

INFLUENCE OF EXTERNAL POTASSIUM ON THE SYNTHESIS AND DEPOSITION OF MATRIX COMPONENTS BY CHONDROCYTES IN VITRO

JON C. DANIEL, ROBERT A. KOSHER, JAMES E. HAMOS,
and JAMES W. LASH

From the Department of Anatomy, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19174. Dr. Daniel's present address is the Department of Anatomy, Northwestern University, Chicago, Illinois 60611. Dr. Kosher's present address is the Department of Anatomy, University of Connecticut Health Center, Farmington, Connecticut 06032.

ABSTRACT

The effect of a high external potassium concentration on the synthesis and deposition of matrix components by chondrocytes in cell culture was determined. There is a twofold increase in the amount of chondroitin 4- and 6-sulfate accumulated by chondrocytes grown in medium containing a high potassium concentration. There is also a comparable increase in the production of other sulfated glycosaminoglycans (GAG) including heparan sulfate and uncharacterized glycoprotein components. The twofold greater accumulation of GAG in the high potassium medium is primarily the result of a decrease in their rate of degradation. In spite of this increased accumulation of GAG, the cells in high potassium fail to elaborate appreciable quantities of visible matrix, although they do retain the typical chondrocytic polygonal morphology. Although most of the products are secreted into the culture medium in the high potassium environment, the cell layer retains the same amount of glycosaminoglycan as the control cultures. The inability of chondrocytes grown in high potassium to elaborate the typical hyaline cartilage matrix is not a consequence of an impairment in collagen synthesis, since there is no difference in the total amount of collagen synthesized by high potassium or control cultures. There is, however, a slight increase in the proportion of collagen that is secreted into the medium by chondrocytes in high potassium. Synthesis of the predominant cartilage matrix molecules is not sufficient in itself to ensure that these molecules will be assembled into a hyaline matrix.

Cartilage matrix consists predominantly of proteoglycans and collagen. The *in vitro* phenotypic expression of these macromolecules can be manipulated by altering the potassium concentration of the culture medium in which cartilage cells are grown.

It was recently demonstrated that *in vitro* chondrogenic differentiation of embryonic chick somites can be profoundly affected by the potassium concentration of the culture medium (Lash, 1972; Lash et al., 1973). There is a striking initial accumulation of chondroitin sulfate in somites

grown in media containing a higher potassium concentration. A difficulty in studying the influence of external ions on chondrogenic differentiation in the somite system is that the somite is a heterogeneous tissue, containing presumptive cartilage, muscle, dermis, and neural crest derivatives, and that therefore, that type of experiment lacks the convenience of a homogeneous cell system.

Manipulations of expression by specific ionic influences can be more readily studied in a homogeneous chondrocyte cell culture. Using chick sternal chondrocytes, we have shown that there is a twofold increase in the amount of chondroitin 4- and 6-sulfate accumulated in the medium when chondrocytes are grown in a high potassium environment. This increase is primarily due to a decrease in the rate of sulfated glycosaminoglycan (GAG) degradation. In spite of the increase in the amount of sulfated GAG accumulated, the cells in the high potassium medium fail to elaborate appreciable quantities of visible matrix. Most of the products synthesized are secreted into the culture medium in the high potassium environment, whereas the cell layer retains the same amount of GAG as the control cultures.

MATERIALS AND METHODS

Cell Culture and Media

Chondrocytes were obtained by dissociation of 13-day White Leghorn embryonic chick sterna as previously described (Coon, 1966; Marzullo and Lash, 1970). The freshly liberated chondrocytes were plated at an initial density of 1.5×10^6 cells per 100-mm plastic petri dish (19 cells/mm²) containing 8 ml of medium. The control culture medium was Ham's F12X (Marzullo and Lash, 1970), which is Ham's (1965) F12 medium containing twice the concentration of amino acids and pyruvate (Grand Island Biological Company, Grand Island, N. Y.). The medium was supplemented with 10% fetal calf serum (Grand Island Biological Company), and 1% bovine albumin fraction V (Miles Laboratories, Inc., Kankakee, Ill.).

To determine the effect of the external potassium concentration on chondrogenesis, a modified F12X medium was prepared in which all of the sodium salts (except sodium bicarbonate and sodium pyruvate) were replaced with potassium salts, giving a total potassium concentration of 134.0 mM/l (see Appendix). The potassium concentration of the "high K" culture medium used in the experiments was 68.4 mM/l, which was prepared by diluting the modified F12X medium 1:1 with F12X (control) medium. The potassium concentration of the F12X (control) medium was 3.0 mM/l (see Appendix). After 24 h of growth in 3 mM K⁺ (F12X) medium,

chondrocyte, were supplied with the 68.4 mM K⁺ (high K) medium and were fed daily by replacing 0.5 of the medium with fresh medium. Cultures were maintained in a humid atmosphere of 95% air-5% CO₂ for periods up to 7 days, depending upon the experimental design.

Analysis of Glycosaminoglycan Synthesis

INCORPORATION OF PRECURSORS INTO GLYCOSAMINOGLYCANS. 5 days after initial plating, the cells were exposed to fresh media containing either 5 μ Ci/ml of Na₂³⁵SO₄ (carrier-free, Amersham/Searle Corp., Arlington Heights, Ill.), or 3 μ Ci/ml of D-[¹⁴C]glucosamine hydrochloride (4.8 mCi/mmol; New England Nuclear, Boston, Mass.). After 12 h of incubation in the presence of the appropriate isotope, the medium was decanted and stored at -20°C before analysis. The cells were scraped from the dish after several washes with Simms' (1942) balanced salt solution, and GAG were extracted by methods previously described (Daniel et al., 1973).

CHARACTERIZATION OF [³⁵S]SULFATE-LABELED GLYCOSAMINOGLYCANS: The relative amounts of chondroitin 4-sulfate, 4-sulfate, and dermatan sulfate synthesized by cell cultures incubated in the presence of Na₂³⁵SO₄ were determined by the enzymatic method of Saito et al. (1968) using chondroitinase ABC and AC (Miles Laboratories, Inc.) as previously described (Kosher and Searls, 1973). The relative amount of [³⁵S]sulfate-labeled heparan sulfate and/or heparin was determined as described by Kosher and Searls (1973) utilizing nitrous acid degradation. Nitrous acid will specifically degrade the N-sulfated GAG heparan sulfate, and heparin (Lagunoff and Warren, 1962; Cifonelli, 1968 a, b; Lindahl, 1970; Kraemer, 1971 a, b).

