Proc. Nat. Acad. Sci. USA
Vol. 69, No. 10, pp. 2865–2869, October 1972

Cytochalasin B: Lack of Effect on Mucopolysaccharide Synthesis and Selective Alterations in Precursor Uptake

(glucose transport/DNA, RNA, and protein synthesis/morphogenesis/microfilaments/plasma membrane)

RONALD H. COHN, SHIB D. BANERJEE, EARL R. SHELTON, AND MERTON R. BERNFIELD

Division of Developmental Biology, Department of Pediatrics, Stanford University School of Medicine, Stanford, California 94305

Communicated by A. Dale Kaiser, July 24, 1972

ABSTRACT **Synthesis and secretion of mucopolysaccharide in mouse 3T3 fibroblasts and in embryonic submandibular glands are unaffected by amounts of cytochalasin B that alter the morphology of these cells and tissues. The drug markedly and reversibly inhibits incorporation of [^{3}H]glucosamine into mucopolysaccharide by preventing cellular uptake of the precursor, but does not affect incorporation of radiosulfate. Cytochalasin does not alter DNA, RNA, or protein synthesis, but stimulates the uptake of orotic acid and markedly inhibits the uptake of glucose. These selective effects on the transport of small molecules suggest that the primary action of the drug may be on cell membranes. Since processes unrelated to microfilament disruption may be altered by cytochalasin, great caution must be exercised in interpreting studies with the drug.**

Cytochalasin B has been reported to inhibit numerous biological functions, including cytokinesis, cell motility, cytoplasmic streaming, phagocytosis, platelet-clot retraction, and morphogenesis (1–4). These biological effects have been suggested to result from the disruptive action of the drug on 50-Å intracellular microfilaments (2, 3, 5, 6). Where studied, the action of the drug was rapidly and completely reversible. Cytochalasin does not have an appreciable effect on DNA, RNA, and protein syntheses (2, 7, 8), and its effect on microfilaments is reversible under conditions where protein synthesis is inhibited 95% (8).

Cytochalasin causes salivary glands of mouse embryos to flatten, lose their clefts, and cease morphogenesis; upon removal of the drug, normal development resumes (4). On the other hand, the normal morphology and morphogenesis of these glands require acid mucopolysaccharide within the epithelial basal lamina (9), and a recent report concluded that mucopolysaccharide synthesis is severely depressed by cytochalasin (10).

To investigate the relationship between the cytochalasin-induced loss of morphology and cessation of morphogenesis, and that caused by removal of surface mucopolysaccharide, we studied the effect of cytochalasin on synthesis of mucopolysaccharide. DNA, RNA, and protein syntheses, as well as the uptake of certain macromolecular precursors, were also examined. The data demonstrate that cytochalasin does not alter mucopolysaccharide synthesis or secretion and that the drug selectively alters the transport of small molecules; they suggest that the primary action of the drug may be on plasma membranes.

Abbreviation: $(CH_3)_2SO$, dimethylsulfoxide.

MATERIALS AND METHODS

Reagents and Isotopes. $Na_2{}^{35}SO_4$ (818 Ci/mol) and [^{3}H] glucosamine (3.6 Ci/mmol) were obtained from New England Nuclear. [^{3}H]Thymidine (10 Ci/mmol), [^{14}C]orotic acid (50 Ci/mol), and [^{3}H]aminoacid mixture were obtained from Schwartz/Mann. Streptococcal hyaluronidase was a gift from Dr. Bryan P. Toole and cytochalasin B was a gift from Dr. S. B. Carter.

Cell and Organ Culture. Submandibular salivary glands from 13-day-old mouse embryos were explanted and cultured without a clot (11) in either control [0.5% $(CH_3)_2SO$] or cytochalasin medium (control medium plus 10 μg/ml cytochalasin B). Radioautography, Alcian Blue staining, and ruthenium red staining for electron microscopy were done as described (9).

