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# Metabolism in *Vitro* of Cartilage Proteoglycans in Rat (Pre)chondrocytes from Different Embryonic Regions

Johan Styrud, Erik Unger* and Ulf J. Eriksson

*Department of Medical Cell Biology, University of Uppsala, Biomedicum, Uppsala, Sweden and*
**Department of Veterinary Medical Chemistry, Swedish University of Agricultural Sciences, Biomedicum, Uppsala, Sweden*

## ABSTRACT

Diabetes in the mother may cause disturbances in the chondrocyte development in the embryo. A rat model was used to investigate whether this was reflected in the production of proteoglycans by cells from two embryonic regions. One of these regions is resistant (limb bud) and the other susceptible (mandibular arch) to malformation in diabetic pregnancy.

Chondroitin sulphate proteoglycans from cultures of day-12 rat embryo limb bud and mandibular arch chondrocytes were extracted with guanidine-HCl and analyzed by gel chromatography after *in vitro* $^{35}$S-sulphate-labeling. Two sizes of proteoglycans ($K_{av}$ 0.26 and 0.66 on CL-2B Sepharose) were found in both types of chondrocytes and in all media. The polysaccharide chain length was the same (Kav 0.36 on CL-6B Sepharose) for both proteoglycans.

Elevated levels of D-glucose or β-hydroxybutyric acid had no effect on either proteoglycan size or proportion, nor on polysaccharide chain length. However, there were differences (in all culture conditions) between limb bud and mandibular arch cultures in that the larger proteoglycan accounted for 80 % of total radioactivity in the limb bud cultures, 53 % in the mandibular arch cultures, and only 25-29 % in the media from both types of cultures. Furthermore, different ratios between radioactive proteoglycans in medium and matrix suggested markedly different efficiencies for matrix formation in the two cell types. These findings indicate differences in the metabolism of the proteoglycans in these two cell types which may be related to the induction of mandibular malformation in diabetic pregnancy.

## INTRODUCTION

Maternal diabetes causes an increased incidence of malformations in the offspring, a great proportion of which involve disturbances in embryonic chondrogenesis. Several experimental studies have indicated that diabetes *per se* affects chondrocyte development and function (1). Rib cartilage from fetuses made hyperglycemic by chronic D-glucose infusion into the mother showed decreased thymidine incorporation *in vivo* and *in vitro* (2, 3). In streptozotocin-diabetic rats the incorporation of sulphate in demineralized bone matrix was decreased and the proteoglycan produced was smaller than that in normal cartilage (4). The synthesis of growth plate proteoglycans was almost abolished in untreated diabetic rats, whereas insulin treatment partly normalized the proteoglycan production (5). Two populations of proteoglycans have been found in costal cartilage from mice (6), and rats (7), and whilst experimental diabetes in rats decreased production of both populations of proteoglycans the larger form was suppressed to a greater extent (7). These alterations in proteoglycan production were more pronounced in diabetic rats from a strain prone to skeletal malformations than in rats from a non-malformation-prone strain (7). Insulin treatment of the diabetic rats normalized proteoglycan production. These results indicate that a diabetic environment inhibits the synthesis of cartilage-associated proteoglycans.

(Pre)chondrocyte cells from two different regions of rat embryos showed markedly decreased thymidine incorporation when exposed to increased levels of β-hydroxybutyric acid in culture (8). Raised D-glucose concentration caused a marginal decrease in thymidine incorporation in mandibular arch chondrocytes but this effect was only found in cells isolated from the malformation-prone rat strain (8).

The aim of the present work was to study the effects of a diabetes-like *in vitro* environment on the production of proteoglycans by the chondrocytes. For this, a "micromass" technique was utilized, in which undifferentiated (pre)chondrocyte cells matured into Alcian blue stainable chondrocytes over six days of culture (9, 10, 11, 12). The (pre)chondrocytes were isolated from day-12 embryos of rats of the

malformation-prone strain (13) and cultured in different D-glucose and β-hydroxybutyric acid concentrations.

## MATERIALS AND METHODS

Sprague-Dawley-derived rats from a colony in Uppsala were used in this study. The fetuses of these rats have an elevated rate of congenital malformations when the mother is diabetic (13, 14, 15). Female rats were caged overnight with male rats from the same strain and the day of conception, *i.e.* presence of sperm in a vaginal smear, was designated day zero of pregnancy. On gestational day 12 the rats were decapitated and the embryos were dissected out. Isolated (pre)chondrocyte cells were prepared and cultured in a micromass *in vitro* system, as described in detail elsewhere (8). In previous studies isolation and culture of (pre)chondrocytes from gestational days 11 and 13 were also attempted, but led to almost no growth of the cells *in vitro* (8).

