Depolymerization of Microtubules Increases the Motional Freedom of Molecular Probes in Cellular Plasma Membranes

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ABSTRACT Depolymerization of microtubules resulted in an increase in the motional freedom of molecular probes in the plasma membranes of Chinese hamster ovary cells expressed by the order parameter, S, measured with two different lipid-soluble spin label probes, 5-doxyl stearic acid and 16-doxyl methylstearate. Treatment with a variety of microtubule-depolymerizing agents, including Colcemid, colchicine, vinblastine, podophyllotoxin, and griseofulvin, all had similar effects on motional freedom of the probes whereas β-lumicolchicine was inactive. Several independent lines of evidence suggest that these changes in motional freedom of the probes were not the direct result of the interaction of these relatively hydrophobic drugs with the plasma membrane: the effects of the drugs were not immediate; the dose response of the Colcemid effect was the same as the dose response for depolymerization of microtubules; taxol, which stabilizes microtubules but does not affect motional freedom in the membranes, blocked the effect of Colcemid on motional freedom; a mutant cell line which is resistant to colchicine because of reduced uptake of the drug showed no effects of colchicine on probe motional freedom; and a Colcemid-resistant mutant cell line with an altered β-tubulin showed no effect of Colcemid on motional freedom in the membrane. These results support the hypothesis that microtubules might affect, directly or indirectly, plasma membrane functions.

Considerable evidence has accumulated indicating that microtubules are able to modulate functions of the plasma membrane including hormone responsiveness (15, 17, 31), capping of cell surface molecules (1, 22), and endo- and exocytosis (26, 35). The mechanism of these effects is not known; however, a direct association of tubulin or microtubules with the cell membrane or membrane-associated structures has been repeatedly observed (5, 6, 10, 27). An early study has demonstrated effects of microtubule depolymerizing agents on phagocytosis-induced alterations in membrane microviscosity (4).

In this study, evidence is presented that indicates that depolymerization of microtubules results in increased motional freedom of fatty acid probes inserted into the plasma membranes of cultured Chinese hamster ovary (CHO)[1] cells as measured with two different spin label probes. The motional freedom of such spin label probes has been used as a measure of "membrane fluidity." CHO cells were chosen for these studies because of the ease with which they could be grown in suspension (12), because previous spin label studies had demonstrated the feasibility of measuring significant alterations in motional freedom of membrane probes in these cells (18), and because of the availability of specific mutants affecting uptake of antimicrotubule drugs (21) and mutants affecting tubulin itself (9) which could be used as biological specificity controls. Our results support models in which microtubules interact directly or indirectly with the cell membrane and alter membrane function by changing the motional freedom of the lipids in the membrane.

MATERIALS AND METHODS

Preparation of Cells for Electron Spin Resonance (ESR) Measurements: CHO cells were maintained in α-modified minimal essential medium (α-MEM) with 10% fetal bovine serum (M.A. Bioproducts, Walkersville, MD), penicillin (50 U/ml), and streptomycin (50 μg/ml) (Flow

[1] *Abbreviations used in this paper:* αMEM, α-modified minimal essential medium; CHO, Chinese hamster ovary; ESR, electron spin resonance; MS, methylstearate; SA, stearic acid.

Laboratories, Inc., McLean, VA) in monolayer culture as previously described (12, 13). The tubulin mutant cell line Cmd-4 (9) and the multiple drug-resistant cell line C5 (gift of V. Ling) (21) have been previously described. 20 h before spin labeling, cells were treated with 0.25% trypsin, 2 mM EDTA in Tris-Dextrose buffer and suspended at a density of 2–4 × 10^5 cells/ml in 20 ml of complete α-MEM medium in 120-ml glass bottles. The bottles were gassed with CO_2, sealed, and incubated at 37°C in a gyrorotatory shaker bath (New Brunswick Scientific Co., Edison, NJ) at 160 rpm. At the time of spin labeling, cells were at a density of 8 × 10^5/ml and were >90% viable as measured by Trypan Blue exclusion.