Mouse 3T3 fibroblasts, obtained from Dr. J. Cassiman, were maintained in 100-mm Falcon plastic culture dishes in an atmosphere of 95% air–5% CO_2. Eagle's minimum essential medium (Earle salts, with $MgCl_2$ in place of $MgSO_4$) contained 10% fetal-calf serum, 0.2 mg/ml streptomycin sulfate, and 200 U/ml penicillin. Cells were grown in this medium (changed every 48 hr) until the addition of isotopes in either control [0.25% $(CH_3)_2SO$] or cytochalasin medium (control medium plus 5 μg/ml cytochalasin B).

Labeling and Assays of 3T3 Fibroblasts. Log phase cells (about 1.3×10^6 cells per 100-mm dish) were incubated with $Na_2{}^{35}SO_4$ (4 μCi/ml) and [^{3}H]glucosamine (5 μCi/ml) for studies of mucopolysaccharide synthesis, and with [^{14}C]orotic acid (0.5 μCi/ml) and [^{3}H]thymidine (1.0 μCi/ml) for studies of nucleic-acid synthesis. For studies of protein synthesis, confluent cultures in 75-cm^2 Falcon flasks were incubated 24 hr with the [^{3}H]aminoacid mixture (1.0 μCi/ml). For measurement of precursor uptake, log phase cells in 60-mm Falcon plastic culture dishes were incubated for 10 min in control or cytochalasin medium (without calf serum or antibiotics) containing 5 μCi/ml [^{3}H]glucosamine, or 2.5 μCi/ml [^{14}C]orotic acid. Cells were harvested by scraping after removal of medium and washing four times with phosphate-buffered saline (pH 7.2). The cell suspensions were disrupted by sonications, and aliquots were taken for assays and for protein analysis (12).

Incorporation into RNA and DNA was measured by addition of 1/3 volume of 40% Cl_3CCOOH (containing 10 mM orotate and thymidine) to the cell sonicates to which 50 μg/ml

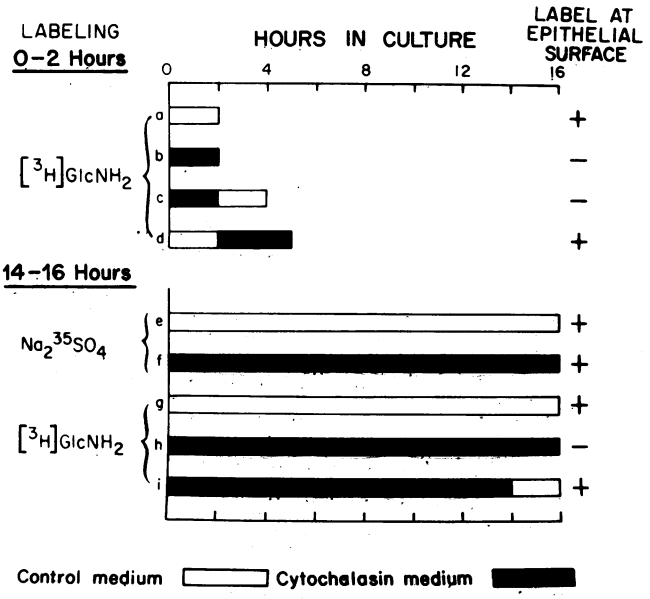

FIG. 1. Summary of radioautographic analyses of salivary glands of 13-day-old mouse embryos. Glands were labeled at 0–2 hr of culture with [^{3}H]glucosamine ([^{3}H]Glc NH_2) (40 μCi/ml) and at 14–16 hr of culture with either $Na_2{}^{35}SO_4$ (100 μCi/ml) or [^{3}H]glucosamine (40 μCi/ml). Glands were maintained in control medium [0.5% $(CH_3)_2SO$; *open bar*] or cytochalasin medium [10 μg/ml, 0.5% $(CH_3)_2SO$; *solid bar*] for the times indicated. At the end of the indicated culture period, the glands were fixed and processed for radioautography. The presence (+) or absence (−) of radioactive material at the epithelial surface is indicated. In experiment *c*, the glands were washed at 2 hr and incubated in 1 mM nonradioactive glucosamine for 2 additional hr.

carrier RNA and DNA had been added. After 30 min on ice, the precipitates were collected on glass fiber filters and washed with 5% Cl_3CCOOH–95% ethanol. The filters were air-dried and counted in toluene scintillant at efficiencies of 31 and 50% for ^{3}H and ^{14}C, respectively. More than 99% of the acid-precipitable [^{3}H]thymidine counts were susceptible to digestion with DNase I (100 μg/ml at 37° for 1 hr in 1 mM $MgCl_2$) and 82% of the [^{14}C]orotic acid counts to RNase A (100 μg/ml at 37° for 1 hr).

