Partial Inhibition by Cycloheximide of Hyaluronate Synthesis in Cell Culture* (32851)

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Anionic polysaccharides of connective tissue are combined with noncollagenous protein to form compounds called proteinpolysaccharides. From bovine nasal cartilage a proteinpolysaccharide has been isolated containing 15% protein (1). Short chains (mol. wt. $< 50 \times 10^3$) of polysaccharides (chondroitin sulfate and keratan sulfate) appear to be bound to a protein core. Proteolytic digestion liberates peptides with attached polysaccharide chains and a drastic fall in viscosity occurs. On the other hand, hyaluronate isolated from normal human synovial fluid has a much larger molecular weight ($>10^6$) and contains only 2% protein which has little influence on the physical properties of the compound in solution; proteolytic digestion leads to no change in viscosity (2).

In view of these differences between proteinpolysaccarides of cartilage and synovial fluid, studies on the interrelation of protein and polysaccharide synthesis by connective tissue cells are of special interest. Cartilage cells from chick epiphysis (3) or vertebral column (4) cannot continue to synthesize chondroitin sulfate chains when protein synthesis is inhibited by puromycin. Incubation of minced chick epiphysis with puromycin resulted in almost complete inhibition of serine-^{14}C incorporation into the protein moiety of the proteinpolysaccharide, and of acetate-^{14}C and $^{35}SO_4$ into the chondroitin sulfate chains. In intact cartilage cells, polysaccharide formation is dependent on continuing synthesis of protein.

The situation with respect to hyaluronate synthesis is not so clear. An established line of rat eye connective tissue cells which appeared to be synthesizing hyaluronate were exposed to acetylglucosamine-^{14}C. The counts subsequently released by hyaluronidase digestion of the cells were regarded as counts incorporated into hyaluronate. The use of actinomycin or puromycin led to a progressive fall in the amount of labeled hyaluronate produced by the cells; after 4 hours the number of counts released from cells incubated with puromycin was only 20% of the controls (5). On the other hand, suspensions of Group A streptococci continued to synthesize hyaluronate when cell protein synthesis was blocked by puromycin (6).

In experiments to be reported here, certain innovations were used in a study of the effects of inhibition of protein synthesis on hyaluronate formation. First, connective tissue cells from animal and human sources were grown in tissue culture. Fibroblast-like cells derived from mouse embryos were chosen as an established "permanent" tissue culture line. Synovial membrane cells from normal and rheumatoid arthritic joints (designated NSM-1, RSM-1, etc.) were cells with a finite life span in tissue culture. Both the mouse fibroblasts (7) and the synovial cells (8) have been shown by us to synthesize hyaluronate and to secrete it into the medium. Second, the use of glucosamine-^{3}H as a specific precursor of the hyaluronate appears not to have been previously reported. This isotope has made it possible to measure amounts of hyaluronate in the medium that could not be detected by colorimetric methods. Studies reported elsewhere (8) have established the identity of hyaluronate-^{3}H and its content of labeled glucosamine. Third, cycloheximide (3, 2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl glutarimide) has been used to inhibit protein synthesis. Cycloheximide inhibits some step or steps in the growth of polypeptide chains by interfering with transfer of amino acids from aminoacyl sRNA to polypeptide (9,10). In the concentrations used, cycloheximide shows minimal toxicity to syno-

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vial cells, as judged by resumption of protein synthesis when the inhibitor is removed, and by the ability of these cells to divide and grow after further subculture. The effects of puromycin (10,11) on protein and hyaluronate synthesis were compared with cycloheximide.

Methods. The fibroblast-like cells (hereafter designated 3T6) derived from mouse embryos were provided by Dr. Howard Green, New York University School of Medicine. Synovial membranes were obtained at arthrotomy from 4 patients with menisceal injuries (normal synovia, NSM-1,3,6, and 7) and from 3 patients with classical or definite rheumatoid arthritis (RSM 4,6, and 7). The tissues were explanated onto sterile plastic petri dishes (60 × 15 mm) and nourished with medium containing 25% calf serum (Dulbecco-Vogt modification of Eagle's medium). The calf serum concentration was decreased to 10% when the cells had formed a confluent monolayer and were ready for subculture. Incubation was carried out at 37°C in an atmosphere of 10% CO_2 and room air. The 3T6 cells were maintained under identical conditions, but these cells grow in multilayers; they have lost the characteristic of "contact inhibition" during the process of becoming an established cell line.

The *d*-glucosamine-6-^{3}H HCl (specific activity 238 mC/mmole) and *l*-leucine-^{3}H HCl (specific activity 500 mC/mmole) were obtained from the New England Nuclear Corp. Cycloheximide (Actidione) and puromycin were purchased from Nutritional Biochemicals Corp.

