

# EFFECTS OF COLCHICINE ON ENDOCYTOSIS AND CELLULAR INACTIVATION OF HORSERADISH PEROXIDASE IN CULTURED CHONDROCYTES

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## ABSTRACT

Horseradish peroxidase (HRP) was used as a marker to study the effects of microtubule-disruptive drugs on uptake and cellular inactivation of exogenous material in cultures of embryonic chick chondrocytes. HRP was ingested by fluid endocytosis, and intracellular enzyme activity subsequently diminished exponentially with time. Cytochemically, reaction product for HRP was found in vesicles often located close to the dictyosomes of the Golgi complex. Colchicine and vinblastine caused disappearance of cytoplasmic microtubules and disorganization of the Golgi complex with concomitant reduction in the cellular uptake of HRP to about half of that in the controls. Lumicolchicine, on the other hand, left cell fine structure and HRP uptake unaffected. These results indicate that microtubules are of considerable importance in the process of fluid endocytosis in cultured chondrocytes although the exact mechanism remains to be elucidated.

The rate of intracellular inactivation of ingested HRP was not affected by colchicine or vinblastine. Double-labeling experiments with colloidal thorium dioxide and HRP likewise indicated that fusion of endocytic vesicles and lysosomes is not dependent on intact microtubules. The total specific activities of the three lysosomal enzymes examined were weakly or not at all changed by treatment of the cultures with colchicine or vinblastine. It therefore seems unlikely that microtubular organization plays an important role in the production or degradation of lysosomal enzymes in cultured chondrocytes.

**KEY WORDS** endocytosis · horseradish peroxidase · microtubules · colchicine · chondrocytes

Macromolecular secretory products are believed to be released from cells primarily by exocytosis. During this process, membranes of secretory vesicles are inserted into the plasmalemma. Endocytosis represents one way by which recycling of membrane back into the cell can take place (19).

Moreover, recent observations suggest that endocytic vesicle membrane may be integrated into the Golgi complex, i.e., the site of secretory vesicle formation (7, 10). If such a coupling between exo- and endocytosis exists, it is possible that the two processes are regulated in a similar way. An inhibition of secretion should then be followed by a decreased endocytic activity possibly coordinated with other changes in the lysosomal system of the cell.

Microtubule-disruptive drugs such as colchicine and vinblastine (40) have been found to inhibit secretion in various cell types (e.g. references 4, 6, 16, 20, 24, 27, 30, 38). The role of microtubules in endocytosis has not been investigated in the same detail, however, and the results published so far are in part contradictory (14, 28). Further studies are therefore needed to clarify whether microtubules are involved in coupling of exo- and endocytosis or not.

It has previously been shown in our laboratory that microtubule-disruptive drugs cause striking changes in the fine structure of cultured chondrocytes, including a characteristic disorganization of the Golgi complex (18, 36). It has also been demonstrated that such drugs inhibit synthesis and secretion of proteoglycans and collagen in chondrocyte cultures (13, 17). In the present study, the effects of colchicine and related drugs on endocytosis of horseradish peroxidase (HRP) by embryonic chick chondrocytes were examined. To determine whether fusion of endocytic vesicles and lysosomes requires the presence of cytoplasmic microtubules, we also followed the cellular inactivation of ingested HRP. Our results indicate that microtubule-disruptive drugs inhibit endocytosis without notably affecting lysosomal enzyme levels and metabolism of HRP within the cells. These findings support the idea that microtubules may be involved in coupling of exo- and endocytosis in secretory cells.

## MATERIALS AND METHODS

### Reagents

Ham's medium F-12 and fetal calf serum were obtained from Gibco Bio-Cult (Glasgow, Scotland), benzylpenicillin and streptomycin sulfate from Glaxo (Göteborg, Sweden), and trypsin (1:250) from Difco Laboratories (Detroit, Mich.). *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES), *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES), collagenase type I, type II HRP, vinblastine sulfate, cytochalasin B, diaminobenzidine tetra-HCl (DAB), *o*-dianisidine di-HCl, *p*-nitrophenyl phosphate, *p*-nitrophenyl-*N*-acetyl- $\beta$ -D-glucosaminide, phenolphthalein glucuronic acid, *p*-nitrophenol, and phenolphthalein were purchased from Sigma Chemical Co. (St. Louis, Mo.). Coomassie Brilliant Blue G-250 and colchicine were products of Serva Feinbiochemica (Heidelberg, West Germany). Lumicolchicine was prepared by irradiation of colchicine, dissolved either in 95% ethanol or in redistilled water, with long-wave ultraviolet light (41). Bovine serum albumin came from Biomed (Cracow,

Poland). Thorotrast (25% stabilized colloidal thorium dioxide by volume) was a product of Testagar & Co. (Detroit, Mich.).

### Cells

Full culture medium consisted of medium F-12 supplemented with 10% fetal calf serum, 50  $\mu$ g/ml of L-ascorbic acid, 150 U/ml of penicillin, and 150  $\mu$ g/ml of streptomycin. To stabilize the pH of the medium 10 mM, each of the organic buffers HEPES and TES was also included (5). Chondrocytes were isolated from sterna of 11-d-old chick embryos by digestion for 5 h at 37°C with 0.05% (wt/vol) collagenase dissolved in full culture medium. The freed cells were passed through a nylon filter (mesh size 40  $\times$  40  $\mu$ m) and rinsed twice with full medium. They were then seeded into 35-mm (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Ca.) or 60-mm (Costar, Data Packaging, Cambridge, Mass.) plastic petri dishes at an initial density of 3  $\times$  10<sup>6</sup> and 7  $\times$  10<sup>6</sup> cells/dish, respectively, and allowed to adhere to the dishes overnight before the experiments.

