CELLULAR DIFFERENTIATION AND THE AGING PROCESS IN CARTILAGINOUS TISSUES

MUCOPOLYSACCHARIDE SYNTHESIS IN CELL CULTURES OF CHONDROCYTES*

BY HERBERT J. SHULMAN,‡ M.D., AND KARL MEYER, M.D.

(From The Department of Medicine, Columbia University College of Physicians and Surgeons, the Edward Daniels Faulkner Arthritis Clinic of the Presbyterian Hospital, New York 10032, and the Belfer Graduate School of Science, Yeshiva University, New York, New York 10033)

(Received for publication 2 August 1968)

The mucopolysaccharides of cartilage are chondroitin-4-sulfate, chondroitin-6-sulfate and keratan sulfate (1, 2). In young animals, chondroitin-4-sulfate predominates whereas the relative proportion of chondroitin-6-sulfate increases with age (2, 3). The glucosamine-containing polymer, keratan sulfate, is almost absent at birth in mammalian species but increases slowly with aging (3, 4).

The advantages of a cell culture system for the study of differential synthesis of these polysaccharides are manifold, yet there has been difficulty in producing sulfated polysaccharides resembling those found in cartilage. Cultures of fibroblasts from various tissues have been shown to accumulate chondroitin sulfate, usually in quantities smaller than the predominant hyaluronic acid (5–8). Glucosamine-containing sulfated polysaccharide is synthesized in culture, but it has not been identified as keratan sulfate or any other compound (9, 10).

Chondrocytes from the chicken embryo have been found by Holtzer (10–14) to develop two forms in tissue culture. In one form ("differentiated"), they appear as rounded cells which synthesize sulfated polysaccharide containing equal amounts of galactosamine and glucosamine, and which is at least partially sensitive to testicular hyaluronidase digestion. In the "dedifferentiated" form, the cells appear as fibroblasts which have only been found to produce nonsulfated, hyaluronidase sensitive compounds (10).

Coon (15, 16) and Holtzer (17) have presented evidence that these two stages can be demonstrated by the same clone of cells with varying environmental conditions.

We have modified the method of Coon (15) and have been able to grow rela-

* A preliminary report of this work has been presented: Shulman, H. J. and K. Meyer. 1968. Chondroitin-4-sulfate synthesis by differentiated chondrocytes in cell culture. *Arthritis Rheumat.* **11**:510. (Abstr.).

‡ Special Fellow, United States Public Health Service.

tively large quantities of primary cell cultures of predominantly differentiated embryonic chondrocytes. Mucopolysaccharide fractions have been isolated and characterized from the cell matrix and culture medium, and have been compared to mucopolysaccharides isolated from embryonic chicken vertebral cartilage.

Methods

Vertebral columns from 10 day chicken embryos (white leghorn eggs) were used to obtain chondrocyte suspensions (10) after a 2 hr incubation in a dissociation medium (15). 5×10^5–3×10^6 chondrocytes were inoculated in 10 ml of a modified Ham's F-10 medium (15) in 100×20 mm Falcon plastic tissue culture dishes, and grown in an atmosphere of 5% CO_2 in a gas-flow humidified incubator. The feeding schedule was that of Coon (15). Early cell populations showed over 95% rounded cells (Fig. 3); when this percentage fell below 50% (10–20 days) the cultures were terminated. The medium was collected and stored under 9 volumes of acetone. After rinsing with phosphate buffered saline (18), the cells and matrix were scraped from the plate and stored under acetone. Vertebral columns from 72 14-day chicken embryos were dissected, cleaned of adhering perichondrial material, and stored under acetone.