In each experiment, duplicate petri dishes containing cells from each group were used, and the plates were prepared so as to be identical with respect to cell count, day of most recent subculture, and number of previous subcultures. At the beginning of each experimental period fresh medium was added to the cultures, and they were incubated for 2 hours. Then, either cycloheximide or puromycin was added at "zero" time, and 5 min later 10 μC of glucosamine-^{3}H was added to both the control and test plates. The concentrations of inhibitor used were 10^{-4} *M* and 10^{-6} *M* cycloheximide (28 and 0.28 μg/ml), and 10^{-4} *M* puromycin (47 μg/ml). Samples of medium were taken at 6,12, and 24 hours, dialyzed in 0.02 *M* potassium acetate, and counted in a Packard Tri-Carb scintillation counter, using a toluene-Cab-O-Sil counting fluid. After correction for volume changes due to dialysis, the cpm/ml of medium were calculated and used as a measure of hyaluronate-^{3}H.

To confirm the identity of hyaluronate-^{3}H, media from control and cycloheximide-treated cells were dialyzed in a NaCl (0.1 *M*)–potassium acetate (0.05 *M*) buffer, adjusted with glacial acetic acid to pH 4.8. Aliquots of dialyzed media were removed for counts, and the remainder of the samples were digested with testicular hyaluronidase (150 turbidity reducing units/ml medium) at 37°C for 16 hours. The media were then dialyzed in potassium acetate (0.02 *M*) and the nondialyzable counts determined as above. Over 75% of the radioactivity was rendered dialyzable by testicular hyaluronidase digestion of the control and cycloheximide treated culture media. Additional studies showed the hyaluronate-^{3}H to be similar. Samples of medium were dialyzed in 0.02 *M* potassium acetate, concentrated by lyophilization, and then subjected to electrophoresis on blocks of polyvinyl chloride (12). In medium from control and cycloheximide-treated plates, the radioactivity was located near the anode in zones corresponding to a hyaluronate standard. The segments of the block containing the hyaluronate-^{3}H were eluted with water. The eluates were concentrated by ultrafiltration through collodion membranes, and then added to a Sephadex G-200 column (41 cm × 1.2 cm) equilibrated with 0.15 *M* potassium acetate. In each sample the radioactivity emerged in the void volume in identical effluent volumes.

To determine the extent of inhibition of cell protein synthesis, one plate of normal and one of rheumatoid synovial cells were incubated with 10^{-4} *M* cycloheximide or puromycin, and leucine-^{3}H for 6 hours. The cells were washed several times with mannitol–Versene–Tris buffer, scraped from the plates, lysed by repeated freeze–thawing, dialyzed in 0.02 *M* potassium acetate, and counted in the same manner as the dialyzed media.

Results. Table I shows that exposure to cycloheximide caused a 92% to 97% inhibition of leucine-^{3}H incorporation into in-

TABLE I. Cycloheximide Inhibition of Leucine-^{3}H Incorporation into Synovial Cell Protein after 6-Hour Incubations.

Cells[a]	Leucine-^{3}H (cpm/plate)		Inhibitory effect (%)
NSM-3	Control	1037	
	Cycloheximide $10^{-4}M$	48	95
RSM-7	Control	1562	
	Cycloheximide $10^{-6}M$	118	92
	Cycloheximide $10^{-4}M$	43	97

[a] NSM and RSM = normal and rheumatoid synovial cells, respectively.

tracellular protein at 6 hours in the normal and rheumatoid cultures. The results with puromycin were the same. Table II records the extent of inhibition of glucosamine-^{3}H incorporation into secreted hyaluronate by several cultured cell strains. The 3T6 cells consistently showed 70–75% inhibition by cycloheximide at 4 hours. Longer incubations led to microscopic signs of cell injury and death. Less inhibition occurred with the synovial cells. Before 6 hours, there was usually an increase in glucosamine-^{3}H incorporation in cycloheximide-treated synovial cells as compared to controls. After 6 hours, a variable amount of inhibition was observed, ranging from 10% to 60%. Additional inhibition of hyaluronate synthesis occurred in the synovial cells incubated for 12 and 24 hours with cycloheximide or puromycin. Hyaluronate synthesis was inhibited to about the same extent after 24 hours when the concentration of cycloheximide was reduced from 10^{-4} M to 10^{-6} M. When the same cells were studied at a later date, similar results were obtained, indicating that the findings are consistent and reproducible. Although the rheumatoid synovial cells appeared to be slightly less susceptible to inhibition of hyaluronate synthesis, this difference was not statistically significant.

Inhibition of hyaluronate-^{3}H synthesis in synovial cells by cycloheximide could be reversed even after 24 hours contact with the inhibitor. When these plates were washed, and fresh medium added without glucosamine-^{3}H, the level of nondialyzable radioactivity observed in the medium after further incubation was similar to that of the controls. This finding suggests that cycloheximide did not modify the entry of glucosamine-^{3}H into the cells, but reduced its utilization for hyaluronate-^{3}H synthesis. In one cell strain incubated with cycloheximide for 12 hours, intracellular incorporation of glucosamine-^{3}H was reduced by 60%, and the hyaluronate-^{3}H in the medium was reduced to the same extent. Thus there appeared to be no retention of partially polymerized molecules within the cell.