Mucopolysaccharides were isolated from cells and media as follows: carrier hyaluronic acid and chondroitin SO_4 (Sigma) were added, and the samples were adjusted to 150 mM Tris·HCl (pH 8.3). The samples were boiled for 10 min, followed by two 8-hr Pronase digestions at 55°. The digests of the cells were adjusted to 3.5 mM Mg^{++}, incubated for 30 min at 37° with DNase I and RNase A (Worthington; 7 μg/ml each), made 0.3 mM in Na_2SO_4 and in glucosamine, and clarified by centrifugation. Digests of the media were centrifuged and then dialyzed against 0.1 M Na_2SO_4 and 10 mM glucosamine for 16 hr and against water for 28 hr. Three volumes of 95% ethanol (containing 1% K acetate) were added to both cell and media samples, and the mixture was kept at −20° overnight.

The precipitates were collected by centrifugation, air-dried, and dissolved in 0.1 M NaCl and Na acetate (pH 5.0). Each sample was divided into two aliquots, one aliquot receiving streptococcal hyaluronidase (100 μg/ml) to assess sulfated mucopolysaccharide, and the other receiving buffer to assess total mucopolysaccharide. The aliquots were incubated at 37° for 3 hr, boiled for 2 min, made 0.08% in cetylpyridinium chloride and incubated at 37° for 1 hr. The precipitates were collected on glass fiber filters and washed with 0.08% cetylpyridinium chloride and ice-cold H_2O. The filters were air-dried and counted at efficiencies of 33 and 50% for ^{3}H and ^{35}S, respectively.

For determination of acid-soluble radioactivity, aliquots of cell sonicates were made 0.2 N in $HClO_4$, kept on ice 10 min, and centrifuged (5000 × *g*, 15 min). Supernatants were neutralized with NaOH and counted in a dioxane-based counting solution. Efficiencies for ^{3}H, ^{14}C, and ^{35}S were 35, 52, and 43%, respectively.

The uptake of radioactive precursors was assessed on 0.5-ml aliquots of cell sonicates dissolved in 1 ml Nuclear Chicago solubilizer (1 hr at 60°). Counting efficiencies for ^{3}H and ^{14}C were 37 and 63%, respectively.

RESULTS

Effect of Cytochalasin on Salivary Rudiments. Submandibular salivary glands from 13-day-old mouse embryos maintained in organ culture form clefts and undergo branching morphogenesis (9). Equivalent development occurs in the presence of 0.5% $(CH_3)_2SO$, however, within 2 hr after addition of cytochalasin B (10 μg/ml) to the culture medium, the glands become flat and the small clefts disappear (4). In cytochalasin medium, the glands remain "wafer-like" and do not form new clefts, but after the glands are rinsed and placed in control medium, they revert to their original shape within 2 hr and resume normal morphogenesis.

To determine the drug's effect on mucopolysaccharide synthesis, salivary glands were incubated in control or cytochalasin medium containing [^{3}H]glucosamine or $Na_2{}^{35}SO_4$ and processed for radioautography (Fig. 1). Glands incubated in control medium immediately after explantation showed maximal incorporation of [^{3}H]glucosamine at the epithelial surface with a distribution identical to that described (9, 11). In marked contrast, glands labeled with [^{3}H]glucosamine in the presence of cytochalasin showed a complete absence of labeled material at the epithelial surface and a slight generalized diminution of label within the epithelium and mesenchyme. When such glands were washed and incubated for 2 additional hr in control medium containing a 35-fold excess of nonradioactive glucosamine, no label was observed at the epithelial basal lamina, indicating that the intracellular label could not be chased into the surface material. Glands labeled with [^{3}H]glucosamine, washed free of residual label, and incubated in cytochalasin medium, showed nearly equivalent amounts of basal laminar radioactivity as glands not treated with the drug, suggesting that the drug does not remove mucopolysaccharide from the epithelial surface. To confirm this point, we stained cytochalasin-treated glands with Alcian Blue at various Mg^{++} concentrations and with ruthenium red. Identical amounts of histochemically and ultrastructurally identifiable mucopolysaccharide were observed within the basal lamina in these glands as in control glands.