For most studies, cells grown in suspension in individual bottles were combined and divided into equal portions. The appropriate amounts of drugs were added and incubation was continued for an additional 45 min. When two drugs were used, as in the case of taxol and Colcemid, the second drug was added 10 min after the first one. Cells were then pelletted in a desk-top laboratory centrifuge and resuspended in phosphate-buffered saline (PBS) to a concentration of 10^6 cells/0.06 ml of PBS. When the mutant cells were used, direct comparison between mutant and wild-type cell lines was not possible since it was shown that cells grown to different densities have membranes with different fluidities, as discussed in Results. 10^6 cells were enough for obtaining good quality ESR spectra.

Spin Labeling of the Cells: To compare the biophysical status of the membranes of CHO cells, we used two types of spin labels: 5-doxyl stearic acid (SA) and 16-doxyl methylstearate (MS), both purchased from Syva Co., Palo Alto, CA. These spin labels are stable in ethanol for several months at −20°C. From such solutions, appropriate volumes were measured into glass conical test tubes and diluted two- to threefold with ether, and the solvents were evaporated in vacuum. The amount of spin labels in each tube was ~8 × 10^{-8} mol and was used to label 10^6 cells. Spin labeling was done by adding the cell suspension in ~0.05–0.07 ml of PBS to the spin label-containing test tubes. The time needed to get practically all the spin label into the cellular membrane is ~30 s for the 5-doxyl SA label and ~90–120 s for the 16-doxyl MS at ambient temperature. We believe that the majority of the spin probes are located in the plasma membrane based on the discussion of Bales et al. (3). After the necessary contact time, the cell suspension was sucked into a 50-μl micropet capillary (Clay Adams Div., Parsippany, NJ) and sealed at the bottom with Critoseal (Syva Co.). Care was taken to avoid direct contact of the cell suspension with Critoseal so as to avoid leakage of Mn^{2+} ions from the seal into the cell suspension.

Instrumentation: ESR spectra were recorded at X-band with a Varian E-9 Century series spectrometer (Varian Associates, Inc., Palo Alto, CA) operated at 9.5 kHz, 100 kHz field modulation, 4 gauss modulation amplitude, 100 gauss sweep range, and 20 mW microwave power. The scan time and receiver gain were changed according to requirements for obtaining a good spectrum. The temperature of the probe was set (±0.5°C) by a Varian variable temperature accessory using N_2 gas flow.

Evaluation of the ESR Spectra: The parameters measured in ESR experiments are $2T_\parallel'$ and $2T_\perp'$, components of the motionally averaged nitrogen hyperfine tensor from which the order parameter (S) and the polarity factor (a_N') are calculated. The equation used for these calculations is from Hubbell and McConnell (16) and was used in experiments similar to ours by Butterfield et al. (8):

$$S = \frac{(T_\parallel' - T_\perp')\, a_{NxL}}{(T_\parallel - T_\perp)_{xL}\, a_N'},$$

where $_{xL}$ refers to single crystal parameters obtained from the data of Hubbell and McConnell. The definitions of $2T_\parallel'$ and $2T_\perp'$ are shown in Fig. 1; $a_N' = 1/3\,(T_\parallel' + 2T_\perp')$. The correction factor 1.6 gauss was used in the estimation of $T_\perp'$ values (16). We have preferred order parameter calculations by this method rather than by the method used by Bales et al. (3), mainly because previous membrane studies with CHO cells also used this calculation (18), allowing comparison of results of our study with this previous study.

ESR spectra obtained from membranes of intact cells with the spin labels 5-doxyl SA and 16-doxyl MS show contributions mostly from spin labels of restricted motion with negligible contributions from free-moving spin labels. Occasionally, some contribution to the spectrum was obtained from free labels and spin-spin interaction in the case of 16-doxyl MS. Therefore, the calculated order parameters are not true order parameters with this probe. Generally, the spin label concentration was kept low enough to avoid spin-spin interaction. Standard deviation of the measurements of $2T_\parallel'$ and $2T_\perp'$ were between 0.1 and 0.3 for 5-doxyl SA and were 0.15 gauss for 16-doxyl MS and are given in Tables I and IV for these two types of cell treatment.