### Endocytosis of HRP

Cells were exposed for 1, 2, or 3 h to HRP dissolved in full medium, usually at a concentration of 1 mg/ml. Triplicate cultures were used for each time point. After the exposures the cultures were rinsed twice with full medium and once with serum-free medium. The cells were removed from the dishes with a rubber policeman, put into plastic tubes, and sedimented by centrifugation. They were then resuspended in phosphate-buffered saline (PBS) and washed three times. After the final rinse, the cell pellets were resuspended in 0.75 ml of 0.05% Triton X-100 and homogenized in glass homogenizers. The homogenates were centrifuged at 4,000 g for 15 min, the supernatants were collected and protein was measured with the Coomassie Brilliant Blue-binding method (3), using bovine serum albumin as standard. The samples were thereafter diluted with 0.05% Triton X-100 to equal protein concentrations.

HRP activity was determined according to Steinman and Cohn (32). Substrate solution was prepared fresh daily by addition of 1.0 ml of 0.3% (vol/vol) H<sub>2</sub>O<sub>2</sub> and 0.82 ml of 1.33% (wt/vol) *o*-dianisidine di-HCl in water to 100 ml of 0.05 M phosphate buffer, pH 5.0. 1 ml of substrate solution was pipetted into 1.25-ml quartz cuvettes of 1-cm path length, and 0.1 ml of the sample to be assayed was added. The cuvettes were then mixed and the increase in absorption at 460 nm was measured for 3 min at 30-s intervals on a Gilford spectrophotometer, model 222 (Gilford Instrument Laboratories Inc., Oberlin, Ohio). No HRP activity was detected in the sedimentable residue of the cell homogenates.

### Cellular Inactivation of HRP

Cultures were allowed to endocytose HRP for 2 h, washed three times with full medium and three times in

serum-free medium, and then followed up to 48 h in HRP-free medium. At each time point, triplicate cultures were assayed for HRP remaining in the cells. To check the possibility of secretion of HRP from the cells, the culture medium was also assayed for HRP in some of the experiments.

### *Drug Effects on Endocytosis and Cellular Inactivation of HRP*

Colchicine, lumicolchicine, and vinblastine were used at final concentrations of  $1 \times 10^{-6}$  or  $1 \times 10^{-5}$  M and cytochalasin B at  $2 \times 10^{-6}$  or  $1 \times 10^{-5}$  M. To study the effects on endocytosis, we treated cultures with the drugs for a total of 4 h, 2 h before exposure to HRP and 2 h together with HRP. In other experiments, cells were exposed to HRP with or without drug treatment, washed three times with full medium and three times in serum-free medium, and then followed up to 48 h in HRP-free medium with or without drugs. In all cases, triplicate cultures were used for each treatment and time point to assay the amount of HRP in the cells.

In double-labeling experiments designed to demonstrate morphologically whether fusion between lysosomes and endocytic vesicles took place, cells were first exposed to Thorotrast (0.03 ml/ml of medium) for 5 h. They were then carefully rinsed three times with full medium and cultured for 18 h before being exposed to HRP for 2 h. Colchicine was added 2 h before or at the same time as HRP. After the exposure to HRP, the cells were sometimes cultured in HRP-free medium, with or without colchicine, for a further 2–18 h.

### *Lysosomal Enzyme Assays*

Activities of the lysosomal enzymes acid phosphatase,  $\beta$ -*N*-acetylglucosaminidase and  $\beta$ -glucuronidase were determined in chondrocytes grown in normal medium or exposed to colchicine or vinblastine for up to 48 h. Cell homogenates were prepared as described above and the supernates were assayed according to Barrett (2), using *p*-nitrophenyl phosphate, *p*-nitrophenyl-*N*-acetyl- $\beta$ -*D*-glucosaminide, and phenolphthalein glucuronic acid, respectively, as substrates. No enzyme activity was detected in the sedimentable residue of the homogenates.

### *Molecular Weight of HRP*

The molecular weights of standard HRP and HRP from culture media were determined on a  $85 \times 0.9$  cm column of Sephadex G-200 fine (Pharmacia Fine Chemicals, Uppsala, Sweden). Elution was done with 0.5 M sodium acetate buffer, pH 7.0, using a flow rate of 3 ml/h and an operating pressure of 15 cm H<sub>2</sub>O.  $V_0$  was 21 ml, and 1-ml fractions were collected. Standard HRP eluted at 37 ml, corresponding to a mol wt of 40,000 according to the standard curve of the column.

### *Electron Microscopy*

Cells were harvested with a rubber policeman or by trypsinization and fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3. After rinsing in cacodylate buffer with sucrose, cell-bound HRP was demonstrated according to Graham and Karnovsky (9). The cells were incubated at 23°C in 50 mg% DAB in 0.05 M Tris buffer, pH 7.6, first for 15 min without H<sub>2</sub>O<sub>2</sub>, and thereafter for 90 min with 0.01% H<sub>2</sub>O<sub>2</sub>. Postfixation was done for 2 h in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.3, and, after rinsing, the material was treated for 1 h at room temperature with 0.5% uranyl acetate in Veronal acetate buffer, pH 5.0 (8). After dehydration in ethanol, the specimens were embedded in Spurr's resin (29). Thin sections were cut on an LKB Ultratome I (LKB Produkter, Bromma, Sweden) and examined in a Philips EM 300 electron microscope either directly or after staining with uranyl acetate and lead citrate (25).

## RESULTS

### *Culture System and Cell Fine Structure*

Chondrocytes were regularly seeded at high density and allowed to adhere to the dishes overnight before the experiments, thereby forming a confluent or partly overlapping layer. Under these conditions, the number of cells and the amount of soluble, cell-associated protein per dish was highly reproducible and changed but little during the course of the experiments, i.e., up to 48 h. Furthermore, these parameters were not distinctly affected by the addition of colchicine to the cultures (Table I).

