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Interaction of Tubulin with Octyl Glucoside and Deoxycholate. 2. Protein Conformation, Binding of Colchicine Ligands, and Microtubule Assembly†

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ABSTRACT: The structural change induced by binding of mild detergents to cytoplasmic calf brain tubulin and the effects on the functional properties of this protein have been characterized. Massive binding of octyl glucoside or deoxycholate monomers induces circular dichroism changes indicating a partial α-helix to disordered structure transition of tubulin. The protein also becomes more accessible to controlled proteolysis by trypsin, thermolysin, or V8 protease. This is consistent with the looser protein structure proposed in previous binding and hydrodynamic studies [Andreu, J. M., & Muñoz, J. A. (1986) *Biochemistry* (preceding paper in this issue)]. Micelles of octyl glucoside and deoxycholate bind colchicine and its analogue 2-methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one (MTC). This impedes the determination of colchicine binding in the presence of detergents. Both detergents cause a reduction in the number of tubulin equilibrium binding sites for the colchicine site probe MTC. Deoxycholate monomers bind poorly to the tubulin–colchicine complex, but deoxycholate above the critical micelle concentration effectively dissociates the complex. Microtubule assembly in glycerol-containing buffer is inhibited by octyl glucoside, which raises the critical protein concentration. Low concentrations of deoxycholate enhance tubulin polymerization, allowing it to proceed without glycerol. The polymers formed are microtubules, pairwise associated open microtubular sheets, and macrotubes possibly generated by helical folding of the sheets, as indicated by the optical diffraction patterns. Saturation of tubulin with octyl glucoside, followed by full dissociation of the detergent, allowed the recovery of binding to the colchicine site and microtubule assembly, indicating the reversibility of the protein structural change.

Cytoplasmic tubulin from bovine brain, a water-soluble protein, is able to show extensive hydrophobic interactions. Tubulin associates to unilamellar dipalmitoylphosphatidylcholine vesicles (Klausner et al., 1981), where it is apparently inserted into the lipid bilayer (Kumar et al., 1981). It has been further reported that vesicle-bound tubulin exhibits normal colchicine and MAP binding and is able to mediate the nonleaky membrane fusion of the vesicles induced by calcium ions

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(Kumar et al., 1982). Hydrophobic regions of soluble tubulin have been probed with mild detergents. This protein binds large amounts of sodium deoxycholate and octyl glucoside (Andreu, 1982). The binding of detergent monomers is cooperative and 95% reversible (deoxycholate) or more than 99.7% reversible (octyl glucoside). Binding is inhibited by the cosolvent 3.4 M glycerol. Octyl glucoside induces a rapidly equilibrating tubulin self-association, while deoxycholate binding causes a marked increase in the frictional coefficient of the protein. The latter effect was interpreted as a not characterized structural change leading to an expansion of the tubulin heterodimer (Andreu & Muñoz, 1986).

Apart from the well-known presence of tubulin in the cytoskeletal system, proteins similar to tubulin have been reported as constituents of a number of membrane systems [introduction of Andreu & Muñoz (1986)]. On the other hand, hydrophobic interactions are probably important in the functions of cytoplasmic tubulin. Microtubule assembly in vivo shows positive apparent standard enthalpy and entropy changes (Inoue & Sato, 1967). In vitro microtubule assembly has similar characteristics (Lee & Timasheff, 1977) and a negative heat capacity change, properties that suggest the loss of ordered solvent during polymerization (Hinz et al., 1980). The binding of several small ligands to tubulin probably involves hydrophobic interactions. This is the case of the trimethoxybenzene ring of colchicine and podophyllotoxin (Andreu & Timasheff, 1982) and the hydrophobic probes anilinonaphthalenesulfonate and bis(anilinonaphthalenesulfonate) (Horowitz et al., 1984). However, the relationship of the particular hydrophobic zones involved in self-assembly and ligand binding to the hydrophobic zones probed with the mild detergents is not known.

The aim of this work is to characterize the conformation of tubulin in the detergent complexes and the involvement of hydrophobic interactions in tubulin functional activities. For these purposes we have examined the effects of binding of octyl glucoside and deoxycholate on the circular dichroism, controlled proteolysis, colchicine binding site, and self-assembly of tubulin.

Materials and Methods

Protein, Ligands, and Binding Measurements. Calf brain tubulin was purified and detergent binding measured as reported (Andreu & Muñoz, 1986). The tubulin–colchicine complex was prepared by room temperature incubation of tubulin with 1 mM colchicine (Andreu & Timasheff, 1982). 2-methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one (MTC)[1] was provided by Dr. T. Fitzgerald (Fitzgerald, 1976), and its binding to tubulin was measured fluorometrically (Andreu et al., 1984).

Spectroscopic Measurements. Circular dichroism spectra were obtained with a Rousell-Jouan Dichrograph II, employing 0.1- and 0.2-cm cells at 25 ± 0.5 °C. The mean residue ellipticity, $[\theta]$, was calculated from the relation $[\theta] = 3300(A_L - A_R)c^{-1}d^{-1}$, where $(A_L - A_R)$ is the difference dichroic absorption, c is the mean residue concentration, and d the light path (Adler et al., 1973). A mean residue weight of 109 was employed (Lee et al., 1978). The experimental spectra were computer-fitted between 203 and 250 nm by a combination of α-helix, β-sheet, and disordered structure (R) contributions (Greenfield & Fasman, 1969). Two different sets of standards were employed, polylysine standards (Greenfield & Fasman, 1969) with disordered histone standards for R (Beaudette et al., 1981) or average protein standards (Chen et al., 1972). Both procedures gave different α- and β-content values for tubulin spectra. The overall fit was better with the polylysine–histone standards, but the shape was not adequately simulated for spectra with two minima at 210 and 220 nm. On the other hand, the Chen et al. (1972) standards simulated better the shape of the two minima at 210 and 220 nm, but the overall fit was poorer, in particular for spectra with a single minimum located between 210 and 220 nm. The percentages of secondary structure given in this paper are average values of the results of fittings with the two sets of standards. This is an arbitrary procedure which was found not to affect the changes in percentage of secondary structure reported. Difference absorption spectra were obtained with a Cary 16 spectrophotometer, employing mixing tandem cells of 0.48 + 0.48 cm light path (Hellma) at 25 ± 0.5 °C, and fluorescence measurements were made with a Fica MKII spectrofluorometer employing 0.5 × 0.5 cm cells at the same temperature.

Controlled proteolysis was made on 2.0 ± 0.1 mg/mL tubulin solutions in PG[1] buffer containing 0.5 mM $MgCl_2$ and 1.5% or 2% (w/w) protease (trypsin, Calbiochem; thermolysin, Sigma; *S. aureus* V8 protease, Miles). After 20 min at 25 °C the solution was made 2 mM in phenylmethanesulfonyl fluoride (Sigma), inmediately 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. A total of 5 or 10 μL of each sample was applied to dodecyl sulfate–polyacrylamide slab gels (Laemmli, 1970). Sodium dodecyl sulfate was from Sigma, containing 25% (w/w) myristyl sulfate and 5% cetyl sulfate (Best et al., 1981). The separation gel was polymerized at pH 9.0 and contained 5 mM β-mercaptoethanol. Apparent molecular weights reported were calculated by interpolation in plots of log (M_r) vs. the relative mobilities of standards of bovine serum albumin and aldolase (Sigma), human immunoglobulin G (Miles), and lysozyme (Calbiochem) at 7.5%, 9% and 10% acrylamide. Molecular weight values were reproducible to ±1000.