After drying the samples with acetone and ether, they were digested with papain (Winthrop Labs., N. Y.) in a cysteine-EDTA buffer, pH 5.5, at 65°C for 48 hr followed by digestion with pancreatin (Mann Research Labs, New York, N. Y.) at pH 7.6, 37°C for 24 hr. The digest was precipitated with 60% ethanol in the presence of calcium acetate (19), followed by redissolving the precipitate in 5% sodium acetate–0.5 M acetic acid. Protein was removed by stirring with chloroform-*n*-amyl alcohol followed by adsorption with a mixture of Lloyd's reagent and kaolin. After reprecipitation with ethanol, the material was digested with Pronase (Calbiochem, Los Angeles, Calif.) in the presence of 0.01 M calcium acetate at 56°C for 24 hr. After a second removal of protein by adsorption on Lloyd's reagent and kaolin, ethanol to 66% was added to the solution. The precipitate was redissolved in 5% calcium acetate, insoluble material was removed by centrifuging at 12,000 rpm, the solution made 0.5 M in acetic acid, and finally precipitated with ethanol to 60%. The precipitate was dissolved in water and lyophilized.

Hexose (20), uronic acid (21), protein (22), ester sulfate (23), and hexosamines, including total hexosamine, were determined, the latter separately in the amino acid analyzer after hydrolysis with 4 N HCl for 12 hr (24) (values uncorrected for hydrolysis loss). Infrared spectra were determined on a Perkin-Elmer, model 257, spectrophotometer (K Br., solid pellet technique). Optical rotation was determined on a Bendix automatic polarimeter.

RESULTS

Cell Matrix.—A very small amount (less than 2 mg) of material was obtained from the cell matrix which showed two spots on electrophoresis at pH 1.8 (Fig. 1). The major spot traveled with the mobility of a sulfated polysaccharide, while a smaller spot moved at the rate of a nonsulfated polysaccharide. Infrared spectroscopy of the combined fractions showed the presence of sulfate (1240 cm^{-1}), carboxylate (1580 and 1410 cm^{-1}), and probably secondary amide (1630 cm^{-1}) groups. An absorption band at 850 cm^{-1}, characteristic of an axial or 4-sulfate was noted, with a very small deflection at 820 cm^{-1}, characteristic of an equatorial or 6-sulfate group (25–28). The two electrophoretic fractions could be separated on DEAE-Sephadex (Fig. 2). The fraction from the second

and larger peak showed one spot on electrophoresis with the mobility of a sulfated polysaccharide, and contained 0.239 μmole of hexosamine, 95% of which was galactosamine, while 5% was glucosamine. The fraction from the first peak showed one spot with the electrophoretic mobility of a nonsulfated

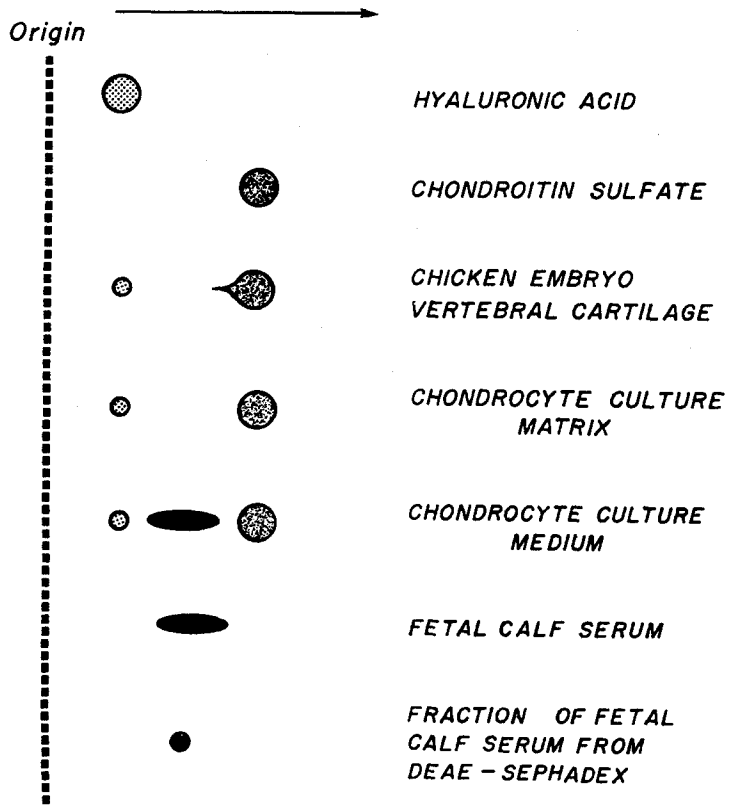

FIG. 1. Electrophoresis on cellulose acetate strips (Sepraphore III) in 4% formic acid (pH 1.78) at 100 v for 1.5 hr. Mucopolysaccharides detected with 0.5% Alcian blue in 3% acetic acid.