Incorporation of $^{35}SO_4$ into epithelial basal laminar mucopolysaccharide is minimal immediately after explanation, but increases with time in culture (11). Therefore, glands were incubated for 14 hr in control or cytochalasin medium before addition of $Na_2{}^{35}SO_4$ (Fig. 1). In contrast to [^{3}H]glu-

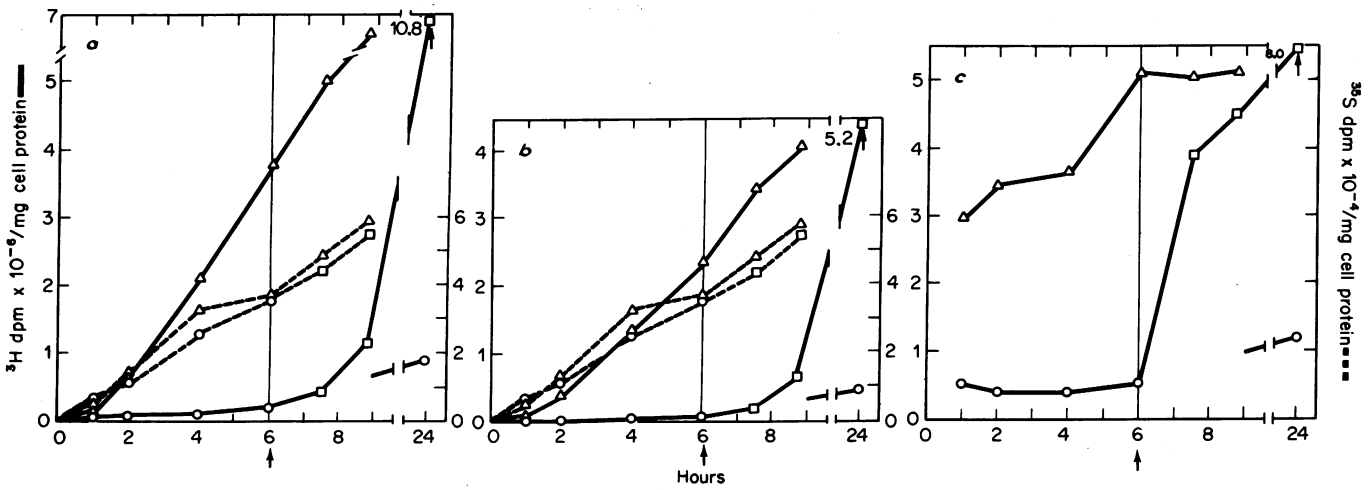

FIG. 2. Effect of cytochalasin on the incorporation of [^{3}H]glucosamine (——) and $Na_2{}^{35}SO_4$ (- - -) in 3T3 fibroblasts. Time of incubation is plotted against mucopolysaccharide isolated from the cells plus medium in *a* and *b*, and against acid-soluble radioactivity in *c*. Control medium contained 0.25% $(CH_3)_2SO$, and cytochalasin medium consisted of control medium plus 5 μg/ml cytochalasin B. To test for reversibility, the original medium was removed at 6 hr incubation, the cells were washed in control medium, and continued in culture in fresh control medium containing radioactive precursors. The incorporation indicated after 6 hr includes the mucopolysaccharide in the medium that was removed at 6 hr. △, control; ○, cytochalasin; □, cytochalasin on reversal; *arrow*, addition of control media.