Ultrastructurally, the cells were analogous to the guinea pig chondrocytes described previously (36). Cells kept in normal medium and cells exposed to lumicolchicine, cytochalasin B, or dimethyl sulfoxide (10  $\mu$ l/ml of medium; control to cytochalasin B) did not differ clearly from one another. The rough endoplasmic reticulum (RER), made up mainly of narrow cisternae, and the Golgi complex, made up of dictyosomes organized in a well-defined juxtannuclear area, were the most prominent cytoplasmic organelles. Microtubules were particularly numerous within the Golgi area and were often seen to radiate out from the pericentriolar region. Colchicine and vinblastine, at both concentrations used, caused complete disappearance of cytoplasmic microtubules. Moreover, the Golgi complex was disorganized, with dictyosomes, often structurally changed, dispersed throughout the cytoplasm (Fig. 1). The RER was not clearly altered, how-

TABLE I  
Cell Number and Amount of Soluble, Cell-Associated Protein in Control Cultures and Cultures Treated with Colchicine

Time of treatment h	Control cultures		Colchicine ( $1 \times 10^{-6}$ M)	
	Cell No. $\times 10^6$	Protein $\mu\text{g}/\text{dish}$	Cell No. $\times 10^6$	Protein $\mu\text{g}/\text{dish}$
0	2.84 (0.05)	179 (1.3)	2.85 (0.07)	181 (3.6)
24	2.88 (0.02)	183 (2.2)	2.82 (0.02)	179 (1.9)
48	2.90 (0.05)	185 (2.2)	2.76 (0.04)	177 (1.5)

Chondrocytes were seeded at an initial density of  $3 \times 10^6$  cells/35-mm dish and allowed to attach to the dishes overnight. After this (time = 0 h), the cultures were grown either in normal full medium or in medium containing colchicine. Cell numbers were counted in a hemocytometer after collecting the cells by trypsinization. For determination of cell-associated protein, the cells were removed from the dishes with a rubber policeman. Data represent means of quadruplicate cultures with standard deviation in parentheses.

ever. After vinblastine treatment, microtubules with associated ribosomal aggregates and paracrystals were also seen (for further details, cf. reference 36).

#### Lysosomal Enzyme Activities

In cultures kept in normal full medium, specific activities in total cell homogenates of the three lysosomal enzymes assayed increased slightly with increasing time of culture. In the case of acid phosphatase and  $\beta$ -glucuronidase, the presence of colchicine or vinblastine in the culture medium did not affect the enzyme activities. In the case of  $\beta$ -*N*-acetylglucosaminidase, enzyme activity was reduced in cultures exposed to the antimicrotubular drugs (Table II). In cultures grown in medium supplemented with heat-inactivated serum (15 min at  $70^\circ\text{C}$ ), this effect was not obtained (Table III). Large amounts of  $\beta$ -*N*-acetylglucosaminidase activity but only small amounts of acid phosphatase and  $\beta$ -glucuronidase activity were present in the serum normally used as supplement to the medium. All these enzyme activities were destroyed by the heat treatment.

#### Drug Effects on Endocytosis and Cellular Inactivation of HRP

No endogenous peroxidase activity was detected in the chondrocytes either biochemically or cytochemically. Cells kept in normal full medium and exposed to HRP at a concentration of 1 mg/ml of medium showed a linear uptake of the marker during the 3-h period studied, with the plot of uptake vs. exposure time passing through the origin (Fig. 2). The rate of interiorization of HRP was of the order of 200 ng/mg protein/h. In

cultures treated with  $1 \times 10^{-6}$  M colchicine for 2 h before and during the exposure to HRP, uptake was also linear. The rate of interiorization of HRP was only about half of that in the control cultures, however, i.e.,  $\sim 100$  ng/mg protein/h (Fig. 2). In both groups, uptake was also found to increase linearly with increasing concentration of HRP in the medium (Fig. 3). The experiments were in all cases highly reproducible, with only small variations within single experiments (triplicates) and from experiment to experiment.

Drug effects on endocytosis were studied in cultures exposed to 1 mg/ml of medium of HRP for 2 h. The length of drug treatment was 4 h, 2 h before, and 2 h together with, HRP. Colchicine and vinblastine reduced the HRP uptake to about half of that in the controls, whereas lumicolchicine and cytochalasin B showed no significant effects (Table IV). These differences in the uptake of HRP were also distinguishable cytochemically. In all groups, reaction product was found in vesicles of varying size distributed throughout the cytoplasm. In both control and experimental cultures, HRP-containing vesicles were frequently located close to dictyosomes of the Golgi complex. No clear reaction product was demonstrated in the Golgi cisternae, however (Figs. 4 and 5).

Cellular inactivation of HRP was studied in two sets of experiments. In the first, cells were exposed to HRP for 2 h and then rinsed six times as described in Materials and Methods. (The two last rinses contained no measurable HRP activity.) The cultures were then followed up to 48 h in HRP-free medium with or without drugs, and the HRP activity remaining in the cells was assayed after varying times. In both control cultures and cultures with colchicine or vinblastine, the amount