Microtubule assembly was performed in 10 mM sodium phosphate, 16 mM $MgCl_2$, 1 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid, 0.1 mM GTP, and 3.4 M glycerol, pH 7.0, buffer (assembly buffer) at 37 °C. The reaction was followed turbidimetrically (Gaskin et al., 1974; Andreu & Timasheff, 1986) at 350 nm by employing a water-jacketed cuvette in a Varian 635 spectrophotometer. Polymers formed were fixed with 0.5% glutaraldehyde (Serva Feinbiochemica, Heidelberg), adsorbed to carbon-coated grids, negatively stained with 2% uranyl acetate, and examined with a JEOL 100B electron microscope equipped with an anticontamination device. The characterization of these structures was carried out by optical diffraction of electron micrographs in a folded beam optical bench (Carrascosa et al., 1982). Spacings in real and reciprocal space were calibrated by measuring the well-established dimensions of normal microtubules (Amos, 1979) as an internal standard.

Results

Tubulin Structural Change Induced by Detergent Binding. The effects of extensive binding of detergents on the secondary structure of tubulin were examined by circular dichroism. The results, shown in Figure 1, indicate that octyl glucoside (spectra b and c) and deoxycholate (spectra e–i) induced moderate changes in the dichroism of tubulin (spectrum a). These changes were very similar for saturating concentrations of both mild detergents and consisted in an approximately 20% re-

[1] Abbreviations: PG, 10 mM sodium phosphate and 0.1 mM GTP, pH 7.0; MTC, 2-methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one.

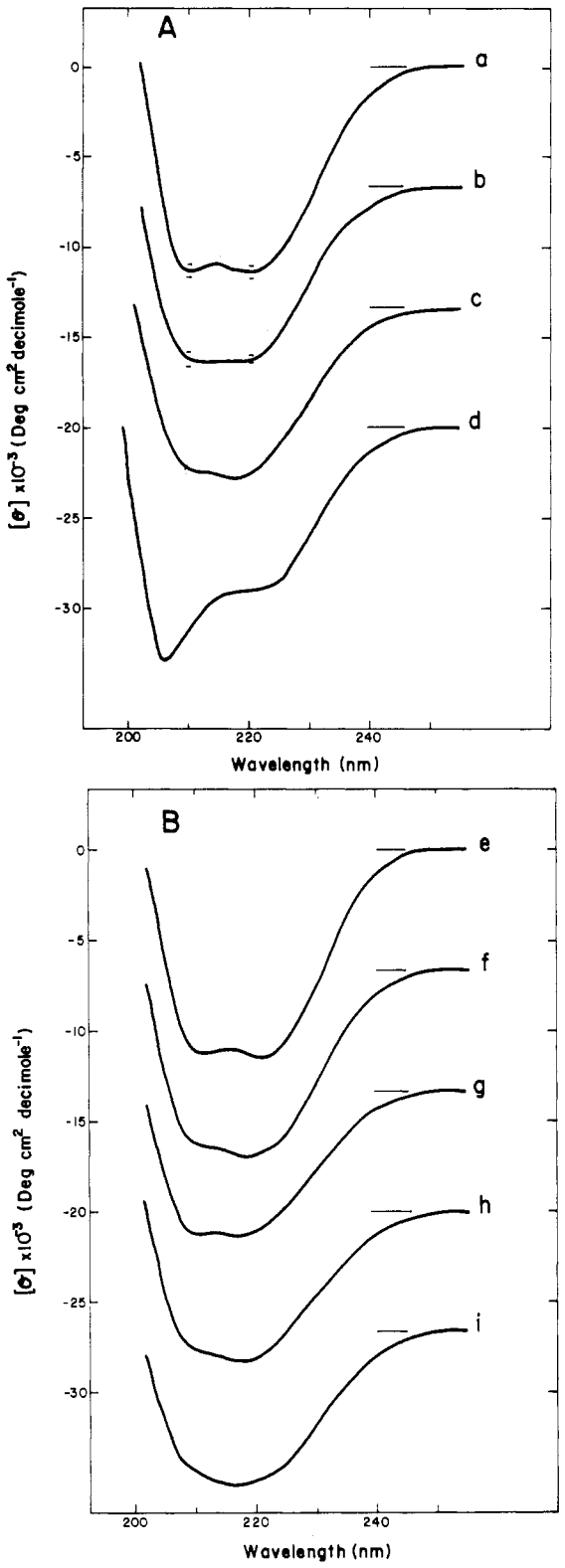

FIGURE 1: Effects of detergents on the circular dichroism of tubulin in PG buffer at 25 °C. (a) Spectrum obtained in the absence of detergent; (b) 40 mM octyl glucoside; (c) 80 mM octyl glucoside; (d) 6.9 mM sodium dodecyl sulfate; (e–i) 2.4, 3.6, 4.8, 6.0, and 12.0 mM deoxycholate, respectively. Tubulin concentration was typically 0.2 mg/mL. Succesive spectra are shifted 6600 deg cm^2 dmol^{-1} to allow comparison.

duction in the absolute value of the ellipticity in the 210–220-nm region, where the shape of the spectrum changed from having minima at these two wavelengths to a single minimum in the vicinity of 217 nm (spectra c and i). The strong denaturing detergent sodium dodecyl sulfate gave a characteristic

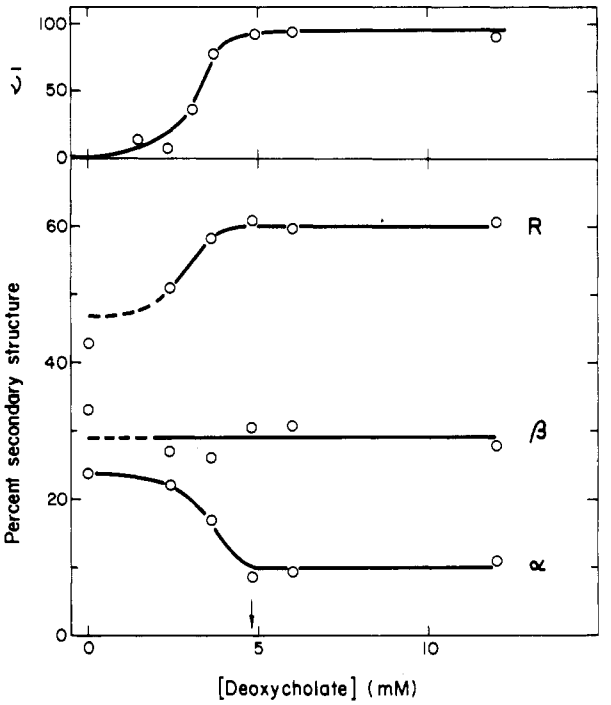

FIGURE 2: Secondary structure changes induced by the binding of deoxycholate to tubulin. Spectra of Figure 1 were computer-fitted by a combination of contributions of α-helix (α), β-sheet (β), and disordered structure (R) (Materials and Methods). The results are compared to the isotherm of binding of deoxycholate (Andreu & Muñoz, 1986) which is reproduced at the top of the figure.