(*a*) Incorporation of [^{3}H]glucosamine and $Na_2{}^{35}SO_4$ into total (sulfated plus nonsulfated) mucopolysaccharide. (*b*) Incorporation of [^{3}H]glucosamine and $Na_2{}^{35}SO_4$ into sulfated mucopolysaccharide only. These data represent the mucopolysaccharide not susceptible to streptococcal hyaluronidase digestion. (*c*) Cellular acid-soluble radioactivity derived from [^{3}H]glucosamine.

cosamine incorporation, the basal lamina of cytochalasin-treated glands contained nearly identical amounts of $^{35}SO_4$ as control glands. The differing effects of the drug on [^{3}H]-glucosamine and $^{35}SO_4$ incorporation were not due to the age of the culture, since cytochalasin prevented the accumulation of surface [^{3}H]glucosamine in glands incubated in an identical manner. The inhibition of glucosamine incorporation was readily reversible—glands incubated in cytochalasin medium for 14 hr, washed, and then incubated in [^{3}H]glucosamine showed substantial label at the epithelial surface.

Mucopolysaccharide Synthesis in 3T3 Fibroblasts. The effect of cytochalasin on mouse 3T3 fibroblasts was studied to investigate the disparity between the inhibition of [^{3}H]-glucosamine incorporation and the lack of effect on $^{35}SO_4$ incorporation into epithelial surface mucopolysaccharide. Cytochalasin caused extensive "arborization" of mouse fibroblasts, similar to that reported for other cell types (2, 10). Mucopolysaccharides were isolated from cells and media of control and cytochalasin cultures labeled for various periods with [^{3}H]glucosamine and $Na_2{}^{35}SO_4$ (Fig. 2*a*). The incorporation of [^{3}H]glucosamine into total mucopolysaccharide was almost completely prevented by cytochalasin. However, the inhibition was readily reversible; after washing the cells and addition of control medium (at 6 hr of labeling), [^{3}H]glucosamine incorporation proceeded at rates similar to that of freshly labeled control cells. In contrast, the incorporation of $^{35}SO_4$ into total mucopolysaccharide was affected only slightly by the drug. These data are qualitatively identical to the results of autoradiographic studies of embryonic salivary rudiments.

In order to clarify the discrepancy between [^{3}H]glucosamine and $^{35}SO_4$ incorporation noted in both cell and organ cultures, we assessed the incorporation of precursors into sulfated mucopolysaccharides after digesting the nonsulfated mucopolysaccharides (hyaluronate, chondroitin) with streptococcal hyaluronidase (Fig. 2*b*). In the presence of the drug there was minimal [^{3}H]glucosamine incorporation into sulfated mucopolysaccharide despite nearly normal radiosulfate incorporation. Removal of cytochalasin was without effect on $^{35}SO_4$ incorporation, but was followed by a prompt rise in the incorporation of [^{3}H]glucosamine.

Quantitative comparison of mucopolysaccharide isolated at 6 hr of culture is presented in Table 1. Sulfated mucopolysaccharide synthesis accounted for a similar proportion of the total [^{3}H]glucosamine incorporation in control and cytochalasin cultures (63 and 54%, respectively), suggesting that the drug does not selectively inhibit nonsulfated mucopoly-

TABLE 1. *Incorporation of precursors into mucopolysaccharides of cells and medium at 6 hr*

	[^{3}H]glucosamine (dpm × 10^{-6}/mg cell protein)			$Na_2{}^{35}SO_4$ (dpm × 10^{-4}/mg cell protein)		
	Control	Cytochalasin	% of control	Control	Cytochalasin	% of control
Sulfated mucopolysaccharide						
Cells	0.18	0.015	8	1.3	1.1	87
Medium	2.2	0.090	4	2.5	2.5	98
Total	2.4	0.10	4	3.8	3.6	94
Total mucopolysaccharide						
Cells	0.24	0.017	7	1.3	1.1	88
Medium	3.6	0.18	5	2.5	2.4	98
Total	3.8	0.19	5	3.8	3.6	95
% in medium	93	90		66	68	

