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Studies on Secretion and Endocytosis of Macromolecules by Cultivated Skin Fibroblasts

EFFECTS OF ANTI-MICROTUBULAR AGENTS ON SECRETION AND ENDOCYTOSIS OF LYSOSOMAL HYDROLASES AND OF SULPHATED GLYCOSAMINOGLYCANS

By KURT VON FIGURA,* HANS KRESSE, ULRIKE MEINHARD and DOROTHEA HOLTFRERICH
Institute of Physiological Chemistry, University of Münster, D4400, Münster, West Germany

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Fibroblasts were incubated in the presence of the anti-microtubular drugs colchicine, vinblastine and vincristine. In concentrations between 10nM and 1mM these drugs stimulated the secretion of β-*N*-acetylglucosaminidase, α-*N*-acetylglucosaminidase and β-glucuronidase, but not of β-galactosidase. The endocytosis of β-*N*-acetylhexosaminidase and α-*N*-acetylglucosaminidase, but not of β-glucuronidase, was inhibited at drug concentrations higher than 0.1 μM. Formation, secretion and association with the cell membrane of sulphated proteoglycans were not affected by anti-microtubular drugs. Endocytosis of sulphated proteoglycans and their subsequent degradation was inhibited by drug concentrations above 0.1 μM. The inhibition of intracellular glycosaminoglycan degradation led to a moderate storage of these compounds. These results suggest that microtubules participate in the control of secretion and endocytosis of lysosomal enzymes, and in the endocytosis and degradation of lysosomal substrates such as sulphated proteoglycans.

Microtubules and microfilaments participate in generating and/or orientating the motion of cellular metabolites and organelles in various cell types [for reviews see Soifer (1975) and Wessels *et al.* (1971)]. Agents that affect microtubules (e.g. colchicine and the *Vinca* alkaloids vinblastine and vincristine) usually lead to an inhibition of movement of cell organelles, thus decreasing secretion of intracellular material as well as endocytosis of extracellular material. During our studies on the metabolism of sulphated glycosaminoglycans in cultured fibroblasts we became especially interested in processes of secretion and endocytosis, since secreted proteoglycans are metabolized only after uptake from the medium by endocytosis and transfer to the lysosomal apparatus (Kresse *et al.*, 1975). Transfer of at least some acid hydrolases involved in degradation of proteoglycans into the lysosomes appears likewise to include secretion and subsequent endocytosis (Hickman & Neufeld, 1972). Treatment of fibroblasts with cytochalasin B, which disrupts microfilaments, simultaneously affected the endocytosis of proteoglycans and of lysosomal enzymes and secretion of lysosomal enzymes (von Figura & Kresse, 1975; von Figura, 1977*a*).

The present paper describes similar effects of anti-microtubular drugs and demonstrates that microtubules are involved in the control of degradation of endocytosed macromolecules.

* To whom reprint requests should be addressed.

Experimental

Materials

$Na_2{}^{35}SO_4$ (specific radioactivity 5Ci/mg of S) was obtained from Amersham Buchler (Braunschweig, Germany). Colchicine, phenyl *N*-acetyl-α-D-glucosaminide, *p*-nitrophenyl β-*N*-acetylglucosaminide, *p*-nitrophenyl β-D-galactoside and *p*-nitrophenyl β-D-glucuronide were purchased from Koch–Light (Colnbrook, Bucks., U.K.). Vinblastine and vincristine were generously provided by Eli Lilly (Giessen, Germany). Lumicolchicine was prepared by irradiation of 10μM-colchicine in 95% (v/v) ethanol for 1h at 315nm (Wilson & Friedkin, 1966). All other reagents were of analytical grade.

Cell culture

Human skin fibroblasts were grown in 75ml Falcon plastic flasks or 60mm×15mm Falcon dishes in modified Eagle's minimal essential medium, supplemented with 10% foetal calf serum, non-essential amino acids and antibiotics as described by Fratantoni *et al.* (1968). All materials were supplied by LS-Labor Service (München, Germany).