Discussion. Cultured human synovial cells thus show only partial inhibition of hyaluronate synthesis when cell protein synthesis is largely shut off by cycloheximide or puromycin. These results are midway between those observed by others, *viz.*, almost complete inhibition of hyaluronate synthesis with

TABLE II. Inhibition of Glucosamine-^{3}H Incorporation into Hyaluronate-^{3}H by Cycloheximide or Puromycin.[a]

	Cycloheximide ($10^{-4}M$) (%)				
Cells[b] Hours:	2	4	6	12	24
3T6	33	74			
NSM-1	+14	25	60		
NSM-3			+21	10	23
NSM-6			12	17	22
NSM-7			23	39	51
NSM-7[c] ($10^{-6}M$)			+3	18	45
RSM-6			11	24	34
RSM-4			+8	8	20
RSM-7[c] ($10^{-6}M$)			+6	1	13
	Puromycin ($10^{-4}M$)				
NSM-6			27	40	51
RSM-6			0	30	43

[a] Nondialyzable radioactivity in the medium (cpm/ml) at equivalent cell densities was determined for control and cycloheximide or puromycin treated plates. Results are expressed as percentage of control values. All + values indicate "stimulation" to a value above the controls.

[b] 3T6 = fibroblast-like cells derived from mouse embryo; NSM and RSM = normal and rheumatoid synovial cells, respectively.

[c] In these 2 experiments, a cycloheximide concentration of $10^{-6}M$ was tested for the purpose of comparing its effect with the higher concentration of $10^{-4}M$.

rat connective tissue cells (5), or no inhibition with streptococci (6). There is one part of our work which is in agreement with that of Davidson and co-workers (5), who used an established line of rat eye cells and found 80% inhibition of hyaluronate synthesis at 4 hours in the presence of puromycin. The 3T6 cells which we used were also established in tissue culture, and in these cells hyaluronate synthesis was also inhibited to the extent of 75% at 4 hours by cycloheximide. It is not clear whether inhibition of hyaluronate synthesis by cycloheximide or puromycin is related to the establishment of these lines in cell culture. The cells derived from human synovial membranes were not established lines, and in general underwent spontaneous degeneration after 4–12 months in continuous subculture. These cells showed variable and less pronounced inhibition of hyaluronate synthesis by cycloheximide or puromycin.

Intact cartilage cells are able to incorporate radioactive serine into the protein moiety of the proteinpolysaccharide (3). Synovial cells in culture have not yet been shown to incorporate a radioactive amino acid into hyaluronate, but it is clear from the present study that at 6 hours, when cell protein synthesis is almost totally inhibited, hyaluronate synthesis is frequently not reduced, and in 24 hours there is only partial inhibition. If hyaluronate made by these cells can be shown to contain a protein moiety, as appears to be the case for human synovial fluid hyaluronate (2), then the failure to reduce appreciably the synthesis of hyaluronate when almost all cell protein synthesis is abolished, would point up a remarkable difference between its formation and that of chondroitin sulfate in cartilage cells (3), or glycoprotein in the liver (13), where continued synthesis of a protein "acceptor" is needed for complete formation of the carbohydrate chains.

Summary. The incorporation of glucosamine-^{3}H into hyaluronate-^{3}H by cultured mouse fibroblasts and human synovial cells is only partially reduced by cycloheximide or puromycin, under conditions where protein synthesis by these cells is almost completely inhibited.

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1. Schubert, M., Federation Proc. **25**, 1047 (1966).
2. Hamerman, D., Rojkind, M., and Sandson, J., Federation Proc. **25**, 1040 (1966).
3. Telser, A., Robinson, H. C., and Dorfman, A., Proc. Natl. Acad. Sci. U.S. **54**, 912 (1965).
4. DeLa Haba, G. and Holtzer, H., Science **149**, 1263 (1965).
5. Davidson, E. H., Allfrey, V. G., and Mirsky, A. E., Proc. Natl. Acad. Sci. U.S. **49**, 53 (1963).
6. Stoclmiller, A. C. and Dorfman, A., Federation Proc. abstr. 51st Ann. Meeting, p. 346 (1967).
7. Hamerman, D., Todaro, G. J., and Green, H., Biochim. Biophys. Acta **101**, 343 (1965).
8. Barland, P., Smith, C., and Hamerman, D., Federation Proc. **26**, 468 (1967).
9. Ennis, H. L. and Lubin, M., Science **146**, 1474 (1964).
10. Bennett, L. L. Jr., Ward, V. L., and Brockman, R. W., Biochim. Biophys. Acta **103**, 478 (1965).
11. Yarmolinsky, M. B. and DeLa Haba, G. L., Proc. Natl. Acad. Sci. U.S. **45**, 1721 (1959).
12. Sandson, J. and Hamerman, D., J. Clin. Invest. **41**, 1817 (1962).
13. Kornfeld, S., Kornfeld, R., Neufeld, E., and O'Brien, P., Proc. Natl. Acad. Sci. U. S. **52**, 371 (1964).

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