CHARACTERIZATION OF [¹⁴C]GLUCOSAMINE-LABELED GLYCOSAMINOGLYCANS: The amount of hyaluronic acid synthesized by cultures incubated with [¹⁴C]glucosamine was determined by utilizing leech hyaluronidase (hyaluronic acid β 1,3 hydrolase; Biotrics, Inc., Arlington, Mass.), an enzyme which specifically degrades hyaluronic acid. Aliquots (100 μ l) of the [¹⁴C]glucosamine-labeled GAG samples were incubated for 4 h at 37°C with 25 μ g of leech hyaluronidase in standard McIlvaine's buffer. The final volume of the reaction mixtures was 0.4 ml at a pH of 5.6. Additional enzyme (6 μ g) was added twice during the incubation period to assure complete digestion. Control reaction mixtures were incubated without enzyme. After treatment, reaction mixtures were placed in a boiling water bath for 3 min, cooled, and supplied with 250 μ g of carrier hyaluronic acid (Sigma Chemical Co., St. Louis, Mo.) and 125 μ g each of carrier chondroitin 4- and 6-sulfate (Miles Laboratories). Undegraded GAG was then precipitated by the addition of 5 mg of cetylpyridinium chloride (CPC) (Toole and Gross, 1971). After centrifugation, the precipitates were dissolved in methanol and supplied with Bray's scintillation fluid

(Bray, 1960), and radioactivity was determined. The radioactivity in the supernates was assayed with the scintillation fluid containing 15% Bio-Solv. In this manner, the amount of leech hyaluronidase-sensitive material (material soluble in CPC after hyaluronidase treatment, i.e. hyaluronic acid) was compared with the amount of leech hyaluronidase-resistant material (material precipitable with CPC after hyaluronidase treatment). These determinations were corrected for radioactivity, not CPC-precipitable, before enzyme treatment, i.e. the amount of untreated material soluble in CPC.

The amount of chondroitin sulfate synthesized by cultures incubated with [¹⁴C]glucosamine was determined by the following procedure. Aliquots (100 μl) of the [¹⁴C]glucosamine-labeled GAG samples were treated with 0.2 units of chondroitinase ABC in "enriched" Tris buffer, pH 8.0 (Saito et al., 1968) for 3 h at 37°C, and the amount of radioactivity associated with chondroitinase-sensitive and resistant material was determined after precipitation with CPC. Since chondroitinase ABC will degrade hyaluronic acid as well as the isomeric chondroitin sulfates, the amount of hyaluronic acid (i.e. leech hyaluronidase-sensitive GAG), was subtracted from the amount of chondroitinase-sensitive GAG to determine the amount of radioactivity associated with chondroitin sulfate. Data were corrected for the amount of radioactivity not precipitable with CPC before enzyme treatment.

Analysis of Collagen Synthesis

PREPARATION OF PROTEASE-FREE COLLAGENASE: Collagenase free of noncollagen proteolytic activity was prepared by gel filtration of partially purified Worthington collagenase (code no. CLSPA) (Worthington Biochemical Corp., Freehold, N. J.), on Sephadex G-200 by a modification of the procedure of Peterkofsky and Diegelmann (1971), as described by Daniel et al. (1973). This purified collagenase failed to degrade any [³H]tryptophane-labeled protein, and was lyophilized and stored at -20°C for future use.

DETERMINATION OF COLLAGEN AND NON-COLLAGEN PROTEIN: 5 days after initial plating, cells grown in either 3 μCi/ml of [³H]glycine (8.6 Ci/mmol; New England Nuclear) or 3 μCi/ml of [¹⁴C]proline (200 mCi/mmol; New England Nuclear). After 12 h of incubation in the presence of the appropriate isotope, total protein was extracted from the cell layers and media as described by Peterkofsky (1972) and Daniel et al. (1973). The amount of labeled collagen and noncollagen protein was determined with the protease-free collagenase as described by Peterkofsky and Diegelmann (1971) and Daniel et al. (1973).

DETERMINATION OF CELL GROWTH RATE: Growth rates were determined by removing cells daily, with 2.5% trypsin, from replicate cultures grown in F12X and high K medium. Cell counts were done with a hemacytometer.

DNA DETERMINATION: DNA was determined by the Santoianni and Ayala (1965) modification of the procedure of Kissane and Robins (1958), except that fluorescence was determined in 1 N HCl instead of 0.6 N PCA (Hinegardner, 1972).

RESULTS

Morphology

To give all cells an equal opportunity to adapt to the culture environment, all cultures were initiated in F12X medium (3.0 mM/1 K⁺). After 24 h, half of the dishes were replaced with fresh F12X medium and half were replaced with medium containing an increased potassium concentration, 68.4 mM/1 (the high K medium). The expression of the differentiated phenotype of chondrocytes in cell culture is typified by polygonal morphology and the accumulation of alcian blue positive hyaline matrix (see Coon, 1966; Marzullo and Lash, 1970). During the first 2 days of culture, there was no discernible difference between chondrocytes grown in the two media. On the 2nd day, the polygonal cells in both types of cultures began accumulating a thin rim of matrix. By the 3rd day, the cells in the F12X (control) medium had perceptibly more hyaline matrix surrounding them (Fig. 1, insets). During subsequent days in culture, the control cells continued accumulating matrix, whereas most of the cells in the high K medium never accumulated more than a thin rim of matrix (Fig. 1). By the 6th day, when the biochemical studies were performed, the control cells had formed clusters of chondrocytes embedded in matrix (Fig. 1 A). The cells in the high K medium, while retaining the chondrocytic polygonal morphology, did not form clusters and did not accumulate appreciable quantities of hyaline matrix (Fig. 1 B). From the appearance of the living cells, there is the intimation that the chondrocytes in the high K medium are impaired in their ability to produce matrix components. An alternative explanation for the appearance of the cells in the high K medium is that whereas the cells can manufacture matrix components, the coalescence of these components to form a hyaline matrix is impaired.

Growth Rate

One of the initial questions to be answered was whether the difference in the morphology and behavior of the cultures was related to any difference in rates of proliferation. Cells that were