Secretion of lysosomal enzymes

The cultures were grown in 4.5ml of the modified Eagle's medium, supplemented with 10% heat-inactivated foetal calf serum (15min at 70°C). The drugs were added dissolved in 0.5ml of 0.15M-NaCl; 0.5ml of 0.15M-NaCl was added to controls. After incubation for various periods the enzyme activities were determined in the medium and the cell suspension obtained as described (von Figura & Kresse, 1974). Cell protein was determined (Kaltwasser *et al.*, 1965), with bovine serum albumin (Serva, Heidelberg, Germany) as standard. The secreted enzyme activity was expressed as a percentage of intra- and extra-cellular enzyme activity. In most experiments of this kind cultures were grown for 16–24h on 5ml of medium before addition of the drugs. The activity present at the time of drug addition was determined in a 0.5ml sample and the secretion was defined as the increase in extracellular enzyme activity in the presence of the drugs.

Endocytosis of lysosomal enzymes

Endocytosis of β-*N*-acetylglycosaminidase (EC 3.2.1.52) from the secretion of human skin fibroblasts and of β-glucuronidase (EC 3.2.1.31) partially purified from human urine was determined as described (von Figura & Kresse, 1975), except that the period of endocytosis was shortened to 16h. Endocytosis of α-*N*-acetylglucosaminidase (EC 3.2.1.50) partially purified from human urine was determined essentially as described by von Figura (1977*b*).

Determination of enzyme activities

Activities of α-*N*-acetylglucosaminidase, β-*N*-acetylglucosaminidase, β-glucuronidase and β-galactosidase (EC 3.2.1.23) were determined as described by von Figura (1977*b*). One unit of enzyme activity was defined as the amount of enzyme required to catalyse the splitting of 1μmol of substrate per min at 37°C. Lactate dehydrogenase (EC 1.1.1.27) was determined with a test pack from Boehringer Mannheim G.m.b.H (Mannheim, Germany). All assays followed zero-order kinetics and the enzyme activities were not affected by the drugs.

Determination of formation, distribution and degradation of ^{35}S-labelled glycosaminoglycans

Incorporation of [^{35}S]sulphate into the cellular and extracellular pool of cultivated fibroblasts and determination of intracellular degradation of sulphated glycosaminoglycans was done essentially as described by Fratantoni *et al.* (1968). The drugs were dissolved in 0.15M-NaCl and added to the medium in one-tenth of the final volume of medium.

Endocytosis of ^{35}S-labelled proteoglycans

Approx. 80000c.p.m. of ^{35}S-labelled proteoglycans prepared from the secretion of skin fibroblasts was added in a volume of 50μl to 5ml of culture medium. Endocytosis was determined as described by Kresse *et al.* (1975) and defined as the sum of intracellular and ethanol-soluble radioactivity of the medium, corrected for the ethanol-soluble radioactivity of medium blanks. The ethanol-soluble radioactivity of the medium blank (^{35}S-labelled proteoglycans added to conditioned medium and incubated at 37°C for the period of uptake) accounted for 1–2% of the total radioactivity added. The rate of degradation is defined as the ratio of ethanol-soluble radioactivity detected intra- and extracellularly and the total radioactivity endocytosed by the cells.

Other methods

Cell viability was monitored for each experimental condition by the Nigrosine test (Kaltenbach *et al.*, 1958). Less than 2% of the cells were stained.

Results

Effect of anti-microtubular drugs on lysosomal enzyme secretion

Medium in contact with fibroblasts contains lysosomal enzymes released by the cells (Neufeld *et al.*, 1975). The enzymes are released from the cells by secretion, as was shown in detail for β-*N*-acetylglucosaminidase (von Figura, 1977*c*). Colchicine, vinblastine and vincristine (all 1μM) increased the secretion of β-*N*-acetylglucosaminidase, α-*N*-acetylglucosaminidase and β-glucuronidase. Only β-galactosidase release was unaffected by the presence of anti-microtubular drugs (Table 1). The high activities of β-*N*-acetylglucosaminidase present in fibroblasts make investigations with this enzyme much easier than with other lysosomal enzymes. β-*N*-Acetylglucosaminidase is released in the initial phase after addition of fresh medium rapidly and the effect of 1μM-colchicine on the secretion is seen clearly only after 12–16h (Fig. 1) In the following experiments the drugs were therefore added after the cells were preincubated for 16–24h on fresh medium. Under these conditions 10nM–1mM-colchicine increased the secretion of β-*N*-acetylglucosaminidase up to 6-fold (Table 2). A similar concentration-dependence was found for vinblastine and vincristine. The stimulatory effect was constant for at least 3 days (Fig. 2). The toxicity of the anti-microtubular drugs was checked by dye-exclusion tests and determination of lactate dehydrogenase release into the medium. In no case did the percentage of stainable cells and the lactate dehydrogenase release in drug-