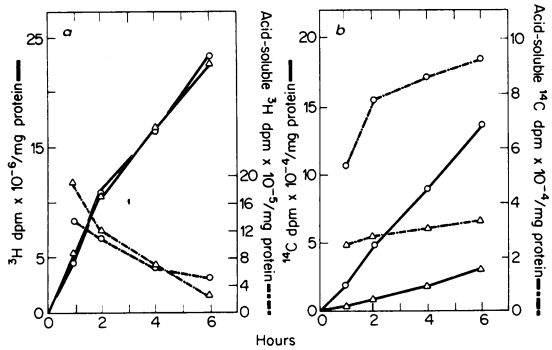

FIG. 3. Effect of cytochalasin on incorporation of [^{3}H]thymidine and [^{14}C]orotic acid in 3T3 fibroblasts. Nutrient media are defined in Fig. 2. △, control; ○, cytochalasin. (*a*) Incorporation of [^{3}H]thymidine into DNA (——) and acid-soluble pools (-·-·-). (*b*) Incorporation of [^{14}C]orotate into RNA (——) and acid-soluble pools (-·-·-).

saccharide synthesis. The extent of inhibition of [^{3}H]glucosamine incorporation was nearly identical in the cells and the medium for sulfated as well as for total mucopolysaccharide, suggesting that cytochalasin does not alter mucopolysaccharide degradation. Moreover, mucopolysaccharide secretion is apparently not affected by the drug, since the proportions of labeled mucopolysaccharide in the media are similar in control and cytochalasin cultures. It is unlikely that qualitatively different mucopolysaccharides are synthesized in the presence of the drug since a similar proportion of sulfate-labeled material from control and cytochalasin cultures was digested with a specific $\beta 1 \rightarrow 4$ hexosaminidase (chondroitinase ABC; 94.3 and 92.7%, respectively).

These results suggest that cytochalasin does not alter mucopolysaccharide synthesis, but prevents either the transport of glucosamine into cells or the use of glucosamine for mucopolysaccharide synthesis. Analysis of cellular acid-soluble radioactivity (Fig. 2*c*) indicated that control cultures contained substantially more label derived from [^{3}H]glucosamine than cytochalasin cultures, and upon cytochalasin reversal, the acid-soluble ^{3}H radioactivity increased immediately. Acid-soluble $^{35}SO_4$ in cytochalasin cultures averaged 94% that of the control cells and did not change appreciably after removal of the drug. Moreover, [^{3}H]glucosamine uptake into cytochalasin-treated cells during a 10-min labeling period was only 6% that of control cells. These data indicate that cytochalasin rapidly and reversibly inhibits intracellular transport of glucosamine.

Protein and Nucleic-Acid Synthesis in 3T3 Fibroblasts. Cytochalasin had minimal effects on [^{3}H]aminoacid incorporation into protein, in agreement with the results of others (7, 8), but caused a slight increase in the labeled protein found in the medium. The incorporation of [^{3}H]thymidine into DNA and into the acid-soluble pool of control and cytochalasin cultures was nearly identical during the 6-hr label period (Fig. 3*a*). In contrast, the rate of [^{14}C]orotic acid incorporation into RNA was stimulated about 4-fold by the drug, and the amount of radioactivity derived from [^{14}C] orotate in the acid-soluble pool was increased to a similar extent (Fig. 3*b*). This increased uptake was confirmed by the finding that during a 10-min labeling period, the uptake of [^{14}C]orotate into cytochalasin-treated cells was 357% that of the control cells. However, since Estensen (7) found that cytochalasin-treated cells incorporate uridine into RNA at a slightly reduced rate relative to control cells, it is very likely that the enhancement of orotate incorporation into RNA is due to augmented transport of the precursor.

Glucose Uptake. Cytochalasin inhibits glucosamine uptake and stimulates orotate uptake, but glucosamine and orotate are not normally present extracellularly, so that effects on the uptake of these compounds may not reflect the usual actions of the drug. Glucose, however, is a normal cell nutrient and is transported into cells by facilitated diffusion (13). Since cytochalasin (10 μg/ml) does not cause microscopic alterations of human erythrocytes (Bernfield, unpublished), the effect of cytochalasin on glucose uptake was compared in 3T3 fibroblasts and in human erythrocytes. Cytochalasin completely prevented the uptake of glucose from the medium by 3T3 fibroblasts, while the uptake of glucose into erythrocytes was not altered by the drug in the presence or absence of inorganic phosphate (Fig. 4). These data suggest that the drug inhibits the uptake of glucose in fibroblasts. The lack of effect of cytochalasin on glucose uptake by erythrocytes suggests that the action of the drug may vary with the type of cell.