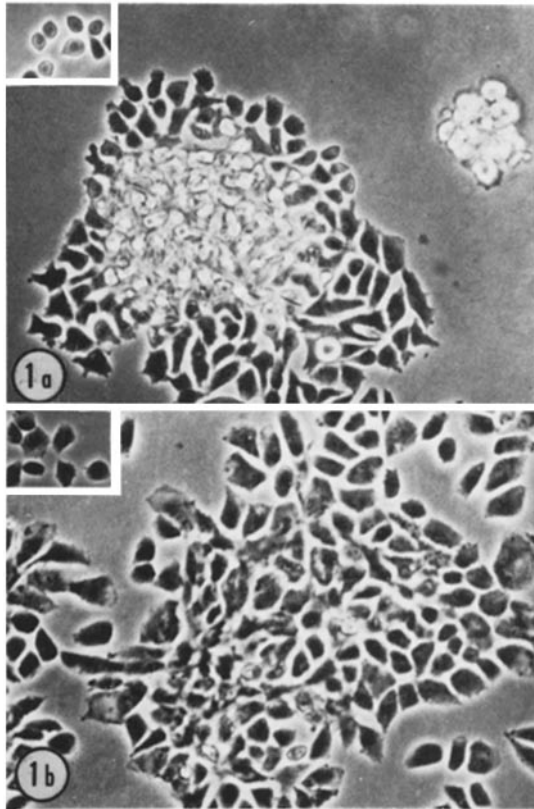


FIGURE 1 Phase micrographs showing the morphology of chondrocytes grown in F12X (3.0 mM/1 K⁺) and high K (68.4 mM/1 K⁺) medium. Fig. 1 A, F12X (control) medium, 6th day of culture (*inset* 3rd day of culture). The chondrocytes are embedded in a considerable quantity of hyaline matrix. Fig. 1 B, high K medium, 6th day of culture (*inset* 3rd day of culture). The polygonal chondrocytic morphology is retained, but the cells have not accumulated more than a thin rim of matrix. $\times 275$.

rapidly proliferating might not be able to accumulate matrix components. That is, there may be expressed here some form of antagonism between proliferation and the expression of the differentiated state. Therefore, the growth rate of cells cultured in F12X (control) or in high K medium was determined by doing cell counts at daily intervals. Duplicate counts were made on each of three series of cultures. The data presented in Fig. 2 indicate that the high K culture environment has no effect on the rate of cell division through the 6th day of culture. After the 6th day, cells grown in the high K medium entered a stationary growth phase as the monolayer of polygonal cells became confluent. Cells in the F12X (control) medium continued in the log phase for another day. This

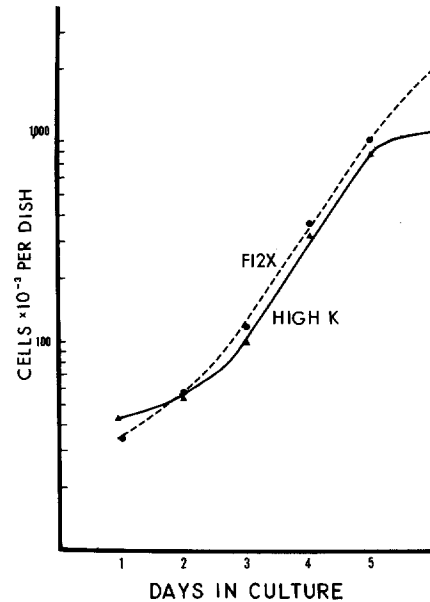


FIGURE 2 Growth rate of chondrocyte cultures in F12X and high K medium. Each point is the average of six determinations.

difference in the attainment of the stationary growth phase appears to be due to the differences in the cellular configurations of the two types of cultures. Whereas the cells in the high K medium reach a monolayer confluency, the cells in the control medium form clusters of multilayer configurations, delaying the onset of confluency.

Glycosaminoglycan Synthesis and Accumulation

In order to examine the synthesis and accumulation of matrix components under the two culture conditions, biochemical analyses were performed. The amounts of sulfated GAG accumulated by cells grown in F12X (control) and cells grown in high K medium in a typical experiment are given in Table 1. There was a twofold increase in the incorporation of [³⁵S]sulfate into sulfated GAG by cells grown in high K medium when compared to controls after a 12-h labeling period. This experiment has been repeated in duplicate six times, and the incorporation of [³⁵S]sulfate into GAG by cells grown in high K medium ranged between 1.8 and 2.3 times greater than the incorporation by cells grown in F12X medium. The increase in sulfate incorporation continued through the culture period, but data are given only for the 6th day of culture.

The distribution of [³⁵S]sulfate-labeled GAG between cell layer and medium indicated that whereas the chondrocytes grown in high K medium retained as much GAG in the cell layer as those in F12X, they secreted more into the culture media than control cells (Tables I and II). Cells grown in high K medium secreted 81% of their [³⁵S]sulfate-labeled GAG into the medium, compared to the 69% secreted by controls. Therefore, the cell layers of both control and high K cultures retained similar amounts of GAG, but control cells possessed considerably more hyaline matrix than cells grown in high K medium (Fig. 1). The intracellular [³⁵S]sulfate-labeled GAG was similar for both control and high K cultures, viz. 2–3% of the values given in Table I.

The analysis of the types of [³⁵S]sulfate-labeled GAG present in the cell layer and medium of F12X and high K cultures indicated that both types of cultures synthesized chondroitin 4-sulfate, chondroitin 6-sulfate, and chondroitinase-resistant GAG (Table III). Furthermore, comparison of radioactivity in 4- and 6-sulfated disaccharides after chondroitinase ABC and AC treatment indicated that control and high K cultures synthesized the various sulfated GAG in similar relative amounts (Table III). The only notable differences were that the cell layer of high K cultures contained less chondroitin 4-sulfate and more chondroitinase-resistant GAG than did the cell layer of F12X cultures (Table III). Approximately 60% of the chondroitinase-resistant GAG produced by both control and high K cultures was sensitive to nitrous acid, and is therefore heparan sulfate

TABLE I
Incorporation of [³⁵S]Sulfate into Glycosaminoglycans by Chondrocytes Grown in F12X (3.0 mM/1 K⁺) or in High K (68.4 mM/1 K⁺) Medium

| Culture | Distribution | dpm/μg | |
|---------|--------------|---------|-------------|
| | | DNA* | high K/F12X |
| F12X | total | 75,533 | 2.0 |
| | cell layer | 23,413 | 1.1 |
| | medium | 52,120 | 2.3 |
| High K | total | 147,439 | — |
| | cell layer | 28,003 | — |
| | medium | 119,436 | — |

* This experiment has been repeated in duplicate six times, and the incorporation of [³⁵S]sulfate into GAG by cells grown in high K medium ranged between 1.8 and 2.3 times greater than the incorporation by cells grown in F12X (control) medium.

TABLE II
Distribution of [³⁵S]Sulfate-Labeled Glycosaminoglycans Between the Cell Layer and Medium of F12X (3.0 mM/1 K⁺) or in High K (68.4 mM/1 K⁺) Cultures

| Culture | Cell layer* | Medium* |
|---------|-------------|---------|
| | % | (%) |
| F12X | 31 | 69 |
| high K | 19 | 81 |

* Each value is the average of 12 determinations. Standard deviation is ± 1–2 in each case.