Table I: Effects of Detergent Binding on the Secondary Structure of Tubulin

detergent	percentage of structure[a]		
	α	β	R
none	24	33	43
12 mM deoxycholate	11	28	61
80 mM octyl glucoside	13	28	59
6.9 mM dodecyl sulfate	23	2	75

[a] Estimated by the circular dichroism data fitting described under Materials and Methods. R stands for unordered structure. Measurements were made in PG buffer, pH 7.0 at 25 °C.

and clearly different effect shown by spectrum d. All these changes were analyzed by computer simulation of the experimental spectra in terms of α-helical, β-sheet and R (disordered) secondary structure contributions (Greenfield & Fasman, 1969; Materials and Methods). The results of applying this procedure to the deoxycholate titration of Figure 1B are plotted in Figure 2. The main observation, apart from minor effects that may be within experimental error, was an α-helix to disordered structure transition, while the β-structure content remained nearly unchanged. The decrease in α-helical content was approximately 12%. The transition essentially followed the binding of the detergent (upper part in Figure 2), was sigmoidal, and was practically completed at the critical micelle concentration.[2] A similar curve was generated by simply plotting the ellipticity ratio $[\theta]_{217}/[\theta]_{220}$ vs. detergent concentration (now shown). Table I summarizes the effects of the detergents and shows that the estimated secondary structures of tubulin saturated with octyl glucoside and deoxycholate are essentially coincident and clearly different from

[2] The terms micelle and critical micelle concentration are employed here for convenience, referring to the process of aggregation of deoxycholate monitored fluorometrically (Andreu & Muñoz, 1986).

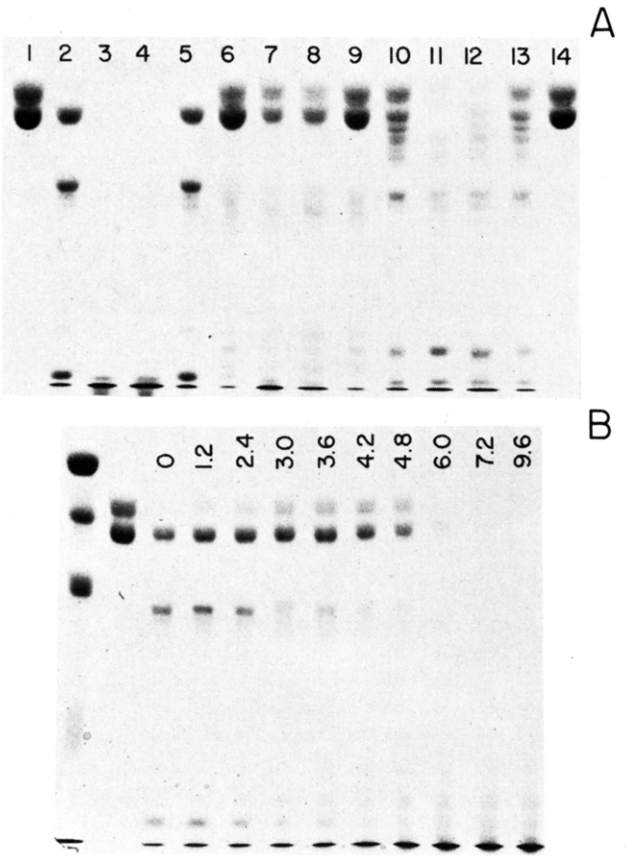

FIGURE 3: (A) Effects of detergent binding on the proteolysis of tubulin (lanes 1 and 13) by trypsin (lanes 2–5), thermolysin (lanes 6–9), and V8 protease (lanes 10–13). Tubulin solutions (2 mg/mL) without any additions (lanes 1, 2, 6, 10, and 14) or with 12 mM deoxycholate (lanes 3, 7, and 11), 50 mM octyl glucoside (lanes 4, 8, and 12), or 2 mM colchicine (lanes 5, 9, and 13) were proteolyzed and subjected to 9% polyacrylamide gel electrophoresis as described under Materials and Methods. (B) Effect of increasing concentration of deoxycholate, shown by the number (mM) above each lane, on the digestion of tubulin by trypsin. The first two unnumbered lanes are the molecule weight standards and undigested tubulin, respectively.

that of the denatured dodecyl sulfate–tubulin complex.

A different and more qualitative manner employed to demonstrate the tubulin structural changes induced by detergent binding was to examine the sensitivity of this protein to controlled proteolysis by trypsin, thermolysin, and *S. aureus* V8 protease. The results are shown in Figure 3A and indicate that trypsin cleaved predominantly native α-tubulin (apparent molecular weight of 54000) into major fragments of M_r 38000 and 16000 (lane 2), whereas the addition of deoxycholate (lane 3) or octyl glucoside (lane 4) resulted in a complete degradation of both α- and β-subunits as well as the primary digestion fragments. The latter effect suggested that both fragments were binding the detergent. Thermolysin (lane 6) cleaved both α and β, generating families of fragments of apparent M_r 29000–37000 and 16000–23000. *S. aureus* V8 protease (lane 10) produced major fragments of M_r 37000, 20000, and 15000 and also a number of products of M_r 41000–48000 generated by close to terminal cleavage of tubulin. Addition of deoxycholate (lanes 7 and 11) or octyl glucoside (lanes 8 and 12) to the two latter systems markedly increased the proteolytic degradation. None of the three proteolysis patterns was sensitive to the addition of 2 mM colchicine in the absence of detergent (lanes 5, 9, and 13). These results were most simply interpreted as a detergent-induced unfolding of the structure of tubulin, making it more accesible to proteolysis. Should the effects observed be due not to increased exposure of the protein substrate but to an activation effect on the protease itself, they would not be expected to take place with three different proteases and the two detergents. Furthermore, the increase in tubulin accesibility to proteolysis could be expected to occur at the detergent concentrations that induced the structural transition detected by circular dichroism (Figure 2). This was verified with trypsin and V8 protease and increasing concentrations of each detergent. The results with trypsin and deoxycholate are shown in Figure 3B, where the detergent concentrations quoted are total concentrations that deviate slightly from the free detergent concentrations. The concentrations plotted in Figure 2 are to a better approximation free concentrations, due to the higher ratio of detergent to protein in the solution. The effects that can be observed in Figure 3B are an initial protection of α at low detergent concentrations, followed by a sharp increase in proteolysis of both α and β at 5–7 mM total deoxycholate. The maximal concentration for bound deoxycholate in this experiment can be estimated in approximately 2 mM. Therefore, the proteolytically detected structural transition took place in excellent semiquantitative agreement with the secondary structure change detected by circular dichroism.