Table 1. *Effect of anti-microtubular drugs on lysosomal enzyme secretion*

All values represent the means of triplicates (ranges in parentheses). The mean total enzyme activity was for β-*N*-acetylglucosaminidase 415munits, for α-*N*-acetylglucosaminidase 0.28munit, for β-glucuronidase 1.57munits and for β-galactosidase 14.8munits per mg of cell protein. Cells were grown in 2ml of medium, containing either drugs or 0.9% NaCl and incubated for 72h before determination of intra- and extra-cellular enzyme activities. The protein content varied between 0.58 and 0.69mg/75ml flask.

	Extracellular enzyme activity (% of total)			
	β-*N*-Acetyl-glucosaminidase	α-*N*-Acetyl-glucosaminidase	β-Glucuronidase	β-Galactosidase
Control	4.4	10.3 (7.6–12.0)	6.7 (6.2–7.1)	0.81 (0.44–1.17)
1 μM-Colchicine	12.6 (12.1–13.4)	18.1 (17.3–19.6)	11.7 (9.9–14.2)	0.85 (0.79–0.91)
1 μM-Vinblastine	13.1 (11.9–14.0)	19.7 (17.3–22.9)	11.5 (9.0–15.4)	0.77 (0.72–0.81)
1 μM-Vincristine	12.6 (12.5–12.8)	18.4 (17.3–20.4)	12.7 (10.4–14.3)	0.97 (0.065–1.22)

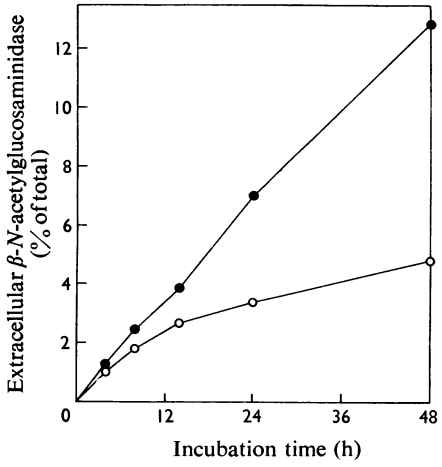

Fig. 1. *Secretion of β-N-acetylglucosaminidase in the presence of colchicine*

Fibroblasts were incubated in the presence of 1 μM-colchicine (●). After various periods samples of the medium were removed for enzyme-activity determination. Intracellular activity was determined after 48h. Controls (○) contained, instead of colchicine, an equal volume of 0.15M-NaCl.

treated cultures exceed the range found in controls. The stimulatory effect of anti-microtubular drugs appears therefore to be specific and cannot be attributed to non-specific cell damage. The great variability of cytoplasmic lactate dehydrogenase release by fibroblasts, which is not due to secretion, was noticed earlier and is so far poorly understood (von Figura, 1977*c*). In the presence of anti-microtubular drugs sparse cultures release relatively twice as much β-*N*-acetylglucosaminidase activity as do dense cultures. The increased secretion is at the expense of the intracellular enzyme activity, whereas the total enzyme activity remains unaffected (not shown).