TABLE III
Relative Amounts of the Various [³⁵S]Sulfate-Labeled Glycosaminoglycans (GAG) Present in the Cell Layer and Medium of Chondrocytes Grown in F12X (3.0 mM/1 K⁺) or in High K (68.4 mM/1 K⁺) Medium as Determined by Chondroitinase Analysis

| Glycosaminoglycan | F12X cultures | | High K cultures | |
|---------------------------|---------------|--------|-----------------|--------|
| | cell layer | medium | cell layer | medium |
| | % | % | % | % |
| Chondroitin 4-sulfate* | 53 | 56 | 40 | 50 |
| Chondroitin 6-sulfate† | 38 | 35 | 37 | 40 |
| Chondroitinase-resistant‡ | 9 | 9 | 23 | 10 |

* Determined as the relative amount of radioactivity associated with 4-sulfated disaccharide residues after chondroitinase ABC and AC treatment.

† Relative amount of radioactivity associated with 6-sulfated disaccharide residues after chondroitinase ABC and AC treatment.

‡ [³⁵S]sulfate-labeled GAG which is not degraded by chondroitinase ABC or AC.

and/or heparin. The very small amount of chondroitinase-resistant, nitrous acid-resistant material has not been characterized, but is very probably keratan sulfate (see Shulman and Meyer, 1970) or sulfated glycoprotein (see Slomiany and Meyer, 1972).

In order to determine whether the increased incorporation of [³⁵S]sulfate into glycosaminoglycans by high K cultures represents synthesis and not just sulfation of pre-existing molecules, the incorporation of [¹⁴C]glucosamine into sulfated GAG was determined. The data presented in Table IV is from a typical experiment and shows that the total incorporation of [¹⁴C]glucosamine into GAG by high K cultures was 1.6 times greater than controls. The incorporation of [¹⁴C]glucosamine into chondroitin sulfates was 1.6 times greater than controls, and the incorporation into chondroitinase-resistant GAG was 1.8 times greater than controls. The incorporation of [¹⁴C]glucosamine into GAG has been determined in duplicate four times, and the incorporation by high K cultures ranged between 1.5 and 1.9 times greater than controls. Therefore, cells grown in high K medium show not only an increase in [³⁵S]sulfate incorporation, but also a comparable increase in [¹⁴C]glucosamine incorporation into sulfated

GAG. The marked increase in the amount of chondroitin sulfate in the high K culture medium was observed using [¹⁴C]glucosamine as precursor (Table V).

The amount of [¹⁴C]glucosamine-labeled hyaluronic acid present in the cell layer and medium of F12X and high K cultures was determined with leech hyaluronidase. There is little difference in the total amount of hyaluronic acid synthesized by control and high K cultures, or in the distribution of [¹⁴C]glucosamine-labeled hyaluronic acid between the cell layer and medium (Tables IV and V). In cultures of both types, the amount of hyaluronic acid synthesized relative to other GAG is quite small, i.e. 4–6% of the total [¹⁴C]glucosamine incorporation (Table IV). The percentage of hyaluronic acid retained by the cell layer of both F12X and high K cultures is considerably greater (47%) than the percentage of sulfated GAG retained by the cell layer of F12X (33%) and high K (19%) cultures (Table V).

The amount of hyaluronic acid synthesized by [¹⁴C]glucosamine-treated cultures was determined by precipitation of undegraded glycosaminoglycans after treatment with leech hyaluronidase. It was observed that whereas 98–100% of untreated or hyaluronidase-treated [³⁵S]sulfate-labeled GAG

TABLE IV
Incorporation of [¹⁴C]Glucosamine into Various Glycosaminoglycans (GAG) by Chondrocytes Grown in F12X (3.0 mM/1 K⁺) or in High K (68.4 mM/1 K⁺) Medium

| Glycosaminoglycan | Culture | dpm/μg DNA* | percent of total | high K/F12X |
|---|---------|-------------|------------------|-------------|
| Total [¹⁴ C]glucosamine-labeled GAG | F12X | 10,420 | 100 | 1.6 |
| | high K | 16,672 | 100 | |
| Chondroitin sulfate‡ | F12X | 6,366 | 61 | 1.6 |
| | high K | 9,920 | 60 | |
| Chondroitinase-resistant§ | F12X | 900 | 9 | 1.8 |
| | high K | 1,624 | 10 | |
| Hyaluronic acid | F12X | 634 | 6 | 1.1 |
| | high K | 710 | 4 | |
| Glycoprotein¶ | F12X | 2,478 | 24 | 1.7 |
| | high K | 4,248 | 25 | |

* Total amount of [¹⁴C]glucosamine-labeled GAG in the cell layer and medium.

‡ Amount of [¹⁴C]glucosamine-labeled material soluble in cetylpyridinium chloride (CPC) after chondroitinase ABC treatment. Since chondroitinase will degrade hyaluronic acid as well as the isomeric chondroitin sulfates, the amount of [¹⁴C]glucosamine-labeled hyaluronic acid (i.e. leech hyaluronidase-sensitive GAG), was subtracted from the total amount of chondroitinase-sensitive GAG to obtain the incorporation into chondroitin sulfate. Data are also corrected for no enzyme control values, i.e. the amount of untreated material soluble in CPC.

§ Amount [¹⁴C]glucosamine-labeled GAG precipitable with CPC after chondroitinase ABC treatment.

|| Amount of [¹⁴C]glucosamine-labeled GAG soluble in CPC after leech hyaluronidase treatment. Data corrected for no enzyme control values.

¶ Amount of untreated [¹⁴C]glucosamine-labeled material soluble in CPC.

TABLE V
Distribution of the Various [¹⁴C]Glucosamine-Labeled Glycosaminoglycans (GAG) Between the Cell Layer and Medium of F12X (3.0 mM/1 K⁺) or in High K (68.4 mM/1 K⁺) Cultures

| Glycosaminoglycan | Culture | Cell layer | | Medium | |
|--------------------------|---------|------------|-------|------------|-------|
| | | dpm/μg DNA | | dpm/μg DNA | |
| | | % | | % | |
| Chondroitin sulfate | F12X | 33 | 2,101 | 67 | 4,265 |
| | high K | 19 | 1,884 | 81 | 8,036 |
| Chondroitinase-resistant | F12X | 33 | 297 | 67 | 603 |
| | high K | 31 | 503 | 69 | 1,121 |
| Hyaluronic acid | F12X | 47 | 298 | 53 | 336 |
| | high K | 47 | 334 | 53 | 376 |
| Glycoprotein | F12X | 41 | 1,015 | 59 | 1,463 |
| | high K | 32 | 1,359 | 68 | 2,889 |

was precipitable with CPC, only approximately 75% of the untreated [¹⁴C]glucosamine-labeled material from cultures of each type was precipitable. In other words, approximately 25% of the [¹⁴C]glucosamine-labeled material produced by both F12X (control) and high K cultures is nonsulfated, protease resistant, TCA soluble, nondialyzable, and CPC soluble. This CPC-soluble material has not yet been completely characterized, but very probably represents the polysaccharide chains of glycoproteins. Preliminary data indicating that this material contains glucosamine but not galactosamine and lacks uronic acid are consistent with this possibility. Similar material from chondrocyte cultures derived from limb anlagen has been observed by Kvist (1973). While there is almost a twofold increase in the amount of this [¹⁴C]glucosamine-labeled "glycoprotein" produced by cells grown in high K medium (Table IV), the cell layer contains approximately the same amount as the control cultures (Table V). Cells grown in high K medium secrete more of this "glycoprotein" into the culture medium than do controls (Table V).