polysaccharide, and contained 0.052 μmole of hexosamine, 77% of which was glucosamine, while 23% was galactosamine.

These data show that a cell culture system of chondrocytes has the main characteristic of in vivo cells, namely the synthesis of a matrix containing chondroitin sulfate as the predominant mucopolysaccharide. 78% of the total polysaccharide present in the culture matrix (from chondrocytes grown in plastic dishes) is chondroitin sulfate. Nameroff and Holtzer (10) growing chondrocyte cultures under different conditions, i.e. on a plasma clot, showed that after 5 days of culture (when cell crowding occurred) chondroitin sulfate was

produced. From their electrophoresis (Fig. 11 in reference 10) and subsequent hexosamine analysis, we estimate that this composed 25–30% of the total polysaccharide produced in their cultures. Chondroitin-4-sulfate was the predominant isomer produced in our cultures. The synthesis of a small amount of chondroitin-6-sulfate cannot be determined.[1] The presence of 5% glucosamine, in the sulfated fraction, along with a small 6-sulfate absorption in the infrared spectrum, is suggestive of keratan sulfate synthesis When chondrocytes were grown on a plasma clot in another study (10), 50% of the hexosamine in the sulfated polysaccharide fraction was glucosamine. Chondrocytes which were

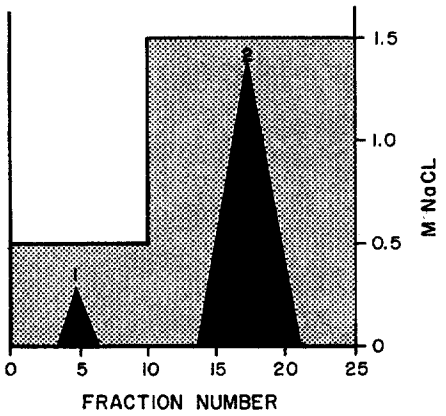

FIG. 2. Mucopolysaccharide from cell matrix fractionated on a DEAE-Sephadex column, 0.5 × 6.5 cm (43). Elution with 0.5 M NaCl, followed by 1.5 M NaCl, 0.01 M in HCl. 0.3 ml fractions collected. Eluted material recovered by adding 3 volumes ethanol to each fraction, chilling at 4° overnight, recovering precipitates which were dissolved in water, pooled, and lyophilized. Size of peaks estimated from distribution of precipitate in the fractions, and from hexosamine content of each peak.

grown in a tightly packed organ-cultured pellet of 2×10^6 cells in that study (10) synthesized chondroitin sulfate and almost no glucosamine-containing sulfated polysaccharide. In a different study (9), when chondrocytes were grown in a more loosely packed pellet of less than 1×10^6 cells, the majority of sulfated polysaccharide contained glucosamine, while a minority was chondroitin sulfate. These seemingly discrepant experimental results will be discussed later.

18% of the total polysaccharide in the culture matrix was nonsulfated. Of the hexosamine in this fraction, 77% was glucosamine and 23% galactosamine. It is presumed that the glucosamine represents mainly hyaluronic acid, shown

[1] Though a small amount of 6-sulfate was seen in infrared spectral analysis of the culture matrix, it cannot be determined if it is on the glactosamine (or possibly galactose) or on the glucosamine present in the sulfated polysaccharide fraction. The 4-sulfate, of course, can only be present on the galactosamine, since glucosamine lacks an axial hydroxyl (or sulfate) group. A galactose-4-sulfate has never been described in animal tissues.

by many investigators to be produced by fibroblasts (5, 6, 29–34); a minority of the cells in our cultures were fibroblastic in appearance (Fig. 3). The galactosamine in this fraction may represent chondroitin; indeed, the latter was apparently the major nonsulfated polysaccharide synthesized when chondrocytes were grown on a plasma clot more than 5 days (10).