Mutual Inhibition of Detergent Binding and Ligand Binding to the Colchicine Site. Having characterized a structural transition of tubulin induced by the mild detergents, it was of interest to know the effects on functional properties of this protein such as binding of ligands to the colchicine site and microtubule assembly. Specifically, we asked whether binding to the colchicine site would be affected by detergent binding and also if the binding of the detergents to tubulin liganded at the colchicine site would differ from that of unligated tubulin. These questions were addressed by examining the effects of detergents on the binding equilibrium of the colchicine analogue MTC, which is a fast and reversible probe for the colchicine site (Andreu et al., 1984), and also the rate of detergent-induced dissociation of the tubulin–colchicine complex. But prior to this and since colchicine ligands have chemical structures capable of hydrophobic interaction, the possible binding of these ligands to the detergents employed was examined by difference absorption spectroscopy. Figure 4A shows how octyl glucoside perturbs the absorption spectra of colchicine and MTC. The difference spectra had maxima at 382–383 nm, shoulders at approximately 366, 343, and 288 nm, and minima at 322 nm. They conspicuously resembled the difference spectra generated by interaction of these drugs with tubulin (Andreu et al., 1984). However, no significant increase of the fluorescence of the ligands induced by the detergents was noticed. Figure 4B shows a titration of the absorbance perturbation of MTC with increasing concentrations of octyl glucoside. The effect was very small below the critical micelle concentration (marked by an arrow), where the plot bends and grows in an apparently saturable manner. These results strongly suggest that the ligand partitions into the detergent micelles. Therefore, detergents are expected to interfere in measurements of binding to the colchicine site, decreasing the free ligand concentration available for binding to tubulin. Bearing this in mind, the interaction of MTC with tubulin in the presence of detergents was examined. The effect observed was a marked reduction in apparent binding. However, since a full characterization of the equilibria of micelle formation and of micelle–MTC interaction was lacking, it was not possible to accurately quantify the activity of this ligand in the MTC–detergent–tubulin solutions, and the binding affinity could not be measured. Therefore, these

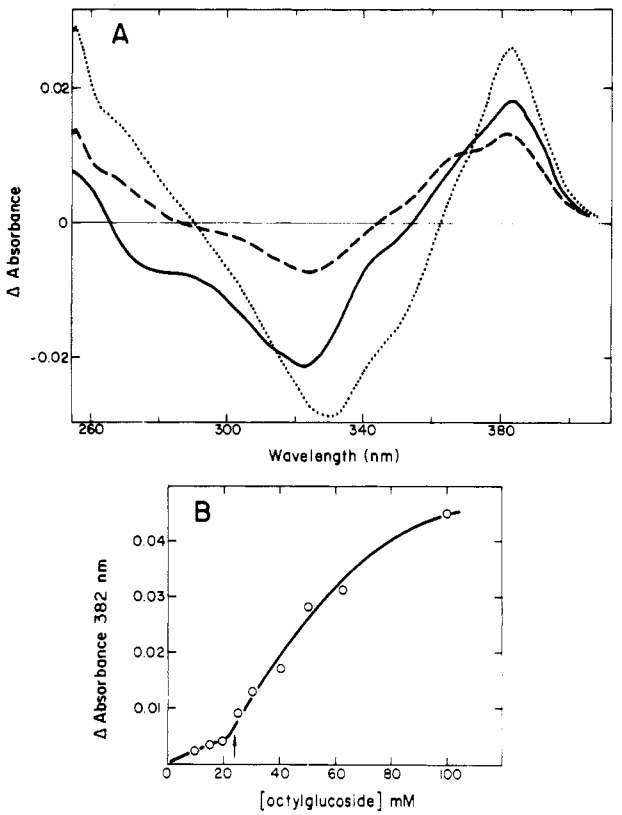

FIGURE 4: Perturbation of the absorption spectra of colchicine and MTC by detergents. (A) Difference spectra generated by interaction of 5×10^{-5} M colchicine with 40 mM octyl glucoside (dashed line), 5×10^{-5} M colchicine with 12 mM deoxycholate (dotted line), and 5×10^{-5} M MTC with 40 mM octyl glucoside (continuous line). (B) A titration of 5×10^{-5} M MTC with octyl glucoside. The arrow marks the critical micelle concentration of the detergent.

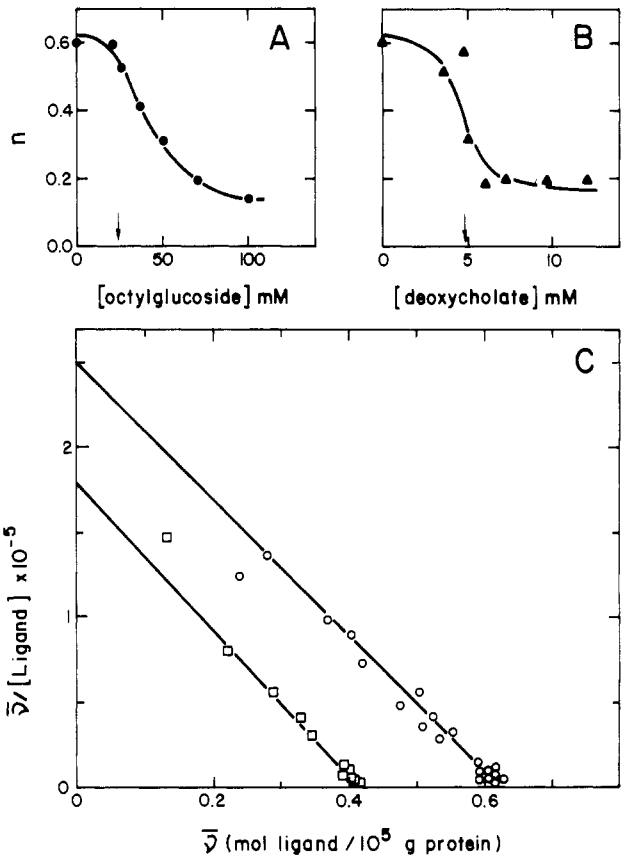

FIGURE 5: Inhibition of the binding of MTC to tubulin by octyl glucoside and deoxycholate in PG buffer at 25 °C. (A and B) Reduction of the number of MTC binding sites by octyl glucoside and deoxycholate, respectively. Arrows mark critical micelle concentration of each detergent. (C) Scatchard plots of fluorometric titrations. The open squares are results of a different experiment in which tubulin was saturated and dissociated from octyl glucoside and titrated in PG–3.4 M glycerol buffer; titration of a nontreated tubulin sample in glycerol-containing buffer gave results superimposable on those obtained in PG buffer (open circles).

experiments were limited to direct fluorometric measurements of the binding of MTC at saturation. The number of sites is plotted vs. detergent concentration in Figure 5A,B, which indicates an inhibitory effect that took place at or above critical micelle concentration. In these experiments tubulin was exposed to detergent and then titrated with MTC, allowing the fluorescence signal to reach equilibrium. Binding of MTC followed by the addition of deoxycholate led to the same results within experimental error, indicating that essentially the equilibrium state of the detergent–MTC–tubulin system had been examined and that both the detergent binding and the binding of MTC were reversible in the time scale of the experiment. The results were most simply interpreted suggesting that (i) the inhibition of MTC binding by detergents was not due to a simple competition for the same site, since this would not cause a reduction in the number of MTC binding sites and (ii) detergent monomers had a decreased affinity for tubulin liganded at the colchicine site, since the effect was observed at concentrations higher than the detergent binding to the unliganded protein.