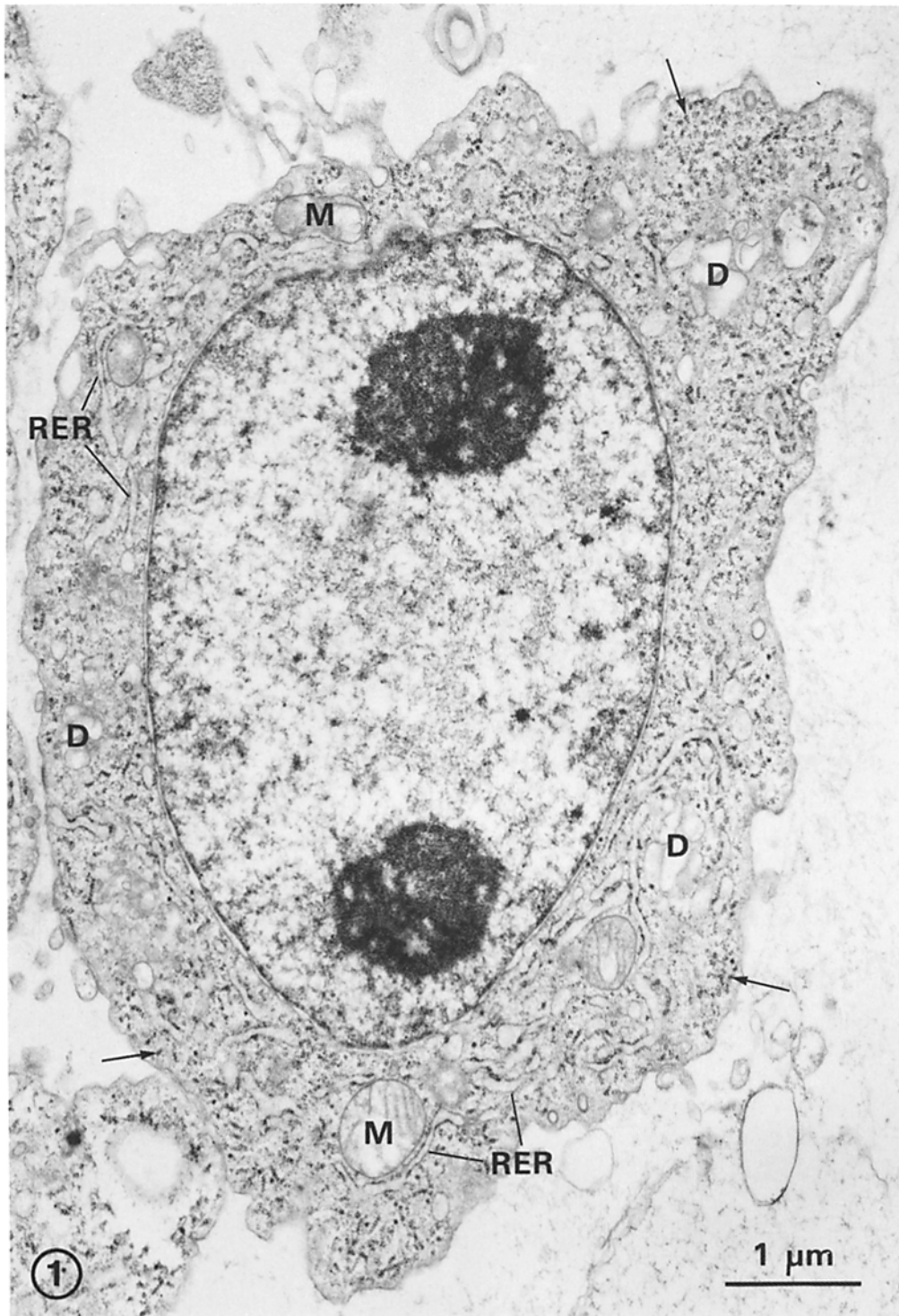


FIGURE 1 Chondrocyte from culture treated with  $1 \times 10^{-6}$  M colchicine for 2 h. The nucleus is located in the center of the cell. Narrow RER cisternae, widely separated dictyosomes (*D*) consisting of distended cisternae, mitochondria (*M*), and numerous free polyribosomes (arrows) are distinguished in the cytoplasm. Section stained with uranyl acetate and lead citrate.  $\times 20,000$ .

of cell-bound enzyme diminished exponentially with time (Fig. 6 and Table V). In the second type of experiment, cultures were exposed to HRP with or without pretreatment with colchicine and then followed up to 48 h in HRP-free medium, again with or without colchicine. Also, in these experiments cell-bound HRP decreased exponentially with time and with the same rate (half-life ~22 h) in both groups (Fig. 6 and Table V). In accordance with the biochemical results, the amounts of HRP demonstrated cytochemically within the cells markedly decreased with time. Because of their frequent association with the dictyosomes, which were randomly spread out in the cytoplasm of drug-treated cells, HRP-containing vesicles appeared to be more widely distributed in cells treated with antimicrotubular drugs than in the corresponding controls. Otherwise, no signs of drug-induced redistribution of HRP within the cells were noted.

Although the last rinses after exposure to HRP were free of enzyme activity, increasing amounts (up to 20% of total amount in cells) appeared in the medium up to ~8 h of continued culture. However, culture medium HRP eluted in the same peak as standard HRP on the Sephadex G-200 column. It is therefore improbable that the HRP appearing in the medium was cycled through the cells, if not released directly from endocytic

vesicles before fusion with lysosomes. Since HRP was not detected cytochemically on the surface of the cells, most of that found in the medium had probably been adsorbed previously to the culture

TABLE III  
Activity of  $\beta$ -N-Acetylglucosaminidase in Cultures Kept in Medium Supplemented with Standard or Heat-Inactivated Serum

Treatment	Time h	Enzyme activity nmol/s/mg protein
Heat-inactivated serum	0	0.77
	24	1.10
	48	1.75
Heat-inactivated serum plus colchicine ( $1 \times 10^{-6}$ M)	0	0.77
	24	1.06
	48	1.65
Standard serum	0	0.77
	24	0.85
	48	1.13

Chondrocytes were seeded out in medium supplemented with heat-inactivated (15 min at 70°C) serum and allowed to attach to the dishes overnight. The cultures were then transferred to the same medium with or without colchicine or to medium supplemented with standard serum (time = 0 h). Data represent means of duplicate cultures (individual values differing <5% from means).

TABLE II  
Lysosomal Enzyme Activities in Control Cultures and Cultures Treated with Colchicine or Vinblastine

Treatment	Time h	Acid phosphatase	$\beta$ -N-acetylglucosaminidase nmol/s/mg protein	$\beta$ -glucuronidase
Control	0	1.12 (0.07)	1.34 (0.02)	0.116 (0.003)
	24	1.28 (0.05)	1.59 (0.07)	0.121 (0.005)
	48	1.43 (0.07)	1.73 (0.05)	0.131 (0.008)
Colchicine ( $1 \times 10^{-5}$ M)	0	1.09 (0.06)	1.34 (0.02)	0.114 (0.008)
	24	1.24 (0.06)	1.39 (0.04)	0.122 (0.004)
	48	1.38 (0.02)	1.47 (0.05)	0.129 (0.006)
Vinblastine ( $1 \times 10^{-5}$ M)	0	1.08 (0.07)	1.34 (0.03)	0.115 (0.006)
	24	1.26 (0.05)	1.41 (0.04)	0.117 (0.007)
	48	1.38 (0.06)	1.48 (0.03)	0.134 (0.008)

Chondrocytes were allowed to attach to the dishes overnight. The medium was then replaced by normal full medium or medium containing colchicine or vinblastine. After 2-h pretreatment with the drugs (time = 0 h), the first samples were taken for determination of enzyme activities, collecting the cells with a rubber policeman. Data represent means of triplicate cultures with standard deviation in parentheses. Colchicine and vinblastine were also used at  $1 \times 10^{-6}$  M (data not given). The results did not differ from those obtained using the drugs at  $1 \times 10^{-5}$  M.

dish. This was supported by the finding that similar amounts of HRP were released from dishes with or without cells (cf. reference 32).