To evaluate the direct and immediate effect of any interaction of the cellular membranes with the applied drugs, we followed the usual cell preparation method and spin-labeled ~10^6 untreated CHO cells as described above, and we obtained an ESR spectrum. The cells were recovered from the capillary and then treated with 0.5 μg Colcemid in a 0.02-ml solvent volume immediately after which an ESR spectrum was obtained. A similar procedure was followed for assessing the effect of 1% dimethylsulfoxide, the solvent used for most of the drugs, and present at a concentration of ≤1% in most experiments. No change was observed in the ESR spectra obtained from spin-labeled cells after a few minutes of drug treatment and no change was seen at all after 1% dimethylsulfoxide treatment.

All experiments were performed in duplicate, i.e., a fresh batch of cells was grown a second time, isolated, and labeled, and ESR spectra were obtained. Some basic experiments, such as those with untreated, Colcemid-treated, and taxol-treated cells, were performed four to nine times and standard deviations were calculated. When only two experiments were done, no statistical data are shown for the calculated ESR parameters, except in Table I.

Immunofluorescence Localization of Microtubules: Cells were grown in 35-mm dishes, washed with PBS, fixed in 1% glutaraldehyde, treated with sodium borohydride (1 mg/ml in PBS), permeabilized with 0.1% Triton X-100 in PBS, and treated sequentially with a mouse monoclonal antibody to chick α-tubulin (40 μg/ml) (Miles Laboratories Inc., Elkart, IN) followed by affinity-purified rhodamine-labeled goat anti-mouse IgG (20 μg/ml) (gift of Mark Willingham, National Institutes of Health). All antibodies were in a PBS solution containing 4 mg/ml normal goat globulin, 1 mM EGTA, and 0.1% saponin. Microtubules were visualized with a Zeiss epifluorescence microscope.

RESULTS

Microtubule-depolymerizing Drugs Affect Membrane Fluidity

Spin label analysis of CHO cells treated with various anti-microtubule agents allowed a determination of the effect of these agents on the motional freedom of the inserted spin probe into the plasma membrane. The evaluated parameters for ESR spectrometric measurements, $2T_\parallel'$ and $2T_\perp'$, are components of the motionally averaged nitrogen hyperfine tensor from which the nitrogen isotropic coupling constant, a_N', and the order parameter, S, are calculated. An S value equal to one represents no motional freedom of the probe, as would be the case in a crystal lattice, whereas S values less than one represent increasing motion of the spin label probe which can be interpreted as increased membrane fluidity. The theoretical explanation and the applicability of these measurements are discussed in the literature (7, 16). Basically, S provides a measure of the mean angular deviation of the hydrocarbon chain of the spin label from its averaged orientation in the lipid bilayer and is a direct measure of the motional freedom of the inserted fatty acid spin probe. This mean angular deviation of the hydrocarbon chain of the spin label, also called the rotational diffusion about the long axis, is rapid and can be assessed within the time frame of ESR measurements.

The ESR spectra obtained with the 5-doxyl SA inserted into the CHO cells treated with either taxol, which stabilizes microtubules, or microtubule-depolymerizing agents, showed significant differences (Fig. 1). The ESR spectrum of taxol-treated cells was not significantly different from that of untreated cells. Table I summarizes the calculated parameters for untreated cells and CHO cells treated with 0.5 μg/ml Colcemid (1.3 μM), 2.5 μg/ml colchicine (6.3 μM), 5 μg/ml podophyllotoxin (12 μM), 2.5 μg/ml vincristine (3 μM), and 125 μg/ml griseofulvin (350 μM). These concentrations were sufficient to cause complete depolymerization of microtubules in CHO cells as measured by indirect immunofluorescence using antitubulin antibodies (data not shown).