Degradation of Sulfated GAG

Since it is possible that the increased accumulation of GAG by the high K cultures could result from a decreased rate of degradation rather than an increased synthesis, the rate of sulfated GAG degradation was determined. High K and control cells were cultured for 5 days and then transferred to [³⁵S]sulfate-containing medium for 12 h. The radioactive medium was then transferred to culture dishes (without cells), and the remaining radioactive cells were washed with excess SBSS. The high K and control cell layers were supplied

with nonradioactive medium of the appropriate constitution. At time 0, and at 6-h intervals, the amount of [³⁵S]sulfate-labeled GAG remaining in the original radioactive medium, which had been transferred to new culture dishes, was determined. At similar intervals, the amount of labeled GAG associated with the prelabeled cell layers (after transfer to nonradioactive medium) was also determined. The total amount of labeled GAG remaining during the chase in nonradioactive medium was determined by adding the values for the cell layer plus the medium.

The rate of disappearance (degradation) of previously labeled GAG in the original medium of the two types of cultures is shown in Fig. 3. It is evident that medium-associated GAG is degraded at a slower rate (approximately twice as slow) in the high K environment. While only 38% of the originally labeled GAG remained in the control medium after a 12-h chase, 70% of the labeled GAG remained in the high K medium (Fig. 3).

The rate of disappearance of labeled GAG from the prelabeled cell layers is shown in Fig. 4. Note that the rate of degradation in the cell layers was followed between 6 and 18 h, rather than between time 0 and 12 h. This was done because a considerable pool of TCA-soluble [³⁵S]sulfate remained at time 0, despite extensive rinsing, but then was rapidly used up, with only traces remaining after 6 h. That this TCA-soluble [³⁵S]sulfate pool was being utilized for synthesis during the first 6 h was indicated by the fact that the amount of [³⁵S]sulfate-labeled GAG doubled between time 0 and 6 h. After the pool of radioactive sulfate was used up, the rate of degradation for the next 12 h was much slower in the high K medium than in the control medium (Fig. 4). Therefore, the two to

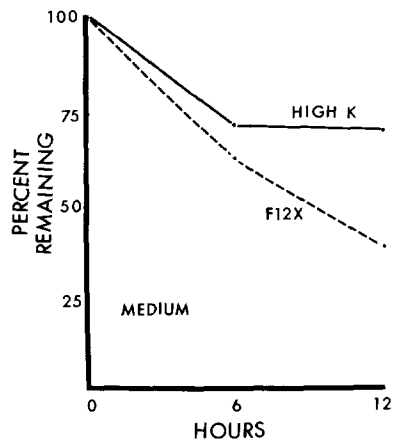


FIGURE 3 Percentage of previously labeled glycosaminoglycan remaining in control (F12X) and high K medium after transfer of medium to new culture dishes in the absence of cells. See text for details of experiment.

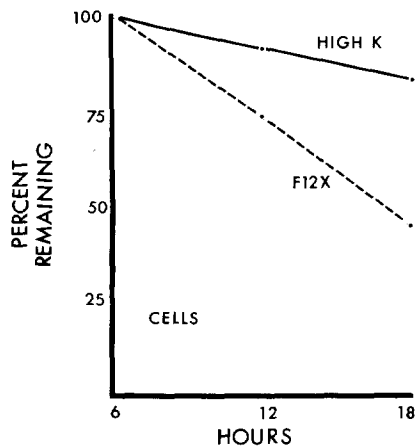


FIGURE 4 Percentage of labeled glycosaminoglycan remaining in prelabeled cell layers after chase in unlabeled medium. Values represent the total amount of labeled GAG in the cell layer and medium of these cultures. See text for details of experiments.

threefold greater accumulation of sulfated GAG in the high K medium is due primarily, if not solely, to a two- to threefold decrease in the rate of degradation.

Collagen Synthesis

The analysis of glycosaminoglycan synthesis indicated that cells grown in high K medium accumulated more GAG than control cells. Since another major component of cartilage matrix is collagen, the synthesis of collagen and noncollagen

protein was examined in cultures of each type to determine if the inability of high K cells to elaborate a matrix could be related to an impairment of collagen synthesis. The data presented in Table VI demonstrates that there was very little difference in the total amount of collagen per DNA synthesized by control cells or cells grown in high K medium using either [³H]glycine or [¹⁴C]proline as precursor. Similarly, there was little difference in the total amount of collagen synthesis relative to noncollagen protein synthesis in either F12X (control) or high K cultures (Table VI). There was however, a slight increase in the amount of collagen that was secreted into the medium by high K cells.

DISCUSSION

The results of this investigation indicate that a high extracellular potassium concentration has several striking effects on the accumulation and deposition of matrix components by chondrocytes in cell culture. There is a twofold increase in the amount of sulfated GAG accumulated by chondrocytes grown in medium containing a high potassium concentration. This increased accumulation is due primarily to a decreased rate of degradation of sulfated GAG. The rate of degradation of sulfated GAG is twice as fast in control (F12X) medium as that in the high K medium. Presumably, a high extracellular potassium concentration affects the activity of enzymes involved in the degradation of sulfated GAG. It is apparent from our results that the accumulation of matrix components can be enhanced by the inhibition of GAG degradation. This raises the interesting point of ionic influences on degradative enzyme activity. Although potassium has been shown to have marked effects upon membrane permeability and secretion (Douglas, 1968), we are not aware of any published work on the effect of potassium on degradative enzymes. It is tempting to relate these potassium-related changes in sulfated GAG degradation with some connective tissue diseases which are marked by aberrant degradation patterns. Some mucopolysaccharidoses are characterized by a decreased degradation of GAG (Fratantoni et al., 1968; Neufeld and Cantz, 1971; Matalon and Dorfman, 1972), and the disease osteoarthritis is characterized by an increased degradation of these molecules (Woessner, 1973; Sapolsky et al., 1973; Ali and Evans, 1973).

