulin (10^{-5} M) was incubated with CDDP (2.5×10^{-4} M) at 27°. Samples were removed and reacted with DTNB as described under Materials and Methods.

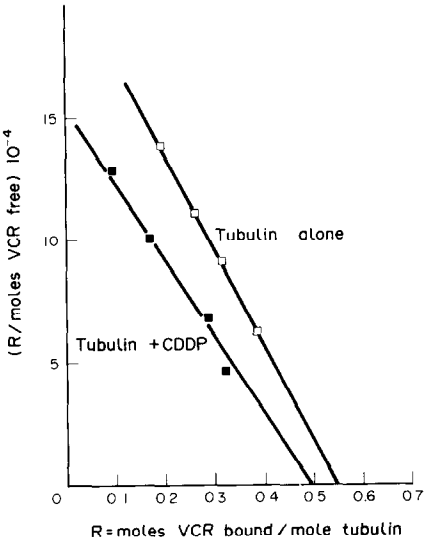

Fig. 6. Scatchard plot. The range of vinca-alkaloids (vinblastine or vincristine) concentration used was from 1.70×10^{-6} M to 10^{-5} M. Tubulin concentration was 5×10^{-6} M.

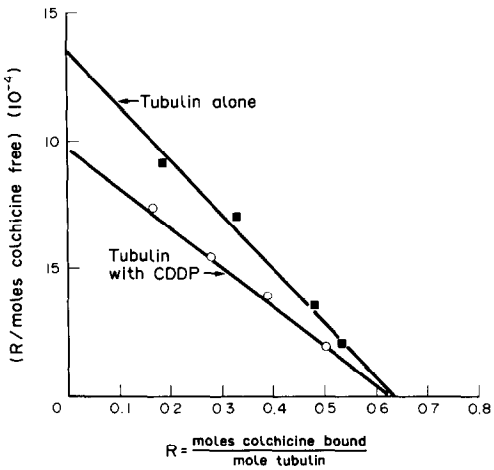

Fig. 5. Scatchard plot. The range of colchicine concentration used was from 4×10^{-6} M to 4×10^{-5} M. Tubulin concentration was 10^{-5} M. Binding values were not corrected for loss of colchicine-binding activity during incubation with colchicine and during purification of tubulin.

assembly. The CDDP–SH binding is followed by a conformational change which slightly affects the colchicine and vinca-alkaloids binding sites.

Acknowledgements—The authors are grateful to H. Bouteille for technical assistance. They thank M. Passerel for assistance in preparing the electron micrographs, Dr D. Braguer for help during binding experiments and Dr A. M. Chauvet-Monges for helpful discussions. This work has been supported by a grant from "La Fédération des Centres de lutte contre le cancer" and by INSERM contract no. 851003.

REFERENCES

1. B. Rosenberg, *Biochimie* **60**, 859 (1978).
2. V. Peyrot, C. Briand, A. Crevat, D. Braguer, A. M. Chauvet-Monges and J. C. Sari, *Canc. Treat. Rep.* **67**, 641 (1983).
3. L. Reinstein, S. S. Ostrow and R. H. Wiernik, *Archs phys. Med. Rehabil.* **61**, 280 (1980).
4. S. W. Thompson, L. E. Davis, M. Kornfeld, R. D. Hilgere and J. C. Standefer, *Cancer* **54**, 1269 (1984).
5. M. L. Shelanski, F. Gaskin and C. R. Cantor, *Proc. natn. Acad. Sci. U.S.A.* **70**, 765 (1973).
6. M. D. Weingarten, A. M. Lockwood, S. Hwo and M. W. Kirschner, *Proc. natn. Acad. Sci. U.S.A.* **72**, 1858 (1975).
7. U. K. Laemmli, *Nature, Lond.* **227**, 680 (1970).
8. M. W. Detrich and R. C. Williams, *Biochemistry* **17**, 3900 (1978).
9. O. M. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
10. J. C. Sari, V. Peyrot, M. Hassid and C. Briand, *Biochimie* **64**, 289 (1982).
11. D. Saltarelli and D. Pantaloni, *Biochemistry* **21**, 2992 (1982).
12. G. L. Ellman, *Archs Biochem. Biophys.* **82**, 70 (1959).
13. G. G. Borisy, *Analyt. Biochem.* **50**, 373 (1972).
14. M. Wallin, M. Larsson and A. Edstrom, *Expl Cell. Res.* **107**, 219 (1977).
15. M. E. Friedman, H. B. Otwell and J. R. Teggins, *Biochim. biophys. Acta* **391**, 1 (1975).
16. R. H. Himes and W. B. Himes, *Biochim. biophys. Acta* **621**, 338 (1980).
17. D. L. Garland, *Biochemistry* **178**, 4266 (1978).
18. B. Drewinko, P. Corry, J. P. Bergerat and B. Barlogie, in *Cisplatinum: Current Status and New Developments* (Ed. S. Crooke). Academic Press, New York (1980).
19. R. Fumarulo, D. Giordano, S. Riccardi and M. Aresta, *Proc. Soc. exp. Biol. Med.* **164**, 164 (1980).
20. S. E. Malawista, *Immunopathy and Inflammation*, pp. 118–120. Excerpta Medica, Amsterdam (1971).