### Double-Labeling Experiments

Ultrastructurally, colloidal thorium dioxide particles were found to be taken up and to accumulate

intracellularly in the same way as HRP (cf. reference 35). In double-labeling experiments, cells were first allowed to ingest thorium dioxide, rinsed, cultured overnight in normal full medium, and then exposed to HRP for 2 h. The rationale behind these experiments was that thorium dioxide particles would first be chased into secondary lysosomes, subsequently making it possible to

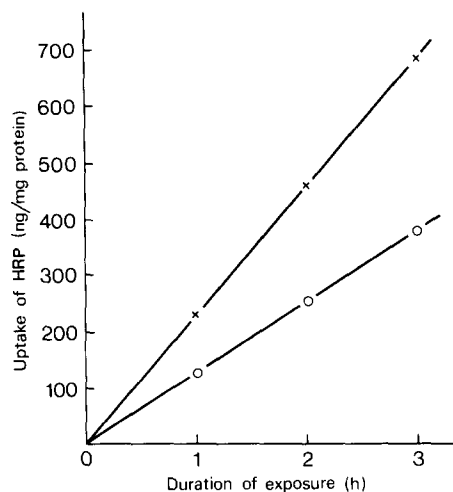


FIGURE 2 HRP uptake in confluent chondrocyte cultures as related to time. Cells were kept in normal medium (x) or pretreated with  $1 \times 10^{-6}$  M colchicine for 2 h (o) and then exposed to 1 mg of HRP per ml of medium for varying times, again with or without colchicine. In both groups, the uptake of HRP increases linearly with time and the plot of uptake vs. exposure time passes through the origin, indicating that there is no adsorption of HRP to the cell surface before ingestion. The rate of uptake in the colchicine-treated cells is only about half of that in the controls.

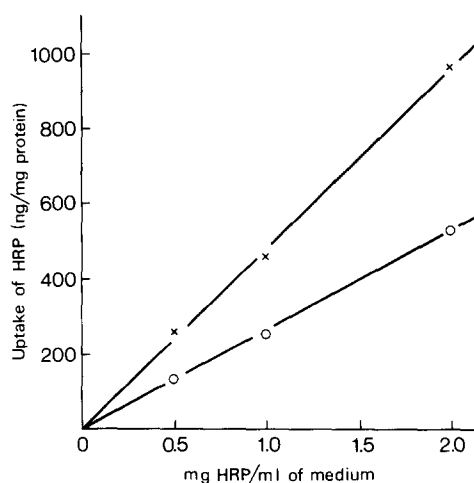
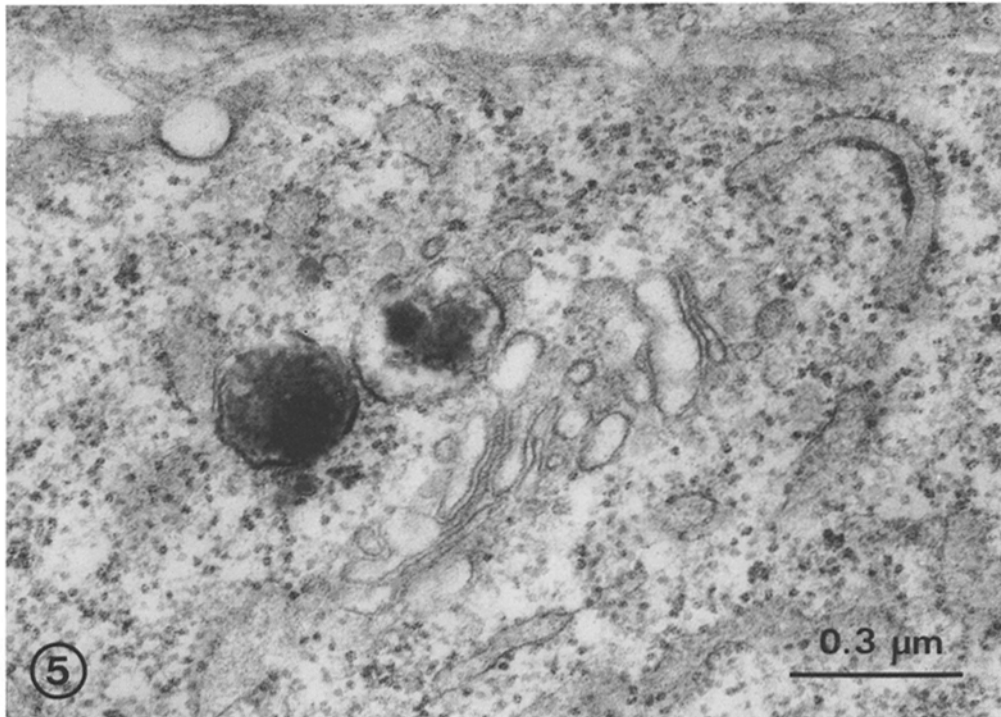
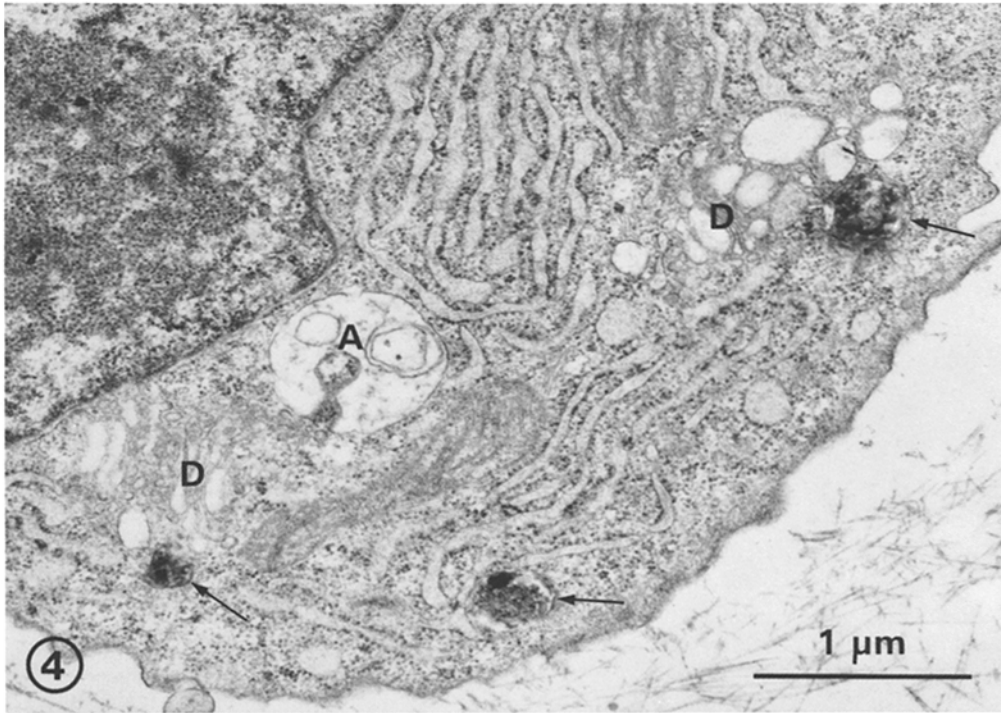