It has been reported that fully differentiated

TABLE VI
Incorporation of [³H]Glycine and [¹⁴C]Proline into Collagen and Noncollagen Protein (NCP) by Chondrocytes Grown in F12X (3.0 mM/1 K⁺) or in High K (68.4 mM/1 K⁺) Medium as Determined by Analysis with Protease-Free Collagenase

| Precursor | Culture | Collagen* dpm/μg DNA | Noncollagen protein† dpm/μg DNA | Relative collagen synthesis‡ | Collagen in cell layer % |
|---------------------------|---------|-------------------------|------------------------------------|---------------------------------|--------------------------------|
| [³ H]Glycine | F12X | 2,513 | 37,376 | 6.3 | 59 |
| | high K | 2,402 | 34,552 | 6.5 | 50 |
| [¹⁴ C]Proline | F12X | 675 | 11,598 | 5.5 | 62 |
| | high K | 640 | 10,788 | 5.6 | 51 |

* Total amount of material in the cell layer and medium which is soluble in 5% TCA-0.25% tannic acid after treatment with protease-free collagenase.

† Total amount of material in the cell layer and medium which is precipitable with 5% TCA-0.25% tannic acid after treatment with protease-free collagenase. The amount of [¹⁴C]proline-labeled noncollagen protein (NCP) was multiplied by 5.4, since collagen is 5.4 times more enriched in proline and hydroxyproline than an average protein (see Peterkofsky and Diegelmann, 1971). For the same reason, the amount of [³H]glycine NCP was multiplied by 4.0.

‡ Calculated as follows:

$$\frac{\text{collagen (dpm/}\mu\text{g DNA)}}{[\text{NCP (dpm/}\mu\text{g DNA)} \times F] + \text{collagen (dpm/}\mu\text{g DNA)}} \times 100$$

F = 5.4 with [¹⁴C]proline as precursor and 4.0 with [³H]glycine as precursor.

cartilage cells, when cultured with various agents, will lose their typical chondrocytic phenotype, i.e. polygonal morphology and extracellular matrix. Agents such as embryo extract (Coon and Cahn, 1966), serum (Marzullo and Lash, 1970), and 5-bromodeoxyuridine (Abbott and Holtzer, 1968) promote the loss of both of these specialized characteristics. The loss of chondrocytic phenotype under the influence of BrdU is accompanied by quantitative and qualitative alterations in glycosaminoglycan and collagen synthesis (Holthausen et al., 1969; Schiltz et al., 1973; Daniel et al., 1973), and the loss of phenotype in embryo serum-treated cells is accompanied by the loss of metabolic intermediates necessary for the synthesis of glycosaminoglycans (Marzullo and Lash, 1970). The lack of matrix production in these instances when glycosaminoglycan and collagen synthesis are impaired is not surprising, since the synthesis of both of these classes of macromolecules is required for the formation of cartilage matrix.

The results of the present investigation indicate that chondrocytes grown in a high potassium environment fail to accumulate appreciable quantities of hyaline matrix despite the fact that they are not impaired in their ability to synthesize glycosaminoglycans and collagen. High K and F12X cultures synthesize chondroitin 4-sulfate,

chondroitin 6-sulfate, chondroitinase-resistant GAG (predominately heparan sulfate), hyaluronic acid, and glycoproteins in similar relative amounts. Although most of the products of the increased accumulation are found in the culture medium in the high K environment, similar amounts of GAG are associated with the cell layers of both high K and control cultures. The amount of this cell layer-associated material which is intracellular (2-3%) is the same in cultures of each type. Although the high K and control cells have similar amounts of *intracellular* and *extracellular* sulfated GAG, the extracellular material in the high K cultures is neither visually nor histochemically detectable as hyaline cartilage. It is possible that visual and histological observations may be deceiving as a result of the differences in the configurations in the two types of cell cultures. Control cells form clusters with easily detectable hyaline matrix, whereas the high K cells remain as monolayers throughout the culture period. The higher degree of refractility of the matrix in the clusters would give the appearance of more hyaline matrix. From our observations however, this is not the case. Even by the 3rd day of culture, when high K and control cells were both sparsely populated, the control cells were contained in a perceptibly more prominent hyaline capsule (see insets, Fig. 1 A, B). Repeated visual observations

with phase microscopy, even by naïve observers, established the fact that the control cells were encapsulated in a typical, refractile hyaline cartilage matrix. The "matrix" of the high K cells was detected neither visually nor histochemically as hyaline, but yet existed as demonstrated biochemically. Ultrastructural studies may be capable of demonstrating the proteoglycan granules and thin, unbanded collagen fibrils which are detected biochemically (see Minor, 1973). The conclusion is that in the high K environment the matrix components are unable to coalesce into an extracellular hyaline matrix, or that a normal matrix is needed for appropriate cell aggregation. This suggests that the proper extracellular ionic environment, or unknown factors that are sensitive to the ionic environment, are required for the normal deposition of cartilage matrix components into the typical hyaline matrix; and that the synthesis of the predominant cartilage matrix molecules is not sufficient in itself to ensure that these molecules will be assembled into a hyaline matrix.

Since the extracellular sulfated GAG in the high K environment is not constituted as hyaline cartilage matrix, its localization must be considered. The observation that there can exist a considerable quantity of extracellular GAG which is not coalesced into a hyaline matrix is not unprecedented. For example, 15–25% of the sulfated GAG synthesized by normal and transformed 3T3 fibroblasts is cell associated, and is certainly not associated with a hyaline matrix (Goggins et al., 1972). It is possible that this material is associated with the cell membrane. Chondroitin sulfate has been shown to be a constituent of cell surfaces (Suzuki et al., 1970; Kojima and Yamagata, 1971). Kramer (1971 *a, b*) has demonstrated that heparan sulfate is also a constituent of the surfaces of a wide variety of cells. In this respect, it is of interest that there is a considerable increase in the amount of heparan sulfate associated with the cell layer in the high K cultures.

In considering the reason why chondrocytes grown in a high potassium environment fail to elaborate appreciable quantities of matrix, a number of other possibilities must be taken into account. The polysaccharide chains of the glycosaminoglycans (proteoglycans) are covalently bound to a protein core. Although we have been unable to detect any significant difference in the types or relative amounts of the various glycosaminoglycans synthesized by high K or control cul-

tures, we cannot eliminate the possibility that the polysaccharide chains of the glycosaminoglycans produced by high K cultures are attached to a different protein core than the polysaccharide chains of control cultures. Possible differences in the number of polysaccharide chains attached to the protein core, or in the length of the polysaccharide chains, must also be taken into consideration. Similarly, although there is not a quantitative impairment in collagen synthesis in the high K environment, an extensive qualitative analysis of collagen synthesis has not yet been undertaken. Chick cartilage contains a unique type of collagen consisting of three $\alpha 1$ type II chains (Miller and Matukas, 1969), while the collagen of most vertebrate tissues, including dermis and bone, consists of two $\alpha 1$ type I chains and one $\alpha 2$ chain (Miller et al., 1967; Kang et al., 1969). Consideration must, therefore, be given to the possibility that chondrocytes in high K are impaired in their ability to synthesize cartilage-specific collagen. Preliminary evidence, however, indicates that high K cultures produce predominantly $\alpha 1$ collagen chains (Daniel, Kosher, and Lash, unpublished observations). Finally, other molecules which may be involved in the maintenance of proteoglycans and collagen in an extracellular matrix must be considered. For example, it has been demonstrated by Hascall et al. (1969) that there exist in the matrix of bovine nasal cartilage glycoprotein link molecules which maintain proteoglycan molecules in an aggregated complex. It will, therefore, be important to examine the synthesis of these glycoprotein link molecules in high K and control chondrocyte cultures.