In a different experiment tubulin was saturated with octyl glucoside in the absence of MTC by incubation of 10 mg/mL protein with 60 mM [^{14}C]octyl glucoside in PG buffer. Then the detergent was dissociated by chromatography through a Sephadex G-50 column equilibrated with PG buffer containing 3.5 M glycerol, a procedure which left 0.12 molecule of ^{14}C-labeled detergent associated per protein molecule. This tubulin was titrated with MTC. The results are depicted by the open squares in Figure 5C and show the same equilibrium constant as for the control of nontreated tubulin, $(4.0 \pm 0.5) \times 10^5$ M^{-1}, and a reduction from 0.63 to 0.42 site. This indicated that approximately 67% of the tubulin molecules were binding MTC like to native protein. When a similar experiment was performed with [^{3}H]deoxycholate, 4.2 residual molecules of detergent were left and less than 5% of the MTC binding sites were recovered.

Finally, in order to further assess the relationship of detergent binding to ligation at the colchicine site, the detergent-induced dissociation of the tubulin–colchicine complex was examined. This kinetic experiment is not subject to the ligand–detergent interaction that hampers equilibrium binding measurements. The results are shown in Figure 6. Deoxycholate produced a biphasic fluorescence decrease (lines c, d, and e). The fast phase could only be recorded at small deoxycholate concentrations above the critical concentration, and its amplitude was approximately 40% of the initial fluorescence. The slow phase had pseudo-first-order kinetics. High concentrations of octyl glucoside produced a very slow fluorescence decrease (line b). The rate constant of the deoxycholate slow phase is plotted vs. detergent concentration in the inset of Figure 6, which once more shows an effect taking place essentially above the critical micelle concentration of the amphiphile, shown by the arrow. At this point the plot bent, and the rate of colchicine dissociation grew steeply with detergent concentration. The lack of binding of detergent monomers was verified as follows. The binding of 3.1 mM deoxycholate, which is below the critical concentration and in the middle region of the isotherm of binding to tubulin

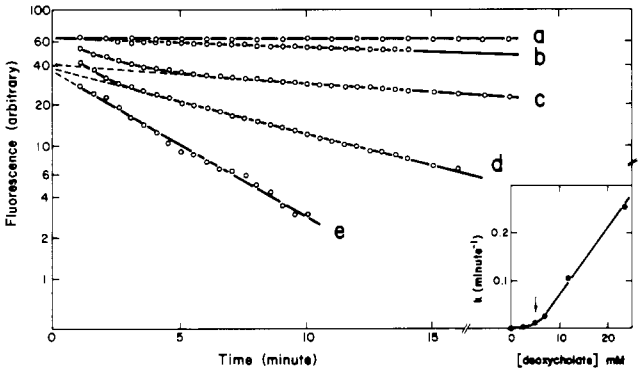

FIGURE 6: Dissociation of the tubulin–colchicine complex by detergents. Tubulin–colchicine complex of 1 mg/mL in PG buffer at 25 °C was excited at 360 nm and the fluorescence of bound colchicine recorded at 430 nm. (a) 0 and 2.4 mM deoxycholate; (b) 4.8 mM deoxycholate and 100 mM octyl glucoside; (c–e) 7.1, 11.8, and 23.6 mM deoxycholate, respectively. The inset is a plot of the pseudo-first-order rate constant of the slow phase of fluorescence decrease vs. deoxycholate concentration.

(Figure 2), was measured by the gel chromatography technique allowing the sample to approach equilibrium for 30 min. Under these conditions tubulin bound 35 ± 5 molecules of deoxycholate, whereas the tubulin–colchicine complex bound only 4.9 ± 4.7 molecules of detergent. These results clearly indicated that massive binding of deoxycholate monomers to the tubulin–colchicine complex did not take place at this detergent concentration. However, deoxycholate bound at higher concentrations since it was able to dissociate the complex, as shown by the fluorescence results of Figure 6. It has not been established whether the biphasic fluorescence decay is due to the existence of two types of tubulin–colchicine complexes (Ide & Engelborghs, 1981) or to two sequential processes, namely, the possible quenching of the fluorescence of bound colchicine by the binding of the detergent followed by the dissociation of colchicine.

Effects of Amphiphiles on Microtubule Assembly. Octyl glucoside was found to inhibit in vitro microtubule assembly. Figure 7A shows the sharp effect of this detergent on the turbidimetric polymerization assay at a fixed protein concentration. Microtubules were observed under the electron microscope at the middle inhibition region (10 mM octyl glucoside), whereas higher detergent concentration (20 mM) caused nonspecific turbidity development in the assembly buffer, and no microtubules could be observed (not shown). The inhibitory effect may be due to an increase in the critical protein concentration needed for polymerization. This is shown by Figure 7B in which the amount of polymer (turbidity) formed is plotted vs. protein concentration of the absence (open circles) and presence of 10 mM octyl glucoside (filled circles). It should be noted that this inhibition was probably exerted by only a few molecules of octyl glucoside bound to each tubulin molecule, since the assembly buffer contained 3.4 M glycerol and this cosolvent inhibits the massive binding of mild detergents to tubulin (Andreu & Muñoz, 1986). In a different experiment tubulin was saturated with octyl glucoside in PG buffer, and the detergent was dissociated by gel chromatography (see above), after which the solution was made up to the composition of assembly buffer and microtubule polymerization assayed. Polymerization proceeded with a critical concentration of 1.25 mg/mL (shown by the open squares in Figure 7B), which was close to that of the control protein (1.13 mg/mL, open circles in Figure 7B). Microtubules and closely related structures were formed, as shown by the electron micrograph in Figure 8A. These results indicated that the