When the deviation between two measurements of $T_\parallel'$ was not more than 0.1 gauss, only two measurements were made. This was found to be adequate since when more measurements were made, as in the case of untreated and Colcemid-

treated cells, the standard deviation of S is 0.004 and 0.005, respectively. Standard errors of similar magnitude were calculated by Lai et al. (18) from their data which were also obtained with 9 GHz ESR measurements. Therefore, the difference in the motional freedom of the probe, expressed by S, of untreated CHO cells, 0.680, and that of Colcemid-treated CHO cells, 0.664, is significant. The same argument holds for the membrane fluidity of CHO cells treated with the other microtubule-depolymerizing drugs.

To show that changes in ESR spectra were related to the ability of the drugs to depolymerize microtubules, we determined a dose response of CHO cells to Colcemid. As shown in Table II, effects of Colcemid on $T_{\parallel}'$ were observed only with concentrations of Colcemid that detectably depolymerize microtubules. Conversely, treatment with taxol and with taxol followed by Colcemid, which blocks microtubule depolymerization, blocked the observed changes in the motional freedom of the probe (Table I). β-Lumicolchicine, a colchicine analog with no biological activity, resulted in no significant changes in membrane fluidity as expressed by the S values (Table I).

Calculations were made only from results obtained at 22°C. The $2T_{\parallel}'$ values obtained varied with temperature, as expected. This variation for untreated, taxol-, Colcemid-, and colchicine-treated CHO cells is shown between 10 and 38°C in Fig. 2. No attempt was made to calculate other parameters such as a_N' and S with data obtained at temperatures other than 22°C, because the largest differences were found at this temperature. Measurements at 10 and 28°C were used only to confirm results obtained at 22°C. Some reduction of the free radical was observed at 28 and 38°C that prevented us from obtaining full spectra in many cases; only the outer hyperfine splitting value could be recorded at these temperatures. No such free radical reduction was observed with artificial membranes by Hubbell and McConnell (16) with which they could produce an S versus temperature graph. The magnitude of changes in the measured $2T_{\parallel}'$ and $2T_{\perp}'$ values for untreated and microtubule-depolymerizing drug-treated cells were of the same order as those found by many investigators who have compared membranes of biologically altered cells (2, 11, 18, 34).

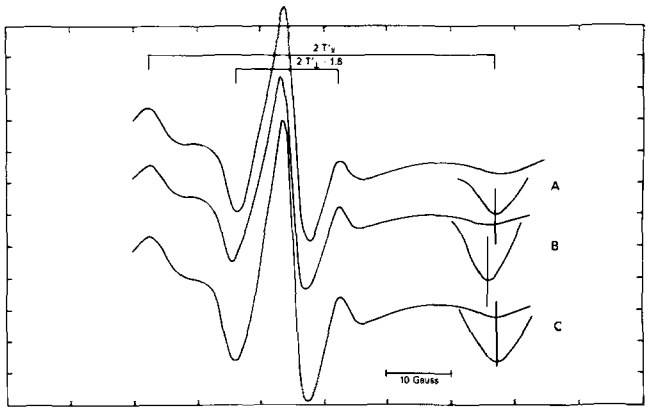

FIGURE 1 ESR spectra obtained with 5-doxyl SA spin label in (A) 10 μg/mg taxol-treated, (B) 0.5 μg/ml Colcemid-treated, and (C) 10 μg/mg taxol-treated followed by 0.5 μg/ml Colcemid-treated CHO cells. Positions of minima of the high field hyperfine splitting are marked with vertical bars. The extrema used in the calculation of the $T_{\parallel}'$ and $T_{\perp}'$ tensors are shown with the spectrum obtained with taxol-treated CHO cells.