The mechanism by which a high extracellular K concentration affects sulfated glycosaminoglycan synthesis and matrix deposition, and the role of K in influencing the chondrocytic phenotype *in vivo* are unknown. It is of interest that Howell et al. (1960) have observed that the K concentration of the zone of hypertrophic (diaphyseal) cartilage at the osteochondral junction of costal cartilage is three times greater than the K concentration of the proliferating (epiphyseal) and resting (articular) zones. It has recently been observed by Kvist (1973) that the rate of chondroitin sulfate accumulation is much greater in hypertrophic diaphyseal cartilage than in either epiphyseal or articular cartilage. An additional correlation between a high K concentration and connective tissue synthesis is seen in the healing of skin wounds in humans. Shortly after injury the wound area shows a rise in K concentration, the result of cell lysis, which is

coincident with the infiltration of synthetically active connective tissue elements (Leif Andersson, University of Helsinki, personal communication).

Regardless of the mechanism of action of K, the results of the present investigation establish that the accumulation of extracellular hyaline cartilage matrix and the synthesis of matrix components are not necessarily coupled. Of the glycosaminoglycans synthesized in the high K environment, there is a disproportionate increase in chondroitinase-resistant material (presumably heparan sulfate and keratosulfate), and an unidentified "glycoprotein". It is possible, but speculative, that this may be a factor in the inability of the high K cells to produce hyaline matrix. Thus, the full expression of the chondrocytic phenotype requires not only the synthesis of matrix macromolecules, but also the coalescence of these macromolecules into hyaline matrix. The synthesis of the predominant cartilage matrix molecules is not sufficient in itself to ensure that a normal matrix will be formed. Manipulations of the chondrocytic phenotype by altering the K concentration of the culture medium may provide a tool for studying the control mechanisms involved in the synthesis and deposition of cartilage matrix components.

This work was supported by grant HD 00380 from the United States Public Health Service to James W. Lash, and by United States Public Health Service postdoctoral fellowships to Jon C. Daniel and Robert A. Kosher.

Received for publication 30 July 1973, and in revised form 8 August 1974.

REFERENCES

- ABBOTT, J., and H. HOLTZER. 1968. The loss of phenotypic traits by differentiated cells. V. The effects of 5-bromodeoxyuridine on cloned chondrocytes. *Proc. Natl. Acad. Sci. U. S. A.* **59**:1144.
- ALI, S. Y., and L. EVANS. 1973. Enzymatic degradation of cartilage in osteoarthritis. *Fed. Proc.* **32**:1494.
- BRAY, G. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Anal. Biochem.* **1**:279.
- CIFONELLI, J. A. 1968 *a*. Reaction of heparitin sulfate with nitrous acid. *Carbohydr. Res.* **8**:233.
- CIFONELLI, J. A. 1968 *b*. Structural features of acid mucopolysaccharides. In *The Chemical Physiology of Mucopolysaccharides*. G. Quintarelli, editor. Little, Brown & Co., Inc., Boston, Mass. 91.
- COON, H. G. 1966. Clonal stability and phenotypic expression of chick cartilage cells *in vitro*. *Proc. Natl. Acad. Sci. U. S. A.* **55**:66.
- COON, H. G., and R. CAHN. 1966. Differentiation *in vitro*: effects of Sephadex fractions of chick embryo extract. *Science (Wash. D. C.)*. **153**:1116.
- DANIEL, J. C., R. A. KOSHER, J. W. LASH, and J. HERTZ. 1973. The synthesis of matrix components by chondrocytes in the presence of 5-bromodeoxyuridine. *Cell Diff.* **2**:285.
- DOUGLAS, W. W. 1968. Stimulus-secretion coupling: the concept and clues from chromaffin and other cells. *Br. J. Pharmacol.* **34**:451.
- FRATANTONI, J. C., C. W. HALL, and E. F. NEUFELD. 1968. The defect in Hurler's and Hunter's syndromes: faulty degradation of mucopolysaccharide. *Proc. Natl. Acad. Sci. U. S. A.* **60**:699.
- GOGGINS, J. F., G. S. JOHNSON, and I. PASTAN. 1972. The effect of dibutyryl cyclic adenosine monophosphate on the synthesis of sulfated acid mucopolysaccharides by transformed fibroblasts. *J. Biol. Chem.* **247**:5759.
- HAM, R. G. 1965. Clonal growth of mammalian cells in a chemically defined synthetic medium. *Proc. Natl. Acad. Sci. U. S. A.* **53**:288.
- HASCALL, V. C., and S. W. SADJERA. 1969. Protein-polysaccharide complex from bovine nasal cartilage. The function of glycoprotein in the formation of aggregates. *J. Biol. Chem.* **244**:2384.
- HINEGARDNER, R. T. 1972. An improved fluorometric assay for DNA. *Anal. Biochem.* **39**:197.
- HOLTHAUSEN, H. S., S. CHACKO, E. A. DAVIDSON, and H. HOLTZER. 1969. Effect of 5-bromodeoxyuridine on expression of cultured chondrocytes grown *in vitro*. *Proc. Natl. Acad. Sci. U. S. A.* **63**:864.
- HOWELL, D. S., E. DELCHAMPS, W. RIEMER, and I. KIEM. 1960. A profile of electrolytes in the cartilaginous plate of growing ribs. *J. Clin. Invest.* **39**:919.
- KANG, A. H., K. A. PIEZ, and J. GROSS. 1969. Characterization of the α chains of chick skin collagen and the nature of the NH₂-terminal cross link region. *Biochemistry*. **8**:3684.
- KISSANE, J. M., and E. ROBINS. 1958. The fluorometric measurement of deoxyribonucleic acid in animal tissues with special reference to the central nervous system. *J. Biol. Chem.* **233**:184.
- KOJIMA, S., and T. YAMAGATA. 1971. Glycosaminoglycans and electrokinetic behavior of rat ascites hepatoma cells. *Exp. Cell Res.* **67**:142.
- KOSHER, R. A., and R. L. SEARLS. 1973. Sulfated mucopolysaccharide synthesis during the development of *RNA pipiens*. *Dev. Biol.* **32**:50.
- KRAEMER, P. M. 1971 *a*. Heparan sulfates of cultured cells. I. Membrane-associated and cell-sap species in Chinese hamster cells. *Biochemistry*. **10**:1437.
- KRAEMER, P. M. 1971 *b*. Heparan sulfates of cultured cells. II. Acid-soluble and precipitable species of different cell lines. *Biochemistry*. **10**:1445.
- KVIST, T. N. 1973. *In vitro* modulation of embryonic chick chondrocytes in different states of differentiation. Ph. D. Thesis. University of Pennsylvania, Philadelphia, Pa.