Since glucose uptake is blocked by cytochalasin, it was of interest to determine whether glucose deprivation mimics the morphologic effects of the drug. However, incubation of embryonic salivary glands and 3T3 fibroblasts in glucose-free medium for 4 hr, a much longer period than that required to observe the morphologic effects of cytochalasin, did not produce analogous changes in cellular or organ morphology.

DISCUSSION

The present report establishes that cytochalasin does not affect synthesis or secretion of mucopolysaccharide in 3T3 fibroblasts. Mucopolysaccharide synthesis in the presence of cytochalasin proceeds at an equivalent rate and is of ap-

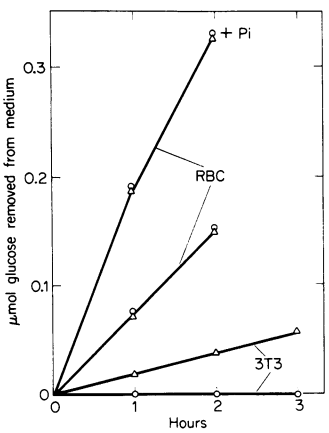

FIG. 4. Uptake of glucose from the medium by human erythrocytes and 3T3 fibroblasts. Fibroblasts in confluent monolayer culture and human erythrocytes in suspension (1.5 × 10^6 cells each) were washed three times in glucose-free medium, and incubated in control [0.20% $(CH_3)_2SO$] or cytochalasin medium [4 μg/ml; 0.20% $(CH_3)_2SO$]. Erythrocytes (*RBC*) were incubated in 5 ml of medium containing 0.55 μmol of glucose (with or without 10 mM Pi) and 3T3 cells, in 5 ml of medium containing 0.187 μmol glucose. At the indicated times, cell-free media were assayed for glucose (14). △, control; ○, cytochalasin.

parently equivalent type as that in the absence of the drug. In addition, the results show that cytochalasin selectively alters the transport of certain small molecules: the uptake of glucose and glucosamine is inhibited and that of orotic acid is stimulated. These results contradict the report that concluded that mucopolysaccharide synthesis was inhibited by cytochalasin (1). This conclusion, based upon glucosamine incorporation studies, was likely reached without appreciation of the rapid inhibitory effect of cytochalasin on glucosamine uptake.

The morphogenetic requirement of embryonic epithelia for surface-associated mucopolysaccharide is apparently unrelated to the action of cytochalasin. The histochemical and autoradiographic results obtained with embryonic salivary epithelia exposed to cytochalasin are fully explained by inhibition of glucosamine uptake.

As assessed by $^{35}SO_4$ incorporation, the rate of mucopolysaccharide synthesis is unchanged by cytochalasin, despite the marked inhibition of glucose uptake. Since glucose provides hexosamine and uronic acid precursors for mucopolysaccharide synthesis, in the presence of cytochalasin these precursors are probably derived solely from an intracellular source of glucose, e.g., endogenous pools, glycogenolysis, or gluconeogenesis. Thus, any interpretation of the cellular effects of cytochalasin must include the possibility that intracellular sources of glucose may be depleted. It is unclear whether the inhibition of glucose uptake, leading to glucose deprivation and its attendant alterations in cellular metabolism, is causally related to the previously reported cellular effects of cytochalasin. However, the morphologic effects of the drug on salivary epithelia and on 3T3 fibroblasts are not duplicated by incubation in glucose-free medium.

The cellular effects of cytochalasin have been closely correlated with disruption of microfilament arrays, and removal of the drug, with resumption of normal function and restoration of microfilament integrity (3, 4, 15). These correlations have implicated the 50-Å microfilaments in many cell functions and have suggested that they are contractile. However, cytochalasin has not been shown to interact directly with microfilaments, and there are reports suggesting that the effects of the drug may be independent of microfilament disruption (16, 17). A major difficulty in these studies is that assessments of microfilament integrity are ultrastructural and, as such, may not accurately reflect the status of microfilament function.