Effect of anti-microtubular drugs on lysosomal enzyme endocytosis

Lysosomal enzymes secreted by the fibroblasts or added to the culture medium become internalized by fibroblasts by adsorptive endocytosis (Hickman & Neufeld, 1972). Endocytosis of lysosomal enzymes can easily be followed if enzyme-deficient cells are used as recipient cells. The kinetics of endocytosis are identical in normal and enzyme-deficient cells (von Figura & Kresse, 1974). The effect of anti-microtubular drugs on endocytosis of β-*N*-acetylglucosaminidase, α-*N*-acetylglucosaminidase and β-glucuronidase was followed (Table 3). Endocytosis of the first two enzymes was significantly inhibited. β-Glucuronidase endocytosis remained almost unaffected. Between 0.1 μM and 1mM the inhibitory effect was related to the drug concentration (Fig. 3).

Formation, distribution and degradation of newly synthesized ^{35}S-labelled glycosaminoglycans in the presence of colchicine

Skin fibroblasts grown in the presence of [^{35}S]-sulphate incorporate the label into sulphated glycosaminoglycans. Newly synthesized polysaccharides are distributed into extra-, peri- and intra-cellular pools (Neufeld & Cantz, 1973). Colchicine in concentrations between 1nM and 1mM had no significant influence on the rate of ^{35}S-labelled-glycosamino-

Table 2. *Effect of colchicine on the secretion of β-N-acetylglucosaminidase and lactate dehydrogenase*
Cells were preincubated for 24h without drug. During this period the fibroblasts released 3.3% of total β-N-acetylglucosaminidase activity into the medium. Secretion was determined after incubation for 48h in the presence of the drug and all values were corrected for the enzyme release during the preincubation period.

	Extracellular β-N-acetylglucosaminidase		Extracellular lactate dehydrogenase (% of total)
	(munits/mg of cell protein)	(% of total)	
Control	8.0	2.6	5.1
Colchicine			
1 mM	54.4	15.2	3.9
0.1 mM	63.4	19.0	2.4
10 μM	73.4	19.2	5.6
1 μM	70.2	21.0	6.0
0.1 μM	65.6	18.6	0.2
10 nM	56.6	16.0	5.9
1 nM	8.8	3.2	1.6
0.1 nM	11.2	3.4	3.2

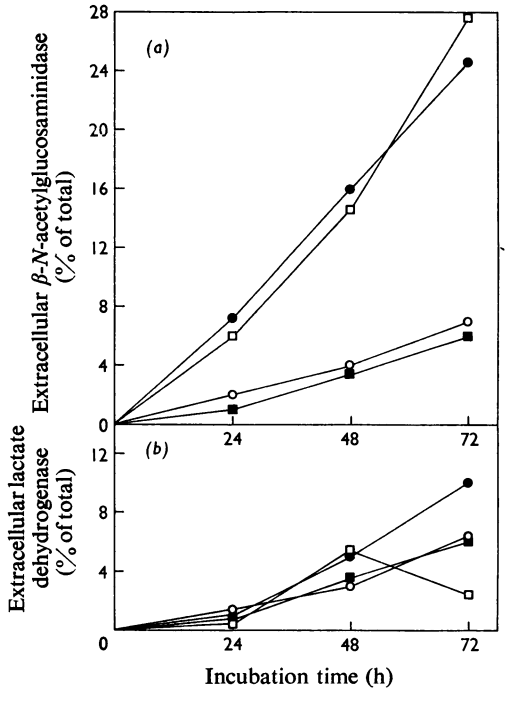

Fig. 2. *Secretion of β-N-acetylglucosaminidase (a) and lactate dehydrogenase (b) in the presence of colchicine*
After a preincubation for 24h, fibroblasts were incubated for up to 72h in the presence of 1 mM- (□), 1 μM- (●) and 1 nM- (■) colchicine, or without the drug (○).

glycan formation. The amount of [^{35}S]sulphate incorporated into sulphated glycosaminoglycans in the presence of 1 nM–1 mM-colchicine after incubation for 24, 48 and 72h varied between 82 and 112% of that of controls. A dose-dependent accumulation of ^{35}S-labelled glycosaminoglycans in the presence of colchicine was found in the intracellular pool, which is the smallest of the three pools (Table 4*a*). Intracellular accumulation of ^{35}S-labelled glycosaminoglycans has been observed in fibroblasts with an impaired catabolism of sulphated glycosaminoglycans due to either a genetic enzyme deficiency (Neufeld *et al.*, 1975) or drugs (von Figura & Kresse, 1975; Lie & Schofield, 1973) or inappropriate medium pH (Lie *et al.*, 1972). Determination of the rate of degradation of intracellular ^{35}S-labelled glycosaminoglycans in a 'chase' experiment revealed that the rate of degradation in the presence of colchicine is initially (0–7h) retarded, but later (7–24h) accelerated (Table 4*b*). Over a period of 24h, the rates of degradation are identical in untreated and colchicine-treated fibroblasts.