Table III contains ESR parameters obtained with 5-doxyl SA in the membranes of CHO cells grown to different densities. The calculated order parameters indicated significant changes in probe motional freedom among membranes of cells between 2×10^5 and 8×10^5 cells/ml. Changes in motional freedom were also observed using another spin label, 16-doxyl MS. A typical ESR curve obtained with 16-doxyl MS spin label in CHO cell membranes, with the measured $2T_{\parallel}'$ and $2T_{\perp}'$ values, is shown in Fig. 3. Order parameter calculations were performed for this probe by the method used for the 5-doxyl SA (8). Results of the spectral parameter measurements and order parameter calculations are shown in Table IV. Results confirmed those obtained with the spin probe 5-doxyl SA, i.e., there was a difference in the membranes among untreated and Colcemid-(0.5 μg/ml) or vincristine- (2.5 μg/ml) treated CHO cells. The observed change in order parameters indicates increased freedom of motion in the membranes of treated cells.

Changes in the Membrane Are Not Due To Membrane-Drug Contact

We have investigated whether any of the tested drugs cause immediate membrane changes upon membrane-drug contact. Such cases were previously reported for other drugs (19, 33). Experiments performed for this purpose as described in Materials and Methods indicated no change in ESR parameters, $2T_{\parallel}'$ and $2T_{\perp}'$, when spectra were obtained immediately after treatment of the cells in PBS with solutions of the drugs. Also, during regular cell preparations, cells were washed twice with PBS to eliminate any possible surface-bound drugs.

TABLE I

*ESR Parameters of 5-Doxyl SA in Untreated CHO Cells**

Treatment (μg/ml)	$T_{\parallel}'$	$T_{\perp}'$	$T' = T_{\parallel}' - T_{\perp}'$	a_N'	S	n
No treatment	27.58 (0.15)	9.06 (0.2)	18.41	15.20	0.680 (0.004)	9
Colcemid (0.5)	27.11 (0.2)	9.20 (0.01)	17.91	15.17	0.665 (0.005)	5
Colchicine (2.5)	27.10 (0.1)	9.10 (0.1)	18.00	15.16	0.662	2
Podophyllotoxin (5.0)	27.15 (0.1)	9.15 (0.1)	18.00	15.16	0.665	2
Vincristine (2.5)	26.75 (0.1)	9.40 (0.1)	17.35	15.20	0.638	2
Griseofulvin (125)	26.75 (0.1)	9.35 (0.05)	17.40	15.10	0.641	2
Taxol (10)	27.66 (0.3)	9.07 (0.2)	18.58	15.20	0.684	4
Taxol (10) followed by Colcemid (0.5)	27.45 (0.1)	9.02 (0.1)	18.42	15.10	0.687	2
β-Lumicolchicine (2.5)	27.35 (0.1)	9.00 (0.1)	18.30	15.12	0.676	2

Standard deviations, where applicable, are given in parentheses, *n* is the number of data points, and S values are calculated as described in Materials and Methods. $T_{\parallel}'$, $T_{\perp}'$, and a_N' are in gauss.

* Experiments performed as described in Materials and Methods, 22°C.

TABLE II

Dose-dependent Effect of Colcemid on $T_{\parallel}'$ of 5-Doxyl SA–Treated CHO Cells Correlates with Microtubule Depolymerization

μg/ml Colcemid	$T_{\parallel}'$	Microtubule depolymerization*
0	27.70	None
0.1	27.45	Few short microtubule segments seen
0.2	27.15	Complete
0.5	27.10	Complete

* Assayed by immunofluorescence.

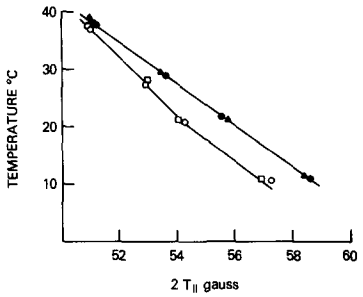

FIGURE 2 Changes of spectral parameter, $2T_{\parallel}$, as function of temperature. Experiments were carried out as described in Materials and Methods. Samples were equilibrated at each temperature in the cavity until no more changes on the detector current could be observed. (○) No treatment; (□) treatment with 10 μg/ml taxol; (▲) treatment with 0.5 μg/ml Colcemid; and (●) treatment with 2.5 μg/ml colchicine.