- LAGUNOFF, D., and G. WARREN. 1962. Determination of 2-deoxy-2-sulfoamino-hexose content of mucopolysaccharides. *Arch. Biochem. Biophys.* **99**:396.
- LASH, J. W. 1972. In *The Comparative Molecular Biology of Extracellular Matrices*. H. Slavkin, editor. Academic Press, Inc., New York.
- LASH, J. W., K. ROSENE, R. R. MINOR, J. C. DANIEL, and R. A. KOSHER. 1973. Environmental enhancement of *in vitro* chondrogenesis. III. The influence of external potassium and chondrogenic differentiation. *Dev. Biol.* **35**:370.
- LINDAHL, U. 1970. Structure of heparin, heparan sulfate and their proteoglycans. In *Chemistry and Molecular Biology of the Intercellular Matrix*. E. A. Balazs, editor. Academic Press, Inc., New York.
- MARZULLO, G., and J. W. LASH. 1970. Control of phenotypic expression in cultured chondrocytes: Investigations on the mechanism. *Dev. Biol.* **22**:638.
- MATALON, R., and A. DORFMAN. 1972. Hurler's syndrome, and α -L-iduronidase deficiency. *Biochem. Biophys. Res. Commun.* **47**:959.
- MILLER, E. J., G. R. MARTIN, K. A. PIEZ, and M. J. POWERS. 1967. Characterization of chick bone collagen and compositional changes associated with maturation. *J. Biol. Chem.* **242**:5481.
- MILLER, E. J., and V. J. MATUKAS. 1969. Chick cartilage collagen: new type of α 1 chain not present in bone or skin of the species. *Proc. Natl. Acad. Sci. U. S. A.* **64**:1264.
- MINOR, R. R. 1973. Somite chondrogenesis: a structural analysis. *J. Cell Biol.* **56**:27.
- NEUFELD, E. F., and M. J. CANTZ. 1971. Corrective factors for inborn errors of mucopolysaccharide metabolism. *Ann. N. Y. Acad. Sci.* **179**:580.
- PETERKOFKY, B. 1972. The effect of ascorbic acid on collagen polypeptide synthesis and proline hydroxylation during the growth of cultured fibroblasts. *Arch. Biochem. Biophys.* **152**:318.
- PETERKOFKY, B., and R. DIEGELMANN. 1971. Use of a mixture of proteinase-free collagenases for the specific assay of radioactive collagen in the presence of other proteins. *Biochemistry.* **10**:988.
- SAITO, H., T. YAMAGATA, and S. SUZUKI. 1968. Enzymatic methods for the determination of small quantities of isomeric chondroitin sulfates. *J. Biol. Chem.* **243**:1536.
- SANTOIANNI, P., and M. AYALA. 1965. Fluorometric ultramicroanalysis of deoxyribonucleic acid in human skin. *J. Invest. Dermatol.* **45**:99.
- SAPOLSKI, A. I., R. D. ALTMAN, and D. S. HOWELL. 1973. Cathepsin D activity in normal and osteoarthritic human cartilage. *Fed. Proc.* **32**:1485.
- SCHILTZ, J. R., R. MAYNE, and H. HOLTZER. 1973. The synthesis of collagen and glycosaminoglycans by dedifferentiated chondroblasts in culture. *Differentiation.* **1**:97.
- SHULMAN, H. J., and K. MEYER. 1970. Proteinpolysaccharide of chicken cartilage and chondrocyte cell cultures. *Biochem. J.* **120**:689.
- SIMMS, H. S., and M. SANDERS. 1942. The use of serum ultrafiltrate in tissue culture for studying fat deposition, and for virus propagation. *Arch. Pathol.* **33**:619.
- SLOMIANY, B. L., and K. MEYER. 1972. Isolation and structural studies of sulfated glycoproteins of hog gastric mucosa. *J. Biol. Chem.* **247**:5062.
- SUZUKI, S., K. KOJIMA, and K. R. ITSUMI. 1970. Production of sulfated mucopolysaccharides by established cell lines of fibroblastic and nonfibroblastic origin. *Biochim. Biophys. Acta.* **222**:240.
- TOOLE, B. P., and J. GROSS. 1971. The extracellular matrix of the regenerating newt limb: synthesis and removal of hyaluronate prior to differentiation. *Dev. Biol.* **25**:57.
- WOESSNER, J. F., JR. 1973. Cartilage cathepsin D and its action on matrix components. *Fed. Proc.* **32**:1485.

APPENDIX

Ingredients of F12X (3.0 mM/l K⁺), Modified F12X (134.0 mM/l K⁺), and High K (68.4 mM/l K⁺) Media

| F12X | | Modified F12X | | high K* | | Constant ingredients of all media† | |
|---|--------|---------------------------------|--------|---|-------|--------------------------------------|-----------------------|
| component | mM/l | component | mM/l | component | mM/l | component | mM/l |
| NaCl | 130.00 | KCl | 132.50 | NaCl | 65.00 | CuSO ₄ ·5H ₂ O | 1 × 10 ⁻⁶ |
| Na ₂ H ₂ PO ₄ ·7H ₂ O | 1.00 | K ₂ HPO ₄ | 1.30 | KCl | 67.75 | ZnSO ₄ ·7H ₂ O | 3 × 10 ⁻³ |
| KCl | 3.00 | | | Na ₂ H ₂ PO ₄ ·7H ₂ O | 0.50 | FeSO ₄ ·7H ₂ O | 3 × 10 ⁻³ |
| | | | | K ₂ HPO ₄ | 0.65 | MgCl ₂ ·6H ₂ O | 6 × 10 ⁻¹ |
| | | | | | | CaCl ₂ ·2H ₂ O | 3 × 10 ⁻¹ |
| | | | | | | Glucose | 1 × 10 ¹ |
| | | | | | | NaHCO ₃ | 1.4 × 10 ¹ |

* Prepared by diluting the modified F12X medium 1:1 with F12X (control) medium.

† All media also contained twice the normal concentration of amino acids and pyruvate, and were supplemented with 10% fetal calf serum and 1% bovine albumin fraction V.