Effect of anti-microtubular drugs on endocytosis of ^{35}S-labelled proteoglycans

Proteoglycans secreted by fibroblasts or added to the medium enter the cells by adsorptive endocytosis (Kresse *et al.*, 1975). The internalized proteoglycans become rapidly degraded and the [^{35}S]sulphate liberated is released into the medium. Within 24–48h degradation is in equilibrium with endocytosis. Endocytosis of ^{35}S-labelled proteoglycans was somewhat more sensitive to inhibition by anti-microtubular drugs than was degradation of endocytosed ^{35}S-labelled proteoglycans (Table 5). The inhibition of degradation of endocytosed ^{35}S-labelled proteoglycans is similar to that of newly synthesized ^{35}S-labelled glycosaminoglycans directly transferred into the lysosomes (see the preceding paragraph).

Table 3. *Endocytosis of lysosomal enzymes in the presence of anti-microtubular drugs*
For β-*N*-acetylglucosaminidase, the endocytosis period was 16h and the clearance of the controls was 0.0058ml/h per mg of cell protein. For α-*N*-acetylglucosaminidase the endocytosis period was 4h and the clearance of the controls was 0.120ml/h per mg of cell protein. For β-glucuronidase the endocytosis period was 16h and the clearance of the controls was 0.027ml/h per mg of cell protein.

	Endocytosis (% of control) of:		
	β-*N*-Acetylglucosaminidase	α-*N*-Acetylglucosaminidase	β-Glucuronidase
Colchicine (0.1mM)	89	75	93
Vinblastine (10μM)	61	55	86
Vincristine (10μM)	48	44	98

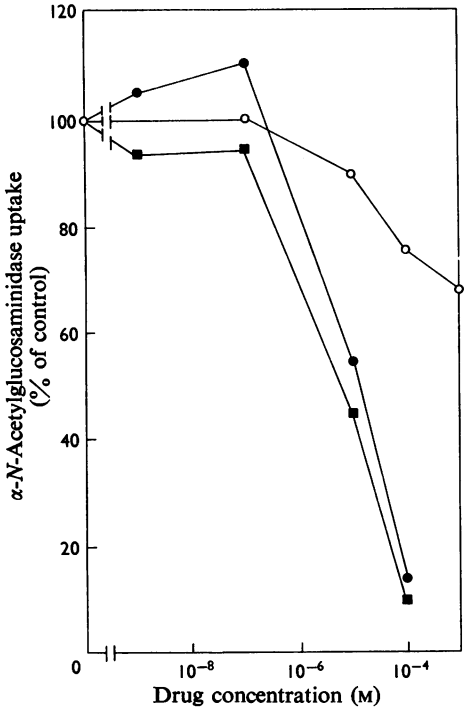

Fig. 3. *Effect of anti-microtubular drugs on the endocytosis of α-N-acetylglucosaminidase*
Fibroblasts from patients with Sanfilippo B disease were incubated with 5ml of α-*N*-acetylglucosaminidase-supplemented medium (1.3munits/ml) in the presence of colchicine (○), vinblastine (●) or vincristine (■) for 4h. Controls internalized in that period 0.64munit of α-*N*-acetylglucosaminidase/mg of cell protein. The protein concentration was 0.32–0.43mg/75ml flask.