Culture Medium.—From the pooled culture medium we isolated 3 mg of a mucopolysaccharide fraction which contained 12.8% uronic acid, 7.8% hexose, 10.4% hexosamine, 84% of which was galactosamine while 16% was glucosamine. The infrared spectrum showed mostly 4-sulfate with a trace of 6-sulfate. An electrophoretic pattern similar to the matrix was found, but which also had a blurred area of intermediate mobility (Fig. 1). This originated from the fetal calf serum composing 10% of the medium. When the serum itself was subjected to the same purification procedure as the medium, a fraction was obtained which showed only this blurred area of intermediate electrophoretic mobility. Further fractionation of this material on DEAE-Sephadex yielded a fraction which showed a single spot with intermediate electrophoretic mobility, containing 17% uronic acid, 3% hexose, 4.9% protein, 14.5% hexosamine, of which 98% was galactosamine and 2% glucosamine, and which showed mostly 4-sulfate with a trace of 6-sulfate on the infrared spectrum. This analysis shows chondroitin-4-sulfate (undersulfated as judged from its slow electrophoretic mobility) as the predominant mucopolysaccharide, but a small amount of 6-sulfated compound is present in addition. It could account for less than 50% of the uronic acid found in the mucopolysaccharide fraction from the medium (after removal from the cultures), thus establishing that net polysaccharide synthesis occurred. The possibility that *some* preexistent undersulfated polysaccharide in the medium was further sulfated by the chondrocytes cannot be excluded.

Embryonic Cartilage.—From the embryonic chicken vertebral cartilage, 4.3 mg of a mucopolysaccharide fraction was obtained with an electrophoretic pattern shown in Fig. 1. On DEAE-Sephadex chromatography, a very tiny peak containing uronic acid was seen in the 0.5 M NaCl elution step (too small to be recovered), while one sharp peak was found in the second elution step with 1.5 M NaCl (0.01 N in HCl). This fraction showed one spot with slight tailing on electrophoresis, corresponding to the mobility of chondroitin sulfate. Chemical analysis of this fraction showed 32.6% uronic acid, 9% hexose, $<1\%$ protein, 9.3% sulfate, and 28.4% hexosamine, of which 99.8% was galactosamine and 0.2% was glucosamine. The infrared spectrum showed mainly 4-sulfate, with a small amount of 6-sulfate; $[\alpha]_D$ was $-31.3°$. This characterization of embryonic chicken cartilage, showing predominantly chondroitin-4-sulfate with some chondroitin-6-sulfate, is consistent with that of Saito et al. (35) who found, using a newly described enzymatic method, 71% chondroitin-4-sulfate and 26% chondroitin-6-sulfate synthesis in intact chicken embryonic limb cartilage.

Keratan sulfate is essentially absent in chicken embryonic cartilage, yet

seems to be present in adult chicken cartilage (approximately 2% of the sulfated polysaccharides at about 1 yr of age, unpublished data). This increase in keratan sulfate concentration with advancing age is consistent with that found in other species (3, 4), though less dramatic than its rise in human rib cartilage from near absence at birth to 50% of the sulfated polysaccharides after 60 yr of age (4).