FIGURE 3 HRP uptake in confluent chondrocyte cultures as related to concentration of HRP in the culture medium. Cells were kept in normal medium (x) or pretreated with  $1 \times 10^{-6}$  M colchicine for 2 h (o) and then exposed to varying concentrations of HRP for 2 h, again with or without colchicine. In both groups, the amount of HRP ingested is linearly related to the concentration of HRP in the medium. The rate of uptake in the colchicine-treated cells is only about half of that in the controls.

TABLE IV  
Drug Effects on Endocytosis of HRP by Cultured Chondrocytes

Treatment	Endocytosis of HRP		
	ng/mg cell-associated protein	% of control	
Control	456 (11)	100	
Colchicine, $1 \times 10^{-6}$ M	249 (7)	55	$P < 0.001$
Colchicine, $1 \times 10^{-5}$ M	246 (10)	54	$P < 0.001$
Lumicolchicine, $1 \times 10^{-5}$ M	459 (12)	101	$P > 0.1$
Vinblastine, $1 \times 10^{-6}$ M	244 (7)	54	$P < 0.001$
Vinblastine, $1 \times 10^{-5}$ M	208 (9)	46	$P < 0.001$
Cytochalasin B, $2 \times 10^{-6}$ M	483 (14)	106	$P > 0.05$
Cytochalasin B, $1 \times 10^{-5}$ M	474 (17)	104	$P > 0.1$

Chondrocytes were allowed to attach to the dishes overnight. They were then pretreated with drugs for 2 h and thereafter exposed for 2 h to HRP (1 mg/ml of medium), again with or without drugs. Data represent means of triplicate cultures with standard deviation in parentheses. The probability ( $P$ ) that the observed difference compared with the controls could arise purely by chance is also given (Student's  $t$  test).



FIGURES 4 and 5 Chondrocytes from cultures treated with  $1 \times 10^{-6}$  M colchicine for 2 h and then exposed to 1 mg of HRP per ml of medium for 2 h, again in the presence of colchicine. In Fig. 4, three vesicles (arrows) with distinct enzyme reaction product are shown. Two of them are located in the immediate vicinity of two dictyosomes (*D*) dispersed in the cytoplasm. *A* indicates an autophagosome-like vacuole without clear reaction product. Fig. 5 shows in further detail the close relationship between HRP-containing vesicles and a dictyosome. Sections stained with uranyl acetate and lead citrate. Fig. 4,  $\times 29,000$ ; Fig. 5,  $\times 75,000$ .



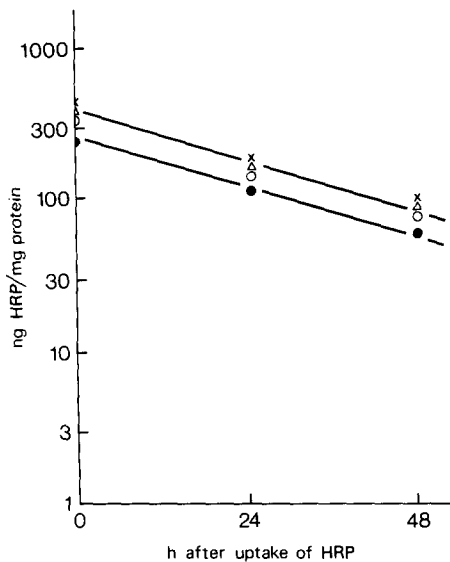


FIGURE 6 Inactivation of HRP ingested by chondrocytes. Confluent cultures were exposed to 1 mg of HRP per ml of medium for 2 h, rinsed, and transferred to HRP-free media. During the subsequent incubation, the amount of HRP diminished exponentially, with no marked differences between control cultures (x) and cultures kept in media with  $1 \times 10^{-5}$  M colchicine (O) or  $1 \times 10^{-5}$  M vinblastine ( $\Delta$ ). If the cells were pretreated with  $1 \times 10^{-5}$  M colchicine for 2 h before exposure to HRP, the uptake was lower but the decline in enzyme activity during continued culture in the presence of colchicine ( $\bullet$ ) proceeded at a rate similar to that in the above-mentioned groups.

judge whether fusion between these structures and incoming, HRP-containing endocytic vesicles takes place or not. Immediately after exposure to HRP, reaction product was found alone in some vesicles, whereas others contained only thorium dioxide or thorium dioxide plus HRP. After 2–18 h of continued culture in HRP-free medium, most vesicles contained both markers. In these respects, there was no difference between control cultures and cultures treated with colchicine (Fig. 7). However, at the later intervals, some vesicles again showed only thorium dioxide particles, probably due to degradation of HRP.