The locus of action of cytochalasin may be directly on cell membranes (1, 16), with the specific effect being dependent upon the type of cell under investigation. Cytochalasin is reported to alter the structure of the plasmalemma (16), and its effects on microfilaments may be a consequence of their attachment to the plasmalemma. Cytochalasin is a neutral lipophilic molecule, and the rapidity with which its cellular effects are seen and are reversed (even in the absence of protein synthesis) imply surface phenomena involving a lipophilic environment. These considerations, taken together with our findings that the drug stimulates and inhibits the transport of small molecules, are consistent with a primary action on cell membranes.

It is clear that cytochalasin has provided an important tool for the cell biologist, and that use of the drug has yielded valuable inferences into the functions of the 50-Å microfilaments. However, since cytochalasin alters processes that may be unrelated to microfilament disruption, great caution must be exercised in interpreting studies with the drug.

NOTE ADDED IN PROOF

While this manuscript was in preparation, similar results on the inhibitory effects of cytochalasin B on glucose and glucosamine transport were reported by Kletzien, R. F., Perdue, J. F. & Springer, A. (1972) *J. Biol. Chem.* **247,** 2964–2966, and by Estensen, R. D. & Plagemann, P. G. W. (1972) *Proc. Nat. Acad. Sci. USA* **69,** 1430–1434.

We thank Dr. K. K. Tsuboi for helpful advice and for the glucose uptake studies, Dr. L. Wilson and S. Mazel for stimulating discussions, and B. Gray for expert technical assistance. This study was supported by grants from the National Institutes of Health (HD 02147 and HD 00049) and from the National Foundation-March of Dimes.

1. Carter, S. B. (1967) *Nature* **213,** 261–264.
2. Spooner, B. S., Yamada, K. M. & Wessells, N. K. (1971) *J. Cell Biol.* **49,** 595–613.
3. Wessells, N. K., Spooner, B. S., Ash, J. F., Bradley, M. O., Luduena, M. A., Taylor, E. L., Wrenn, J. T. & Yamada, K. M. (1971) *Science* **171,** 135–143.
4. Spooner, B. S. & Wessells, N. K. (1972) *Develop. Biol.* **27,** 38–54.
5. Schroeder, T. E. (1968) *Exp. Cell Res.* **53,** 272–276.
6. Schroeder, T. E. (1969) *Biol. Bull.* **137,** 413–414.
7. Estensen, R. D. (1971) *Proc. Soc. Exp. Biol. Med.* **136,** 1256–1260.
8. Yamada, K. M., Spooner, B. S. & Wessells, N. K. (1970) *Proc. Nat. Acad. Sci. USA* **66,** 1206–1212.
9. Bernfield, M. R., Banerjee, S. D. & Cohn, R. H. (1972) *J. Cell Biol.* **52,** 674–689.
10. Sanger, J. W. & Holtzer, H. (1972) *Proc. Nat. Acad. Sci. USA* **69,** 253–257.
11. Bernfield, M. R., & Banerjee, S. D. (1972) *J. Cell Biol.* **52,** 664–673.
12. Oyama, V. I., & Eagle, H. (1956) *Proc. Soc. Exp. Biol. Med.* **91,** 305–307.
13. Stein, W. D. (1967) in *The Movement of Molecules Across Cell Membranes* (Academic Press, New York), pp. 164–168.
14. Lowry, O. H., Passonneau, J. V., Hasselberger, F. X. & Shultz, D. W. (1964) *J. Biol. Chem.* **239,** 18–30.
15. Wrenn, J. T. & Wessells, N. K. (1970) *Proc. Nat. Acad. Sci. USA* **66,** 904–908.
16. Bluemink, J. G. (1971) *Z. Zellforsch.* **121,** 102–126.
17. Goldman, R. D. (1972) *J. Cell Biol.* **52,** 246–254.