DISCUSSION

We suggest that there are three stages of differentiation in chondrocytes. In one stage (undifferentiated) the cells have a fibroblastic form synthesizing only nonsulfated polysaccharides. According to Coon (15, 16) and Holtzer (17), this stage is interchangeable with one showing a rounded morphology of cells (differentiated stage) in which sulfated polysaccharides are produced. The data reviewed suggests there are two differentiated stages. One type, called "young" is produced in well-packed organ-cultured pellets (10), or in cell cultures on plastic (15, 17, and the present study) and synthesizes nearly all chondroitin sulfate and very little keratan sulfate. The other type of chondrocyte, called "old," forms in more loosely packed pellets (9), or on a plasma clot as crowding occurs after 5 days (10). These cells synthesize chondroitin sulfate, but at least as much keratan sulfate in addition.

There is evidence that keratan sulfate and chondroitin-6-sulfate are linked to the same protein backbone in a "protein-polysaccharide" (36, 37), whereas a chondroitin-4-sulfate protein-polysaccharide has been found exclusively in fetal or newborn human rib cartilage (38). This would suggest that young chondrocytes synthesize chondroitin-4-sulfate protein-polysaccharides, while old chondrocytes produce complexes containing keratan sulfate and chondroitin-6-sulfate.[2]

The factors which determine the stage of differentiation of the chondrocyte are not well understood, but seem to involve electrical charges at the cell membrane. Only specially treated plastic tissue culture dishes (Falcon Plastics, Los Angeles, Calif.), where a negative charge is characteristic of the surface, seem to produce the effects mentioned; ordinary plastic (non–tissue culture) Petri dishes do not allow chondrocyte differentiation (17). In tightly packed pellet cultures (10), a minimal amount of anionic matrix (mucopolysaccharide) would yield a high negative charge density. Here, as on the treated plastic, young chondrocytes form. On plasma clots, chondrocytes usually appear fibroblastic (undifferentiated) until a certain cell density is reached, whereupon rounded cells appear and sulfated polysaccharide synthesis begins (13). Cell crowding here, as in the loosely packed pellets (9), might be expected to provide an environment of negative charge, but much less dense than tightly packed pellets or treated plastic. In the conditions of a lesser negative charge density, old chondrocytes tend to form. The (negatively charged) chondroitin sulfate we have described in fetal calf serum (4.4 mg/liter) may also be an important factor in the differentiation of cells in culture.

[2] A relative rise in chondroitin-6-sulfate with a fall in chondroitin-4-sulfate has been noted in human costal cartilage with increasing age (3, 4).

Since only the products of young chondrocytes are found in intact embryonic cartilage, it is suggested that certain culture conditions may induce the chemical expression of aging (keratan sulfate) by embryonic cells, or the conversion of young into old chondrocytes.[3] For intact cartilage to show a progressive increase in keratan sulfate would require a progressive decrease in the negative charge surrounding chondrocytes throughout life. The decrease in chondroitin sulfate concentration, also noted with aging cartilage (4), might explain this in part. Also, the rise in calcium in older cartilage (39, 40) might contribute to this lowered pericellular negative charge; calcium can reduce the net negative charge on sulfated polysaccharides (41) and on cell surfaces (42).

The hypothesis presented to explain the aging process of cartilage in terms of cellular differentiation remains to be tested. Other explanations may exist for the data reported by ourselves and others. It should be emphasized that keratan sulfate synthesis is suggested, but not proved, by the data reported. Direct measurements of the electrical charge have not been made in the situations described. Experiments designed to yield further information in this area are now in progress.

SUMMARY

Primary cell cultures of differentiated chondrocytes were shown to produce chondroitin-4-sulfate as the predominant mucopolysaccharide, with suggestive evidence for the synthesis of keratan sulfate and possibly chondroitin-6-sulfate.

Chicken embryonic cartilage was shown to be composed mainly of chondroitin-4-sulfate, with a small amount of chondroitin-6-sulfate, but essentially no keratan sulfate.

These findings were compared to the data of others, and a hypothesis explaining the aging process in cartilage in terms of cellular differentiation was presented.

This investigation was supported by grants from the John A. Hartford Foundation, the National Institute of Arthritis and Metabolic Diseases, United States Public Health Service, and by Research Grant HD-02646 of the National Institute of Child Health and Human Development, United States Public Health Service.