TABLE III

*ESR Parameters of 5-Doxyl SA in CHO Cells Grown to Different Densities in Suspension**

Cell density	$T_{\parallel}'$	$T_{\perp}'$	$T' = T_{\parallel}' - T_{\perp}'$	S
8×10^5 cells/ml (regular density)	27.75	9.20	18.5	0.676
4×10^5 cells/ml	27.00	9.30	17.7	0.652
2×10^5 cells/ml	16.10	9.90	16.1	0.591

S values were calculated as described in Materials and Methods. $T_{\parallel}'$ and $T_{\perp}'$ values are in gauss.
* Experiments performed as described in Materials and Methods, 22°C.

Another way to determine whether drugs needed to enter the cells to affect the membrane was by examining a CHO mutant resistant to colchicine because of a defect in permeability to this drug (21). The CHO cell mutant, C5, resistant to a colchicine concentration of 2.5 μg/ml because of reduced uptake of colchicine (21), was tested with the 5-doxyl SA probe with or without drug treatment. The results are shown in Table V. The calculated order parameters, 0.669 for untreated and 0.663 for treated cells, indicated no significant effect of colchicine in this cell line.

Membrane Fluidity Changes Are Blocked in a Colcemid-resistant Tubulin Mutant

Another CHO cell mutant, Cmd-4, resistant to Colcemid because of an alteration in β-tubulin (9) was evaluated in parallel to the parent cell line for membrane fluidity changes

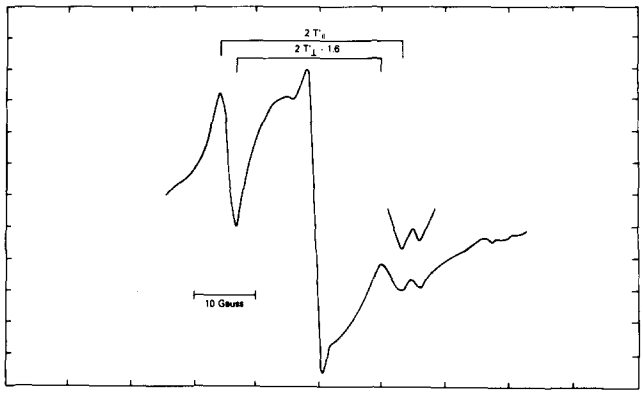

FIGURE 3 ESR spectra obtained with 16-doxyl MS spin label in untreated CHO cells. The extrema used in the calculation of the $T_{\parallel}'$ and $T_{\perp}'$ tensors are shown.

TABLE IV

*ESR Parameters of 16-Doxyl MS in Untreated Colcemid and Vincristine-treated CHO Cells**

Treatment (μg/ml)	$T_{\parallel}'$	$T_{\perp}'$	$T' = T_{\parallel}' - T_{\perp}'$	a_N'	S	*n*
No treatment	14.87 (0.1)	12.25 (0.05)	2.62	13.11	0.110 (0.002)	6
Colcemid (0.5)	14.55 (0.05)	12.40 (0.05)	2.15	13.11	0.092 (0.002)	4
Vincristine (2.5)	14.60 (0.05)	12.45 (0.05)	2.15	13.16	0.091 (0.001)	4

S values are calculated as described in Materials and Methods. $T_{\parallel}'$, $T_{\perp}'$, and a_N' are in gauss. Standard deviations are in parentheses. *n* is the number of data points.
* Experiments performed as described in Materials and Methods, 22°C.

TABLE V

ESR Parameters of 5-Doxyl SA in a Colchicine-resistant CHO Permeability Mutant, Untreated and Treated with 2.5 μg/ml Colchicine

Treatment	$T_{\parallel}'$	$T_{\perp}'$	$T' = T_{\parallel}' - T_{\perp}'$	a_N'	S
Untreated	27.32	9.13	18.20	15.18	0.669
Treated	27.40	9.25	18.15	15.30	0.663

S values were calculated as described in Materials and Methods. $T_{\parallel}'$, $T_{\perp}'$, and a_N' are in gauss.