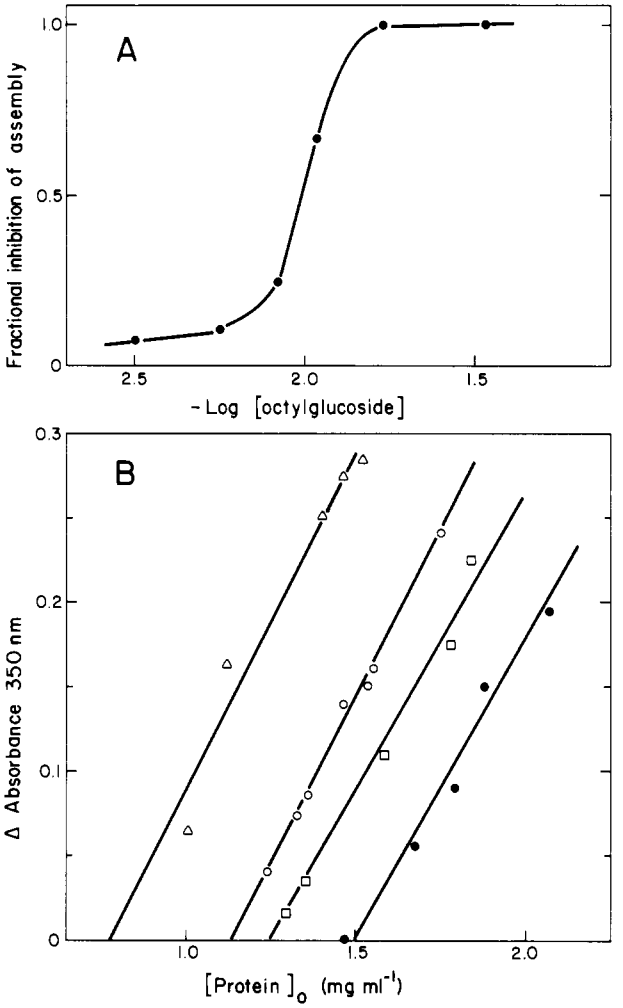

FIGURE 7: Effects of octyl glucoside and deoxycholate on microtubule assembly. (A) Inhibition of the assembly of 1.7 mg/mL tubulin by octyl glucoside. The assembly reaction was started by heating to 37 °C a solution of tubulin and octyl glucoside in assembly buffer (Materials and Methods). The reaction was followed turbidimetrically until it reached the plateau and the sample cooled for disassembly. The inhibition of the reversible plateau turbidity relative to controls without detergent is plotted vs. detergent concentration. (B) Effects of octyl glucoside and deoxycholate on the critical protein concentration. (Open circles) No detergent; (filled circles) 10 mM octyl glucoside; (triangles) 0.24 mM deoxycholate. The squares represent an experiment in which tubulin was saturated with octyl glucoside in PG buffer, the detergent dissociated, and the protein transferred to assembly buffer (see Results) and assembled.

majority of the protein (approximately 90%) had recovered the ability of self-assemble, while the rest had probably denatured during the experimental handling. A more marked tendency to nonspecific aggregation was noticed in the samples that had been dissociated from octyl glucoside.

High concentrations of deoxycholate precipitated in assembly buffer. Sodium cholate, which did not precipitate, effectively inhibited microtubule assembly at a concentration of 20 mM. Other steroidal amphiphiles such as digitonin and saponin also inhibited microtubule assembly. Saturation of tubulin with deoxycholate in PG buffer, followed by partial detergent removal and transfer to assembly buffer, did not allow microtubule polymerization. Low concentrations of cholate and deoxycholate had the characteristic effect of enhancing polymerization. This was due to a decrease in the critical protein concentration, as shown by the experiment indicated by the open triangles in Figure 7B, which was made at 0.24 mM deoxycholate (total concentration). Electron

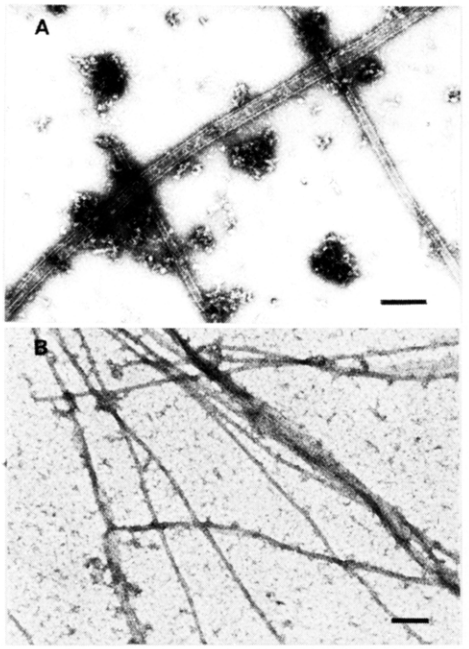

FIGURE 8: (A) Electron micrograph of polymers assembled from tubulin previously saturated and dissociated from octyl glucoside (Results and Figure 7B); protein concentration was 2.2 mg/mL. (B) Assembly products of 1.5 mg/mL tubulin with 0.24 mM deoxycholate in assembly buffer containing glycerol. Bars indicate 200 nm.

microscopy showed microtubules and ribbons (Figure 8B and 9A). Increasing deoxycholate concentrations in assembly buffer gave larger slopes in the critical concentration plot and poorer reversibility upon cooling, suggesting the formation of other polymers or aggregates. The effect of deoxycholate on the critical concentration was most clearly shown when 2.4 mg/mL tubulin was induced to assemble in buffer without glycerol by 2.41 mM deoxycholate. It was verified that the ordered structures formed dissapeared when the sample was cooled before fixation. Under these conditions a concentration of 8–10 mg/mL tubulin is required for assembly without deoxycholate (Lee & Timasheff, 1977). Apart from the microtubules the structures induced by deoxycholate were ribbons, and the larger tubes are shown in lower Figure 9. The structural relationship of the deoxycholate-induced ribbons and macrotubules to microtubules was examined. The ribbons were 130 nm wide and typically had 26 protofilaments, and it was possible to visualize a singular line dividing them into two parts, 13 filaments each (Figure 9A). The optical diffraction patterns of the ribbons result from the superimposition of two independent diffraction patterns, each one mirror symmetric to the other along the axis defined by the protofilaments (not shown). Each diffraction pattern arises from half of the ribbon, as shown in Figure 9 B,C. This suggests that the two half ribbons show opposite faces, giving rise to the diffraction patterns characteristic of microtubules (Amos, 1979) but mirror symmetric to each other. Whether the polarities of each half ribbon are equal or opposite cannot be determined at the present level of resolution. The larger structures induced by deoxycholate appear as flattened tubes which have a roughly constant width around 80 nm, with very little surface morphology (lower part of Figure 9). The diffraction pattern (inset) shows two sets of spots (corresponding to 5 and 4 nm) resulting from the superimposition of two sets of parallel protofilaments rotated 82° from each other. It is possible that these microtubules would arise from the helicoidal folding of the ribbons, giving rise to the protofilament line sets superimposed in projection. The process is easily visualized

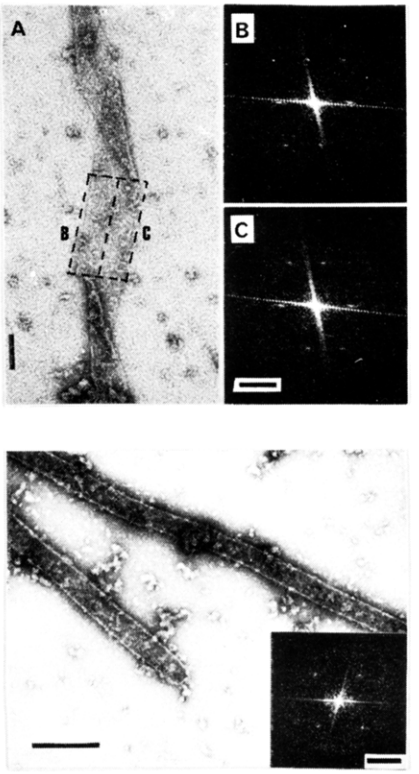

FIGURE 9: (Upper part) (A) Typical ribbon formed by 1.0 mg/mL tubulin with 0.24 mM deoxycholate in assembly buffer containing glycerol. The bar indicates 100 nm. The optical diffraction patterns B and C correspond to the regions of the half ribbons marked in the micrograph; the bar represents $(5\ nm)^{-1}$. (Lower part) Macrotubes formed by 1.9 mg/mL tubulin with 2.4 mM deoxycholate in buffer without glycerol. The bar indicates 200 nm. (Inset) A representative optical diffraction pattern of these structures. Bar indicates $(5\ nm)^{-1}$.

imagining the closure of the twisted ribon of Figure 9A. From the 82° angle between front and back protofilaments and the width of the ribbons, disregarding the ribbon thickness, a width of 86 nm can be calculated for fully flattened macrotubules. The approximate experimental measurement clearly supports this possibility.