We are grateful to Dr. Howard Holtzer, in whose laboratory many of the techniques used in this study were learned by one of us (H. J. S.), to Dr. Charles L. Christian whose encouragement has been invaluable, and to George Crawford and Rose Meyers for technical assistance.

BIBLIOGRAPHY

1. Meyer, K., E. Davidson, A. Linker, and P. Hoffman. 1956. The acid mucopolysaccharides of connective tissue. *Biochim. Biophys. Acta.* **21**:506.
2. Meyer, K., P. Hoffman, and A. Linker. 1958. Mucopolysaccharides of costal cartilage. *Science.* **128**:896.

[3] Evidence has not yet been obtained as to whether the "young" and "old" stages are potentialities of the same cell clone. If they are found to be products of separate clones, which seems unlikely, then the data could be explained by the favored growth of a previously dormant old chondrocyte population.

3. Mathews, M. B., and S. Glagov. 1966. Acid mucopolysaccharide patterns in ageing human cartilage. *J. Clin. Invest.* **45:**1103.
4. Kaplan, D., and K. Meyer. 1959. Ageing of human cartilage. *Nature.* **183:**1267.
5. Berenson, G. S., W. M. Lumpkin, and V. G. Shipp. 1958. Study of the time-course production of acid mucopolysaccharides by fibroblasts in a synthetic medium. *Anat. Record.* **132:**585.
6. Morris, C. C. 1960. Quantitative studies on the production of acid mucopolysaccharides by replicate cell cultures of fibroblasts. *Ann. N. Y. Acad. Sci.* **86:** 878.
7. Grossfeld, H., K. Meyer, G. Godman, and A. Linker. 1957. Mucopolysaccharides produced in tissue culture. *J. Biophys. Biochem. Cytol.* **3:**391.
8. Grossfeld, H. 1963. Production of chondroitin sulfate in tissue culture of cartilage. *Biochim. Biophys. Acta.* **74:**193.
9. Glick, M. C., and F. E. Stockdale. 1964. Differences in glycosaminoglycans derived from chick embryo chondrocytes grown in vitro and in vivo. *Biochim. Biophys. Acta.* **84:**61.
10. Nameroff, M., and H. Holtzer. 1967. The loss of phenotypic traits by differentiated cells. IV. Changes in polysaccharides produced by dividing chondrocytes. *Develop. Biol.* **16:**250.
11. Stockdale, F. E., J. Abbott, S. Holtzer, and H. Holtzer. 1963. The loss of phenotypic traits by differentiated cells. II. Behavior of chondrocytes and their progeny *in vitro*. *Develop. Biol.* **7:**293.
12. Holtzer, H. 1964. Control of chondrogenesis in the embryo. *Biophys. J.* **4**(Suppl.): 239.
13. Abbott, J., and H. Holtzer. 1966. The loss of phenotypic traits by differentiated cells. III. The reversible behavior of chondrocytes in primary cultures. *J. Cell Biol.* **28:**473.
14. Holtzer, H., J. Abbott, J. Lash, and S. Holtzer. 1960. The loss of phenotypic traits by differentiated cells *in vitro*. I. Dedifferentiation of cartilage cells. *Proc. Natl. Acad. Sci.* **46:**1533.
15. Coon, H. G. 1966. Clonal stability and phenotypic expression of chick cartilage cells in vitro. *Proc. Natl. Acad. Sci.* **55:**66.
16. Coon, H. G., and R. D. Cahn. 1966. Differentiation in vitro: effects of Sephadex fractions of chick embryo extract. *Science.* **153:**1116.
17. Abbott, J., and H. Holtzer. 1968. The loss of phenotypic traits by differentiated cells. V. The effect of 5-bromodeoxyuridine on cloned chondrocytes. *Proc. Natl. Acad. Sci.* **59:**1144.
18. Dulbecco, R., and M. Vogt. 1954. Plaque formation and isolation of pure lines with poliomyelitis viruses. *J. Exptl. Med.* **99:**167.
19. Meyer, K., A. Linker, E. A. Davidson, and B. J. Weissman. 1953. The mucopolysaccharides of bovine cornea. *J. Biol. Chem.* **205:**611.
20. Seifter, S., S. Dayton, B. Novic, and E. Manturyler. 1950. The estimation of glycogen with the anthrone reagent. *Arch. Biochem.* **25:**191.
21. Dische, Z. 1947. A new specific color reaction of hexuronic acids. *J. Biol. Chem.* **167:**189.
22. Lowry, O. H., N. J. Roseborough, A. C. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:**265.