## DISCUSSION

### *Microtubules and Endocytosis of HRP*

Uptake of HRP by cultured chick embryonic chondrocytes proceeded linearly for at least 3 h, without signs of adsorption to the cell surface

TABLE V  
*Cellular Inactivation of HRP*

Treatment	Amount of HRP		
	0 h	24 h	48 h
	<i>ng/mg cell-associated protein</i>		
Control	456 (14)	177 (3)	112 (12)
Colchicine, $1 \times 10^{-6}$ M	"	143 (4)	89 (2)
Colchicine, $1 \times 10^{-5}$ M	"	136 (11)	94 (3)
Vinblastine, $1 \times 10^{-6}$ M	"	134 (2)	86 (3)
Vinblastine, $1 \times 10^{-5}$ M	"	145 (11)	100 (11)
Control	461 (4)	182 (3)	98 (4)
Colchicine, $1 \times 10^{-6}$ M	254 (12)	102 (5)	66 (4)
Colchicine, $1 \times 10^{-5}$ M	249 (7)	103 (3)	67 (2)

Chondrocytes were allowed to attach to the dishes overnight. In the upper group of experiments, the cells were then exposed to HRP (1 mg/ml of medium) for 2 h, rinsed, and transferred to HRP-free media with or without drugs (time = 0 h). In the lower group of experiments, the cells were pretreated with drugs for 2 h, exposed to HRP (1 mg/ml of medium) with or without drugs for 2 h, rinsed, and transferred to HRP-free media, again with or without drugs. Data represent means of triplicate cultures with standard deviation in parentheses.

before ingestion. Moreover, uptake of HRP was directly related to its concentration in the medium. These data indicate that the enzyme was interiorized by fluid endocytosis (12). Analogous results have previously been obtained in studies of HRP uptake by mouse macrophages (32) and fibroblasts (33).

Cytochemically, HRP was found in vesicles of varying size, often located close to the dictyosomes of the Golgi complex. Golgi cisternae were unreactive, however. Similar observations were made in the studies on macrophages and fibroblasts mentioned above (32, 33). Stereologic analyses on these two cell types showed that fluid endocytosis may bring in as much as 10 times more fluid and membrane than can be accounted for in the secondary lysosome compartment (31). It was postulated that interiorized fluid, but not HRP, rapidly leaves the lysosomes and then the cell itself, with incoming endocytic vesicle membrane being recycled back to the cell surface (cf.

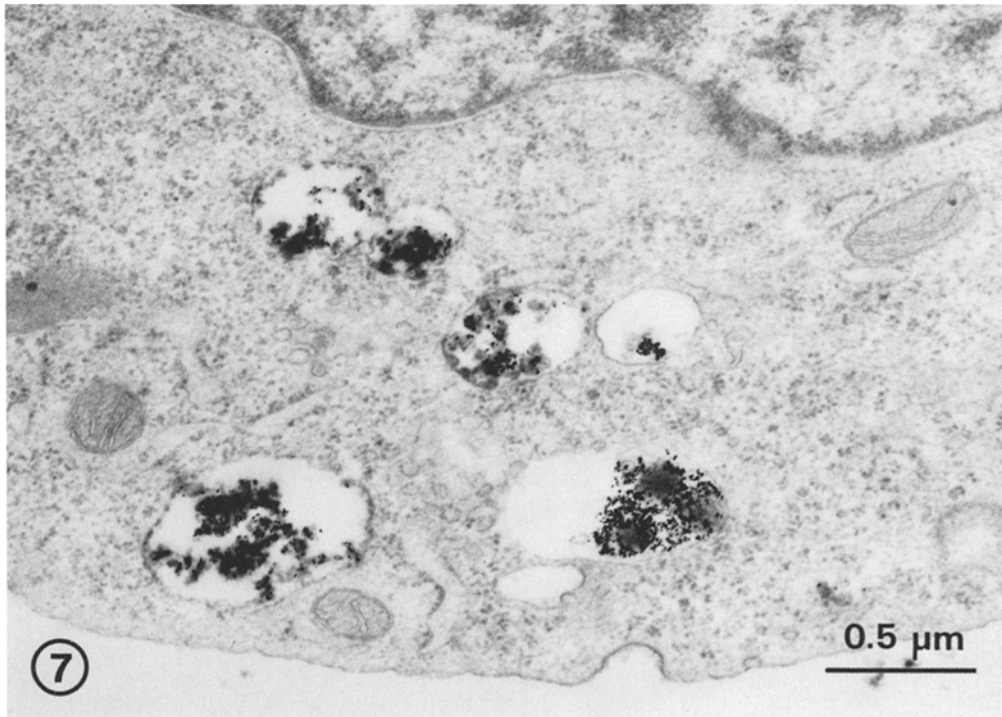


FIGURE 7 Chondrocyte from a culture exposed to colloidal thorium dioxide for 5 h, rinsed, cultured overnight in normal medium, treated with  $1 \times 10^{-6}$  M colchicine for 2 h, exposed to HRP (1 mg/ml of medium) for 2 h in the presence of colchicine, rinsed, and finally incubated in medium with colchicine for another 8 h. All the vesicles shown contain both thorium dioxide particles and reaction product for HRP, indicating that fusion between different vesicles belonging to the lysosomal system can take place in the absence of intact cytoplasmic microtubules. Unstained section.  $\times 40,000$ .

reference 26). The possibility was also discussed that endocytosis represents a means for retrieval of membrane inserted into the plasmalemma during release of secretory products by exocytosis.