TABLE VI

*ESR Parameters of 5-Doxyl SA in a Colcemid-resistant Mutant with an Altered β-Tubulin Compared with Wild-type CHO Cells**

Cells (treatment)	$T_{\parallel}'$	$T_{\perp}'$	$T' = T_{\parallel}' - T_{\perp}'$	a_N'	S
Resistant mutant (no treatment)	27.25	9.25	18.00	15.25	0.659
Resistant mutant (0.1 μg/ml Colcemid)	27.20	9.20	18.00	15.20	0.661
Wild-type (no treatment)	27.45	9.10	18.30	15.20	0.675
Wild-type (0.1 μg/ml Colcemid)	27.00	9.15	17.85	15.10	0.659

S values are calculated as described in Materials and Methods. $T_{\parallel}'$, $T_{\perp}'$, and a_N' are in gauss.
* Experiments performed as described in Materials and Methods, 22°C.

when both cell lines were exposed to this drug. Results of the calculations are shown in Table VI. The parent cell line responded to 0.1 μg/ml Colcemid treatment; its membrane fluidity changed from S = 0.675 to S = 0.659. Contrary to this, the treated and untreated mutant cell lines indicated no significant difference in the order parameter, i.e., S = 0.661 and 0.659, respectively. This lower S value obtained for the mutant is probably due to a slightly lower density of mutant cells in this experiment (see Table III).

DISCUSSION

The analysis of the motion of spin label probes in the plasma membrane is a sensitive and reliable indicator of the physical state of the membrane of CHO cells (18). In this study, significant increases in the motional freedom of membrane probes have been shown by this technique to result from depolymerization of microtubules with a variety of antimicrotubule agents. These changes do not appear to be the result of a direct effect of the drugs on the cell membranes since they are not seen immediately after addition of the drugs and are not seen with β-lumicolchicine, a structurally similar but nonfunctional analog of colchicine.

Uptake of the drug by the cells is a prerequisite for these effects on membranes as indicated by the time delay in response and the failure of the colchicine-resistant drug permeability mutant C5 to respond to colchicine (Table V). This mutant belongs to a class of multi-drug-resistant CHO mutants (21) with reduced uptake of a variety of hydrophobic drugs, many of which affect microtubule function (for review see reference 20). Evidence that the antimicrotubule agents must interact with microtubules after entering the cells to alter the motional freedom of the spin label in the membrane is of three kinds: (*a*) the dose response of the effect of Colcemid is similar to the dose response for depolymerization of microtubules for this drug (Table II); (*b*) taxol, a drug known to stabilize microtubules in cultured cells (23), blocks the effect of Colcemid on membranes; and (*c*) a CHO mutant resistant to Colcemid because of an alteration in β-tubulin (9) shows no effect of Colcemid on the motional freedom in the membrane. The results make it highly likely that the effects on membranes we have observed are actually mediated by microtubule depolymerization, rather than resulting from toxic, secondary effects of these drugs at the relatively high concentrations used in these studies.

A previous study using the same spin label probes to study the status of CHO membranes indicated that there are differences in the motional freedom of the spin probe in the membrane throughout the cell cycle with an increase during mitosis and decreases in G1 and S (18). This increase in probe freedom of motion observed during mitosis could be related to the general reorganization of microtubules which is a prerequisite for spindle formation. These results are consistent with our finding that depolymerization of microtubules increases motional freedom of the spin labels in unsynchronized cell populations. Both studies are consistent with the morphological observations of Porter, who found cell cycle-specific changes in the appearance of the cell surface in CHO cells (28), and suggest a possible role for microtubules in the mediation of some of these morphologic phenomena.

Lai et al. (19) reported earlier that they found no differences for order parameters obtained with 5-doxyl SA in CHO cells grown to different density in suspension culture, but they did find such differences when these cells were grown on microcarriers to different densities. In contrast, we have found differences in the order parameter of 5-doxyl SA in CHO cells grown to different densities. Table III shows the spectral parameters and the calculated S values obtained with CHO cells grown to a density used in most of our experiments (8 × × 10^5 cells/ml), and those grown to one-half (4 × 10^5) and one-fourth (2 × 10^5 cells/ml) of the usual density. Because of this dependence of the S value on cell density, we made the inoculation and culturing time identical for each experiment wherever possible. Furthermore, homogeneous cell suspensions were used for different drug treatments when these were compared. When this was not possible, as with the mutant cell lines, the results were compared only for individual cell lines undergoing different treatments.