DISCUSSION

Tubulin Structural Change and Reversible Inhibition of Functional Properties Induced by Binding of Mild Detergents. The binding of large amounts of mild detergents to cytoplasmic tubulin was proposed in a previous study to induce a change from water-soluble to amphipathic protein. It was suggested on thermodynamic grounds that the structure of the detergent-bound tubulin would be somehow looser. The structural change was detected hydrodynamically as a large increase in the frictional coefficient of tubulin bound to deoxycholate (Andreu & Muñoz, 1986). In the present work the change has been characterized by circular dichroism. Analyzed by the Greenfield and Fasman (1969) procedure, it consisted in a partial helix to coil transition. This was equally induced by deoxycholate and octyl glucoside and was clearly different from denaturation by dodecyl sulfate. At the present level of accuracy the results indicated that the structural transition was induced by bulk detergent binding and not by high-affinity binding of the first detergent molecules. No distinction can be made between detergent-mediated or detergent-facilitated pathways. The β-sheet and R values given here for native tubulin and tubulin–dodecyl sulfate (Table I) are not coincident with those reported in a previous conformational study of calf brain tubulin (Lee et al., 1978). They do not have to

coincide, since different sets of standards were employed in both studies (Materials and Methods). It has to be remarked that analyses of circular dichroism in terms of secondary structure are necessarily very simplifying procedures which give parameters lacking a reliable absolute value, as discussed by Lee et al. (1978). However, these arbitrary parameters are very useful to investigate changes as exemplified in the present study. The changes reported were independent, within experimental error, of the set of standards employed.

Under defined conditions of limited proteolysis of tubulin by trypsin, thermolysin, or V8 protease, octyl glucoside or deoxycholate induced a marked increase in degradation. This indicated a more accessible or unfolded protein structure in agreement with the dichroism analysis. The trypsin and thermolysin proteolysis patterns in the absence of detergents are not the same as reported in other studies (Brown & Erickson, 1983; Serrano et al., 1984). These patterns may obviously depend on the tubulin preparation procedure and exact solution conditions, which, in our case, were chosen to be as close as possible to the rest of the study and to keep within limits of tubulin stability (Andreu & Timasheff, 1982; Prakash & Timasheff, 1982).

The conformational change induced by mild detergents is apparently different from the change elicited by incorporation of tubulin into phospholipid vesicles which was reported to increase the helical content of the protein, to hinder collisional quenching of fluorescence, and to partially protect tubulin against proteolysis (Kumar et al., 1981). The present change is also different from the effects of trifluoroethanol on microtubule protein, which were mainly a β-sheet to α-helix transition (Bayley et al., 1983). However, the change induced by mild detergent binding resembles those reported for the binding of the tranquilizing drug chlorpromazine. Nine or ten molecules of this amphiphilic compound bind cooperatively to tubulin (Hinman & Cann, 1976). Binding of the first amphiphile molecule induced a circular dichroic change that was analyzed as a marked decrease in α-helical content, with increase in β and R structures (Appu Rao et al., 1978).

Which features of the tubulin molecule are responsible for the water-soluble/amphipathic protein transition? Typical water-soluble proteins do not interact extensively with nondenaturing amphiphiles (Tanford, 1980). However, the high-density plasma apolipoproteins AI and AII, which are water-soluble, have a few high-affinity binding sites for amphiphiles. Binding to those sites induces a conformational change that allows the cooperative binding of a large number of amphiphile molecules, apparently to fill a fixed volume per polypeptide chain. In contrast to this, integral membrane proteins have hydrophobic regions which appear to nucleate whole detergent micelles (Tanford, 1980, pp 165–180). The hydrophobic regions of typical membrane proteins can be directly recognized in their amino acid sequences as significantly apolar segments, which do not appear in the soluble proteins examined (Kyte & Doolittle, 1982). Soluble proteins that interact with lipid bilayers are generally lytic components that either are arranged in the form of transmembrane helices forming oligomeric pores or bind to the polar–apolar interface at the surface of the membrane, thus perturbing its structure (Eisenberg, 1984; Bhakdi & Tranum-Jesen, 1983; Podack, 1985). These transmembrane or surface helices typically show an uneven distribution of polar and apolar residues at opposite faces, giving them an amphipathic character (Eisenberg et al., 1984). There are no strongly hydrophobic tracts in the amino acid sequences of α- and β-tubulin (Postingl et al., 1981), although its potential to form strongly amphipathic secondary structure elements has not been documented. The Fourier transforms of the hydrophobicities of the amino acids (Finer-Moore & Stroud, 1984) in the sequences of α- and β-tubulin reveal defined segments that show a significant deviation from a random distribution at periods of 2 amino acids (β-sheet) and 3.5 amino acids (α-helix). Comparison of the intensity of these putative amphipathic elements with other known soluble and membrane protein parts (Eisenberg et al., 1984) might indicate possible regions of binding of amphiphiles to tubulin (Medrano and Andreu, unpublished results). It is conceivable that natural and model amphiphiles may bind to the apolar faces of tubulin folding domains, which are normally in contact with each other. In the case of mild detergents, separation of these faces need not impose strong structural constraints, but favor contact with the solvent, a loosening of tertiary structure, and a partial randomization of secondary structure. Such regions could be involved in the formation of contacts among protomers during normal microtubule assembly. Similar regions could conceivably interact with the hydrocarbon core for the assembly of tubulin-like proteins into membranes.

The detergent-bound state of tubulin entails the inhibition of binding to the colchicine site as well as microtubule assembly. It is important that when a full dissociation of detergent is achieved in the case of octyl glucoside, both the native hydrodynamic characteristics (Andreu & Muñoz, 1986) and these functional properties are regained by 70–90% of the protein (Results and Figures 5C, 7B, and 8A). This strongly suggests that the structural change induced by mild detergent binding is essentially reversible and not a denaturing one, which would be very difficult to reverse in such a labile protein as tubulin. On the other hand, the conformational effects and reversibility of mild detergent binding can only be met by a large degree of protein flexibility, which may also be suggested by the reported low level of secondary structure potentials of large parts of the amino acid sequence of tubulin (Postingl et al., 1981).