After treatment of the chondrocytes with colchicine and vinblastine, two microtubule-disruptive drugs (40), no cytoplasmic microtubules were detected ultrastructurally. Furthermore, the cells showed morphological changes of the same type described previously, i.e., most characteristically a disorganization of the Golgi complex (36). Lumicolchicine, a mixture of photoisomers of colchicine not binding to microtubule protein but exerting effects of colchicine unrelated to the antimicrotubular action (40), did not clearly affect cell fine structure, nor did cytochalasin B, a drug believed to interfere with the integrity and function of microfilaments (23).

Colchicine and vinblastine both reduced chondrocyte uptake of HRP to about half of that in the controls. Lumicolchicine and cytochalasin B, on

the other hand, did not influence the rate of interiorization of the enzyme. We have recently obtained similar results in studies of HRP uptake by rat macrophages and chondrocytes (unpublished observations). These findings indicate that microtubules, but not microfilaments, are of considerable importance in the process of fluid endocytosis. However, in the case of microfilaments, the lack of clear ultrastructural effects of cytochalasin B and the uncertainty still existing regarding the site of action of this drug (23) must be kept in mind.

Previous studies concerning the role of microtubules and microfilaments in endocytosis have given somewhat contradictory results (for reviews see references 14 and 28). Nevertheless, microtubules are believed to be implicated in the ingestion of thyroglobulin from the follicular lumen of the thyroid gland (39). Moreover, Pesanti and Axline (21) found colchicine to retard pinocytic uptake of sucrose by mouse macrophages. Likewise, the

adsorptive endocytosis of lysosomal enzymes and sulfated proteoglycans by human fibroblasts was recently shown to require intact microtubules (37).

The mechanism by which microtubules participate in the control of fluid endocytosis in chondrocytes remains unclear. Production of the macromolecular components of the extracellular matrix constitutes the principal function of these and other secretory connective tissue cells. Previous studies have shown that colchicine and other antimicrotubular agents inhibit secretion of matrix components in chondrocyte cultures (13, 17). Colchicine also causes a morphological disorganization of the Golgi complex in the cells, with dispersion of the dictyosomes throughout the cytoplasm (18, 36). Hence, the secretory disturbances may be due to a defective function of the Golgi complex and a partial interruption in the transport of material to or from the dictyosomes. Independent of the exact function of microtubules, the end result is a decreased rate of exocytosis. The finding that microtubule-disruptive drugs likewise inhibit fluid endocytosis by cultured chondrocytes supports the concept that endocytosis is used by the cell to recover membrane from the plasmalemma after exocytosis and that microtubules are involved in this coupling of exo- and endocytosis. A further confirmation of this concept may be obtained by studying the effects of microtubule-disruptive drugs on endocytosis in other cell types in which these drugs have been found to inhibit secretion.

#### *Microtubules and Fusion between Endocytic Vesicles and Lysosomes*

Colchicine and vinblastine did not decrease the rate of intracellular inactivation of HRP in the chondrocytes, indicating adequate mixture of endocytic vesicle content and lysosomal enzymes. Double-labeling experiments with colloidal thorium dioxide and HRP likewise suggested that fusion between endocytic vesicles and lysosomes does not require intact cytoplasmic microtubules. Similar conclusions have been reached in studies of intracellular degradation of exogenous material (21) and transfer of lysosomal enzymes to phagocytic vesicles (22) in cultured mouse peritoneal macrophages. Contrarily, in a study on human fibroblasts, von Figura et al. (37) reported that the intracellular degradation of proteoglycans is impaired in the presence of antimicrotubular

drugs and suggested that this is due to decreased fusion of substrate and enzyme-containing vesicles.

In previous studies on guinea pig chondrocytes treated with microtubule-disruptive drugs, increased numbers of autophagosomes were observed (18, 36). Antimicrotubular drugs have also been found to produce signs of increased autophagocytosis in other cell types (1, 11, 15, 34). This effect was less prominent in the present investigation. Nevertheless, the results of this study suggest that the accumulation of autophagosomes in cells exposed to colchicine or vinblastine is more likely to be ascribed to a stimulation of autophagocytosis per se than to a decreased rate of fusion between autophagosomes and lysosomes.

The total specific activities of the three lysosomal enzymes measured increase slightly (10-30%) during the experimental period of 48 h. In the case of acid phosphatase and  $\beta$ -glucuronidase, this increase was unaffected by colchicine and vinblastine. The specific activity of  $\beta$ -*N*-acetylglucosaminidase increased less distinctly in the presence of these drugs. It is not probable, however, that this effect was due to inhibition of endocytosis of enzyme from the culture medium (enzyme present in serum). Hence, in cultures kept in medium supplemented with heat-inactivated serum, the specific activity of  $\beta$ -*N*-acetylglucosaminidase increased to a similar extent in control cultures and cultures treated with colchicine. In view of these results, it seems unlikely that microtubular organization has any significant influence on the production or degradation of lysosomal enzymes in cultured chondrocytes. However, the possibility that these two processes were in fact affected to the same extent by the drugs, with no overall effect on the lysosomal enzyme activities, cannot be excluded.

#### *Concluding Remarks*

The present study shows that uptake of HRP by cultured chick embryonic chondrocytes is distinctly reduced by the drugs colchicine and vinblastine. Cellular inactivation of ingested HRP was not inhibited by the presence of these drugs, however. Nor did these drugs have any clear effect on the total specific activities of the three lysosomal enzymes measured. The results indicate that microtubules are of considerable importance in the process of fluid endocytosis in chondrocytes,

although the exact mechanism remains unknown. Contrariwise, no support was obtained for a role of microtubules in the fusion of endocytic vesicles and lysosomes, or in the overall metabolism of lysosomal enzymes.

We thank Karin Blomgren for excellent technical assistance and Ingrid Wäälma for typing the manuscript.

Financial support was obtained from the Swedish Medical Research Council (project No. 12X-03355), the King Gustaf V 80th Birthday Fund, the Swedish Society of Medical Sciences, the Å. Wiberg Foundation, the M. Bergvall Foundation, the A. O. Swärd Foundation, and from the funds of Karolinska Institutet.

Received for publication 11 October 1978.

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