23. Antonopoulos, C. A. 1962. A modification for the determination of sulphate in mucopolysaccharides by the benzidine method. *Acta Chem. Scand.* **16:**152.
24. Hashimoto, T., and W. Pigman. 1962. A comparison of the composition of mucins and blood-group substances. *Ann. N. Y. Acad. Sci.* **93:**541.
25. Orr, S. F. D. 1954. Infrared spectroscopic studies of some polysaccharides. *Biochim. Biophys. Acta.* **14:**173.
26. Mathews, M. B. 1958. Isomeric chondroitin sulfates. *Nature.* **181:**421.
27. Lloyd, A. G., and K. S. Dodgson. 1959. Infra-red specta of carbohydrate sulfate esters. *Nature.* **184:**548.
28. Hoffman, P., A Linker, and K. Meyer. 1958. The acid mucopolysaccharides of connective tissue. III. The sulfate linkage. *Biochim. Biophys. Acta.* **30:**184.
29. Grossfeld, H., K. Meyer, and G. Godman. 1955. Differentiation of fibroblasts as determined by mucopolysaccharide production. *Proc. Soc. Exptl. Biol. Med.* **88:**31.
30. Castor, C. W. 1957. Production of mucopolysaccharides by synovial cells in a simplified tissue culture medium. *Proc. Soc. Exptl. Biol. Med.* **94:**51.
31. Green, H., and D. Hamerman. 1964. Production of hyaluronate and collagen by fibroblast clones in culture. *Nature.* **201:**710.
32. Vaubel, E. 1933. The form and function of synovial cells in tissue cultures. II. The production of mucin. *J. Exptl. Med.* **58:**85.
33. Davidson, E. H., V. G. Allfrey, and A. E. Mirsky. 1963. Gene expression in differentiated cells. *Proc. Natl. Acad. Sci.* **29:**53.
34. Grossfeld, H. 1962. Production of hyaluronic acid in tissue culture of Rous sarcoma. *Nature.* **196:**782.
35. Saito, H., T. Yomagata, and S. Suzuki. 1968. Enzymatic methods for the determination of small quantities of isomeric chondroitin sulfate. *J. Biol. Chem.* **243:**1536.
36. Rosenberg, L., M. Schubert, and J. Sandson. 1967. The proteinpolysaccharides of bovine nucleus pulposus. *J. Biol. Chem.* **242:**4691.
37. Seno, N., K. Meyer, B. Anderson, and P. Hoffman. Variations in keratosulfates. *J. Biol. Chem.* **240:**1005.
38. Hoffman, P., T. A. Mashburn, Jr., K. Meyer, and B. A. Bray. 1967. Proteinpolysaccharide of bovine cartilage. I. Extraction and electrophoretic studies. *J. Biol. Chem.* **242:**3799.
39. Silberberg, M., and R. Silberberg. 1961. Aging changes in cartilage and bone. *In* Structural Aspects of Aging. G. H. Bourne, editor. Hafner, New York. 86.
40. Hass, G. M. 1956. Pathological calcification. *In* Biochemistry and Physiology of Bone. G. H. Bourne, editor. Academic Press, New York. 802.
41. Farber, S. J., and M. Schubert. 1957. The binding of cations by chondroitin sulfate. *J. Clin. Invest.* **36:**1715.
42. Abercrombie, M., and E. J. Ambrose. 1962. The surface properties of cancer cells: A review. *Cancer Res.* **22:**525.
43. Schmidt, M. 1962. Fractionation of acid mucopolysaccharides on DEAE-Sephadex anion exchanger. *Biochim. Biophys. Acta.* **63:**346.