Detergent Binding and the Colchicine Site. Since the binding of colchicine ligands is in part hydrophobic and induces a conformational change of tubulin (Andreu & Timasheff, 1982; Andreu et al., 1984), it was of interest to examine the relation of the colchicine site to the binding of mild detergents. It was first shown that colchicine ligands interact with mild detergent micelles (Figure 4). The expected effects on colchicine or analogue binding assays are interference by binding to micelles and also a reduction in free ligand available for interaction with tubulin. Therefore, any measurements of binding of colchicine ligands in the presence of detergents like deoxycholate or octyl glucoside such as those performed in the characterization of membrane tubulins [see introduction in Andreu & Muñoz (1986)] have to take into account the possible artifactual effects of ligand binding to the detergent micelles. This may be achieved by (i) looking at the very specific enhancement of the fluorescence of colchicine ligands upon binding to tubulin, which has not been observed with octyl glucoside or deoxycholate, and (ii) performing an adequate characterization of the several equilibria involved. Fluorometric equilibrium measurements at saturation of the colchicine site probe, MTC, in the presence of the detergents showed a decrease in the number of available sites above critical micelle concentrations. This indicated an inhibition of binding to the colchicine site by the detergents and suggested a different mode of binding of the detergents to tubulin liganded at the colchicine site. The latter was verified by examining the rate of dissociation of the tubulin–colchicine

complex, which was induced by deoxycholate above critical micelle concentration, and also by direct measurement of the binding of deoxycholate to tubulin–colchicine, which showed an inhibition of detergent monomer binding to the liganded protein. These results indicate that while the binding of mild detergents to tubulin is essentially of detergent monomers (Figure 2; Andreu & Muñoz, 1986), the binding to tubulin liganded at the colchicine sites takes place at higher detergent concentration. This suggests a decrease in the affinity of detergent monomers for the protein (Reynolds, 1979) in its liganded state. Alternately, the species binding to the liganded protein could be the detergent micelles, into which tubulin would insert. The two simplest interpretations are (i) the unliganded colchicine site is an area that participates in the nucleation of the cooperative binding of detergent monomers to tubulin and (ii) colchicine ligands and the mild detergents bind preferentially to different tubulin conformations. These two possibilities, which are not exclusive, are fully consistent with the hydrophobic character of part of the colchicine site and the different structural changes detected by circular dichroism that are induced by colchicine (Andreu & Timasheff, 1982) and the detergents (this paper).

Tubulin Self-Assembly. Octyl glucoside has been found to inhibit microtubule assembly by raising the critical protein concentration. This indicates a small reduction in the affinity of the growth reaction of these polymers, whose thermodynamic characteristics are consistent with the participation of hydrophobic interactions (Lee & Timasheff, 1977; Hinz et al., 1980). The inhibition could be related to the fact that octyl glucoside induces a tubulin self-association different from microtubule polymerization (Andreu & Muñoz, 1986). However, the assembly buffer employed contains 3.4 M glycerol, and this cosolvent has been shown to restrict the binding of deoxycholate and octyl glucoside to tubulin to possibly a few molecules. Therefore, the inhibitory effect is most probably due to the binding of detergent to a few sites that might be involved in the formation of the contacts between tubulin molecules in the microtubule and is not due to the large binding and acompanying structural transition observed in PG buffer. Since other amphiphiles (cholate, digitonin, and saponin) were shown to inhibit microtubule assembly, the inhibitory effect is probably general and not due to the particular chemical structure of octyl glucoside.

Very low concentrations of cholate and deoxycholate had the interesting effect of enhancing microtubule polymerization and lowering the critical tubulin concentration. At these detergent concentrations the not rigorously corresponding measurements of binding in PG buffer indicated less than one molecule of deoxycholate bound per soluble tubulin heterodimer. The enhancement of protein polymerization by a ligand implies that the ligand binds more to the polymer that to the monomer (Wyman, 1964). Such linkages have been quantitatively studied in the case of tubulin polymerization with a variety of ligands like protons, magnesium ions, and vinblastine (Timasheff, 1978). The detailed mechanism by which deoxycholate enhances tubulin polymerization is not known at present. Binding of this anionic detergent may add negative charge to tubulin, and therefore, a repulsion among protomers might be expected, unless the carboxy groups of deoxycholate associated to tubulin bind magnesium ions, leading to an increase in the preferential interaction of this metal with the polymerized protein. However, the detailed characterization of such possible linkages is technically difficult due to the need to know the activities of deoxycholate and magnesium in the polymerization solution and to the fact that deviations from the correct microtubule polymerization geometry appeared at increasing detergent concentrations. Actually, it is the structure of the deoxycholate-induced polymers, in particular under solution conditions in which tubulin does not polymerize in the absence of the detergent, that gives some clues on the mechanism of the process. Apart from microtubules these polymers have been shown to consist to opened microtubular sheets of 13 protofilaments which are associated in pairs by the lateral interaction of elements of opposite orientation (Figure 9A). Similar structures have been previously reported to form from microtubule protein (Mandelkow & Mandelkow, 1979). These ribbons probably folded helically to form the macrotubules whose walls were closed by lateral interaction of protofilaments. It is not known whether the association of half ribbons was made by interaction of protofilaments of the same or opposite polarity; in any case, simple geometrical considerations indicate that the protofilament interaction that closes the macrotubules has to be of the same type. This suggests that low concentrations of deoxycholate not only have the general effect of enhancing tubulin polymerization but also slightly favor microtubule opening and/or the lateral association of protofilaments at the ribbon borders. Deoxycholate-induced ribbons are clearly different from zinc sheets, in which the protofilaments have alternating opposite polarities (Amos, 1979), but may be related to other forms of tubulin polymerization more similar to microtubules such as the "S"-shaped structures (Burton & Himes, 1978) or calcium-induced twisted ribbons and macrotubes (Matsumura & Hayashi, 1976). All these arise by the inherent property of tubulin of making not only interprotomer bonds leading to the formation of the microtubule wall but also different bonds forming junctions between microtubule walls (Mandelkow & Mandelkow, 1979).

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Registry No. MTC, 60423-21-4; colchicine, 64-86-8; octyl glucoside, 29836-26-8; deoxycholate, 83-44-3.

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No Phospholipid Monolayer–Sugar Interactions†

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ABSTRACT: Studies by a number of workers using the Langmuir film balance have shown that when carbohydrates, such as sucrose or glycerol, are dissolved in a subphase on which a phospholipid is spread, film expansion occurs (Cadenhead & Demchak, 1969; Cadenhead & Bean, 1972; Maggio et al., 1976; Maggio & Lucy, 1978). Recently such effects have been observed again, particularly with the carbohydrates galactose and trehalose (Johnston et al., 1984). The origin of these film expansions was uncertain, and various suggestions have been made to explain them. One idea was that they might be due to interactions which these carbohydrates have with the water molecules close to the polar head groups of the lipids. Recent studies in our two laboratories, described here, show that the magnitude of the expansion effects is variable and that in general they arise from surfactant impurities in the sugars. These impurities are observed in carbohydrates which are reputedly of high grade; the amount of impurity present can vary from batch to batch, and sometimes they can be difficult to remove. Film balance techniques or subphase preparation can mask the detection of minor impurities. The presence of surfactant impurities in reputedly pure carbohydrates needs to be considered in other biochemical and biophysical studies of lipids and cell membranes.

Measurements of the relationship between the surface pressure and area of monomolecular films have long been used to obtain information about the forces operating within the film and by extrapolation to biomembranes made up of the same type of lipid molecules (Phillips & Chapman, 1968). However, study of surface pressure–area isotherms can also throw light on the interaction between film molecules and other

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