Mode of action of colchicine and reversibility of colchicine-mediated effects

Colchicine can be converted by a photochemical reaction into lumicolchicine. Lumicolchicine neither binds to tubulin nor prevents microtubule assembly (Wilson *et al.*, 1974), but still exerts some of the effects of colchicine, which are unrelated to the anti-microtubular action of colchicine (Mizel & Wilson, 1972). Lumicolchicine has no effect on the lysosomal enzyme secretion, endocytosis of ^{35}S-labelled proteoglycans and their intracellular degradation (Table 6). The parallel effects produced by colchicine and the *Vinca* alkaloids and the inability of lumicolchicine to exert these effects strongly suggest that the microtubular depolymerization is the common cause for the observed drug effects. All effects mediated by colchicine are completely reversible within 24h of removal of the drug.

Discussion

Microtubules have been detected in almost every cell type. A diversity of functions has been attributed to microtubules, such as participation in cell motility, cell-shape determination, arrangement of cell-membrane components and transport of subcellular organelles and proteins (for review see Soifer, 1975). Whereas the relation of microtubules to endocytosis has so far not been investigated, several reports describe the involvement of microtubules in secretory events in a variety of cellular systems. In most cases anti-microtubular agents inhibit secretion (Malawista, 1968; Zurier *et al.*, 1973; Gillespie & Lichtenstein, 1972; Stein & Stein, 1973; Redman *et al.*, 1975; Taylor *et al.*, 1973; Wolff & Williams, 1973; Jansen & Bornstein, 1974; Lohmander *et al.*, 1976), whereas a few secretory processes are either stimulated (Temple & Wolff, 1973; Gordon & Werb, 1976; Edwards & Howell, 1973) or remain unaffected (Parkhouse & Allison, 1972; Douglas & Sorimachi, 1972; Temple *et al.*, 1972) by depolymerization of microtubules. In fibroblasts the secretion of procollagen is inhibited by colchicine and related drugs (Dehm & Prockop, 1972; Diegelmann & Peterkofsky, 1972).

The results of the present study suggest that, in fibroblasts, microtubules participate in the control of secretion of lysosomal enzymes but not of proteo-

Table 4. *Intracellular accumulation (a) and degradation (b) of ^{35}S-labelled glycosaminoglycans in the presence of colchicine*
Fibroblasts were incubated for up to 72h in the presence of [^{35}S]sulphate and 1nM–1mM-colchine or without drug. For determination of the degradation rate, radioactive medium was removed after 72h and replaced by medium without [^{35}S]sulphate but containing the drug at the concentration used in the labelling period. Intracellular ^{35}S-labelled glycosaminoglycans were determined after 'chase' periods of 7 and 24h.

(*a*) Incubation period (h)	Intracellular ^{35}S-labelled glycosaminoglycans (c.p.m./mg of cell protein)			
	Control	+1mM-Colchicine	+1μM-Colchicine	+1nM-Colchicine
24	24030	30810	29690	23340
48	27080	37480	31940	25500
72	27490	43570	34140	24400
(*b*) 'Chase' period (h)	Degradation of intracellular ^{35}S-labelled glycosaminoglycans (% of that present at 72h)			
	Control	+1mM-Colchicine	+1μM-Colchicine	+1nM-Colchicine
0–7	61.1	40.7	54.0	62.0
7–24	20.2	38.8	24.8	20.0
0–24	81.3	79.5	78.8	82.0

Table 5. *Endocytosis and degradation of ^{35}S-labelled proteoglycans in the presence of anti-microtubular drugs*
The period of endocytosis was 16h. All values were referred to the mean cellular protein contents of 0.88mg, 0.35mg and 0.71mg per flask in Expts. I, II and III respectively. All values represent means of duplicates.

Experiment	Internalized ^{35}S-labelled proteoglycans (% of added)	Degraded ^{35}S-labelled proteoglycans (% of internalized)
I Colchicine		
1mM	12.6	69.4
10μM	14.6	71.1
0.1μM	15.6	68.2
1nM	18.8	91.8
Control	19.2	92.7
II Vinblastine		
0.1mM	11.4	64.3
10μM	10.3	62.4
0.1μM	12.5	84.0
1nM	15.4	84.2
Control	15.6	84.5
III Vincristine		
10μM	13.8	58.5
0.1μM	15.2	90.1
1nM	17.9	91.0
Control	17.0	92.2

Table 6. *Effect of lumicolchicine on secretion of β-N-acetylglucosaminidase and on endocytosis and degradation of ^{35}S-labelled proteoglycans*
The period of endocytosis was 16h. All values were referred to the mean cellular protein content of 0.57mg/flask (range 0.52–0.65).