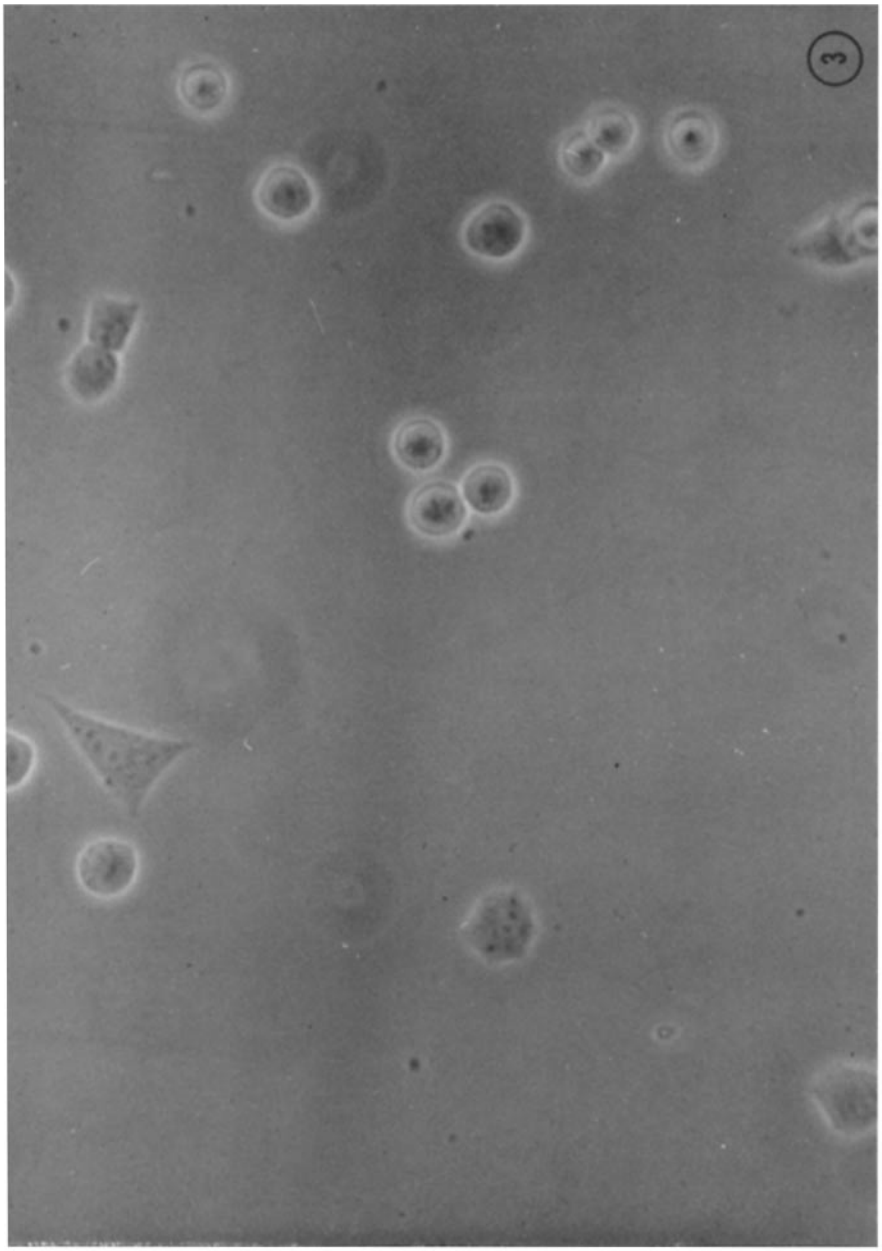

Fig. 3. Typical chondrocyte culture. Rounded, differentiated cells with a fibroblastic cell at upper border. × 300.