These variations in fluidity with cell density could reflect a difference in the percentage of cells in various stages of the cell cycle at low (exponential growth) and high density, or could reflect other changes in the growth environment (cell-cell contact, medium pH, toxic metabolites). In either case, this finding of altered motional freedom of a spin label probe as a function of cell density might explain previous puzzling observations on the striking differences in sensitivity of cultured cells to toxic agents (i.e., drugs used to select mutants) as a function of cell density (14) (Gottesman, M. M., unpublished observations).

We have found a change in $2T_{\parallel}$ value of 1 gauss brought about by a 5°C difference under our experimental conditions, in good agreement with the experiments of Lai et al. (18). The change of $2T_{\parallel}$ value versus temperature for untreated, Colcemid-, colchicine-, and taxol-treated cells is shown in Fig. 2.

The interpretation of these membrane fluidity changes is based on calculations of the order parameter, S, as described by Hubbell and McConnell (16). We used this method for purposes of comparison since other studies on the membrane fluidity of CHO cells also used this method. For the same reason, we have not calculated rotational correction times, τ'_R, either by the method of Mason and Freed (24) or by that of Schroit et al. (32). Because of the dependence of the nitrogen hyperfine tensor on the polarity of its environment, the a_N' constant is calculated from the measured values of $2T_{\parallel}'$ and $2T_{\perp}'$ to compensate for $(T_{\parallel}$ and $T_{\perp})_{xL}$ values which are given for a hydrophobic environment. Calculated values, which are given in Tables I, V, and VI for 5-doxyl SA at 22°C, are generally between 15.1 and 15.2 gauss. The similarity in the a_N' values for the differently treated cells indicates an environment of similar polarity for this spin label in each case. Also, these values agree well with those of Hubbell and McConnell (16) and Butterfield et al. (8) for the same type of nitroxide spin labels in lecithin-cholesterol membranes and erythrocytes (15.2 and 15.9, respectively). In comparison, the a_N' values we calculated for 16-doxyl MS at 22°C are 13.11 gauss both in untreated and in 0.5 μg/ml Colcemid-treated CHO cells, 13.6 gauss in vincristine-treated CHO cells, and 13.9 gauss calculated by Butterfield et al. (8) in erythrocytes. The polarity of the environments of these two nitroxide probes, 5-doxyl SA and 16-doxyl MS, is different but does not change significantly with treatment of the CHO cells with microtubule-depolymerizing drugs. The change in polarity of these two spin labels is expected since cellular membranes are known not to be uniform in their hydrocarbon and water content through the lipid bilayer (29).

There is a considerable difference in the values of the order parameter, S, for 5-doxyl SA (0.680) and that for 16-doxyl MS (0.110), in untreated CHO cells at 22°C. This difference is expected because it was shown before that deeper in the hydrocarbon region, where the nitroxide group of 16-doxyl MS penetrates, exists a more "fluid" environment than that closer to the membrane surface where the nitroxide group of 5-doxyl MS probes (25, 30). Similarly, an increase in the motional freedom of spin probes, although of a smaller magnitude, was reported for spin labels probing at the same membrane depths as in our study in erythrocyte membranes by Butterfield et al. (8).

We can confirm the finding of Lai et al. (18) that spin labeling does not effect viability of the CHO cells as judged from dye exclusion tests. However, we prefer the spin labeling technique described above over that of Lai et al. because ours requires much shorter time and because by our technique the possibility of having free label in the sample is less. Spin labels attached to the glass wall of a test tube are picked up by cell membranes directly and do not become suspended in aqueous media easily.

Our results indicate that spin labeling is a powerful technique for studying tubulin-membrane interactions and support models which predict that such interactions affect membrane function.

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