	β-*N*-Acetylglucosaminidase secreted (munits/48h per mg of cell protein)	Internalized ^{35}S-labelled proteoglycans (% of added)	Degraded ^{35}S-labelled proteoglycans (% of internalized)
Control	7.1	21.9	89.5
Lumicolchicine			
0.1μM	10.7	19.9	88.3
10nM	8.7	22.6	90.9
Colchicine			
0.1μM	39.4	16.4	73.2
10nM	29.9	20.9	71.8

glycans. Furthermore intact microtubules facilitate endocytosis of lysosomal enzymes and of sulphated proteoglycans. The intracellular degradation of glycosaminoglycans is impaired in the presence of anti-microtubular drugs, regardless of whether the polysaccharides stem from the extracellular space or whether they are directly transferred into secondary lysosomes after their formation. Analysis of the kinetics of polysaccharide degradation reveals that the impaired degradation results from decreased fusion of substrate and enzyme-containing vacuoles rather than from inhibition of lysosomal enzymes. Microtubules may control the fusion rate by participation in intracellular translocation of vesicles and/or by involvement in the process of fusion itself. Colchicine has been shown to inhibit the fusion of phagosomes with lysosomes in polymorphonuclear leucocytes (Malawista, 1968; Zurier *et al.*, 1973).

There is no obvious explanation available for the mechanism by which microtubules participate in the control of lysosomal enzyme secretion and in the control of endocytosis of lysosomal enzymes and of proteoglycans. If some acid hydrolases are transferred in fibroblasts into lysosomes only after secretion and endocytosis (Hickman & Neufeld, 1972), inhibition of endocytosis could explain an increased extracellular accumulation of these enzymes. The diversity of effects of anti-microtubular agents on secretion and endocytosis may be explained more generally by the hypothesis that microtubules control the exposure of specific receptors in membranes to which lysosomal enzymes and proteoglycans bind. Microtubules closely associated with membranes (Franke, 1971*a*,*b*; Jarlfors & Smith, 1969) and membrane-incorporated tubulin (Bhattacharyya & Wolff, 1975, 1976) have been postulated to control the arrangement of surface receptors for lectins and immunoglobulins (Yahara & Edelman, 1975; Edelman, 1976; McClain *et al.*, 1977). Association of lysosomal hydrolases with membranes has been described for primary lysosomes (Goldstone & Koenig, 1973), secondary lysosomes (Tappel, 1969) and cell membranes (Neufeld *et al.*, 1975). In the presence of anti-microtubular drugs membrane-attached enzymes could become solubilized within the vesicles and, after fusion of these vesicles with the cell membrane, diffuse into the extracellular space, whereas release of non-membrane-attached enzymes should remain unaffected. Endocytosis of acid hydrolases would be impaired by the same mechanism. Normal secretion and impaired endocytosis of proteoglycans would indicate that these macromolecules, which are destined for secretion, are freely diffusible in secretory vacuoles and require a receptor-mediated attachment to membranes only for endocytosis (Kresse *et al.*, 1975). The observation that secretion of β-glucuronidase is stimulated and its endocytosis unaffected in the presence of anti-microtubular drugs is so far not understood. Further studies with other macromolecules and methods allowing the direct measurement of binding are required to elucidate the mechanism by which microtubules control secretion and endocytosis in fibroblasts. The present study was restricted to the effects of anti-microtubular drugs in cultivated fibroblasts on the processing of two functionally related kinds of macromolecules, proteoglycans and lysosomal enzymes involved in their degradation. None of the conclusions or hypotheses may be generalized, since anti-microtubular drugs inhibit the secretion of lysosomal enzymes in polymorphonuclear leucocytes (Zurier *et al.*, 1973, 1974) and inhibit the synthesis and secretion of sulphated proteoglycans in chondrocytes (Jansen & Bornstein, 1974; Lohmander *et al.*, 1976).

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