Briefly, the limb bud and the mandibular arches were dissected free, pooled separately and trypsinized for 8 to 9 minutes at 37°C until single cell suspensions were obtained. Non-viable embryos or embryos with less than 30 somites were discarded. The cells were carefully suspended in the medium (RPMI 1640, supplemented with 10 % fetal calf serum) (Flow Laboratories, Irvine, UK) by vortex mixing for 5 minutes and filtered through a Nitex monofilament screen (mesh diameter 20 μm). The cell concentration was determined using a haemocytometer and adjusted to $1.8\text{-}2.2 \cdot 10^7$ cells/ml for limb bud and $1.5\text{-}2.0 \cdot 10^7$ cells/ml for mandibular arch (pre)chondrocyte cells. In each experiment 20 μl of a cell suspension was placed as a single droplet on the dry base of a 35 mm tissue culture dish (Cluster 3406, Costar, Cambridge, MA, USA).The dishes were then maintained in an incubator (95 % air and 5 % $CO_2$; ASSAB, Sundbyberg, Sweden) at 37°C for 90 minutes, to allow the cells to attach to the substratum, before each dish was carefully flooded with 2 ml of the medium (11.1 mmol/l D-glucose). After 24 hours culture, the medium was changed and the (pre)chondrocytes were subsequently

cultured for five days in either 11.1 mmol/l D-glucose, 44.4 mmol/l D-glucose or with 32.0 mmol/l β-hydroxybutyric acid (in the presence of 11.1 mmol/l D-glucose). On day 3 of culture the medium was changed and 100 μCi $Na_2$ $^{35}S$-$SO_4$ (carrier-free) (Amersham International plc, Amersham, Buckinghamshire, UK) was added, and the culture was continued for a further three days. The medium was then saved and pooled for each culture condition and 200 μl guanidine buffer (4 M guanidine HCl in 0,05 M acetate buffer, pH 5.8, containing 0.2 % Triton X-100, 1 mM N-ethyl-maleimide, 0.1 M ε-amino-n-caproic acid, 5 mM benzamidine and 10 mM EDTA, all from Sigma Chemical Co., St. Louis, MO, USA) (16) was added to each dish and the cultures were scraped off with a "rubber policeman" into plastic tubes. The cultures were extracted for 72 hours at 4°C, centrifuged at 15 000 x g for 15 minutes and dialyzed against 2 x 50 volumes (approximately 50 ml) of guanidine buffer for 24 hours. Cartilage extracts were chromatographed in guanidine buffer on a Sepharose CL-2B (Pharmacia Fine Chemicals, Uppsala, Sweden) column (88 x 1 cm). Dextran blue (Pharmacia Fine Chemicals) and DNP-alanine (Sigma Chemical Co.) were used to indicate the void ($V_O$) and the total ($V_t$) volumes of the column. To each 1 ml fraction 4 ml Emulsifier Scint 299 (Packard AB, Stockholm, Sweden) was added and the radioactivity was estimated in a liquid scintillation counter (No. 300-C, Packard AB). Disintegrations per minute (d.p.m.) were calculated by external standardization.

The pooled medium samples were diluted with a 0.14 M NaCl 10 mM Tris-buffer, pH 8, applied to a 0.5 ml DEAE-Sepharose (Pharmacia Fine Chemicals) column, washed with the Tris-buffer, then with a 0.14 M NaCl 20 mM Na-acetate buffer, pH 4, and subsequently eluted with 1.5 M NaCl 20 mM Na-acetate buffer, pH 4. The medium samples were then chromatographed in the same manner as the extracts.

Chondroitinase ABC digestion followed by gel chromatography was performed to determine chondroitin sulphate content. Proteoglycans were degraded by incubation for 15 hours at 4°C with 0.05 units of chondroitinase ABC (Sigma Chemical Co.) in 0.05 M Tris/HCl, pH 8.0, containing 0.03 M sodium acetate and 0.1 mg/ml

bovine serum albumin. The samples were chromatographed on a Sepharose CL-6B column (Pharmacia Fine Chemicals) with Blue dextran and DNP-alanine as markers for void and total volumes (17).

For polysaccharide chain length determination the samples were incubated overnight in 0.5 M NaOH at 4°C. After neutralization with Tris-HCl, pH 8.0, and HCl the samples were chromatographed on a Sepharose CL-6B column, eluted with 0.01 M Tris buffer, pH 8.0, containing 0.14 M NaCl and 0.02 % $NaN_3$. Blue dextran and DNP-alanine were used as markers for void and total volumes, respectively.

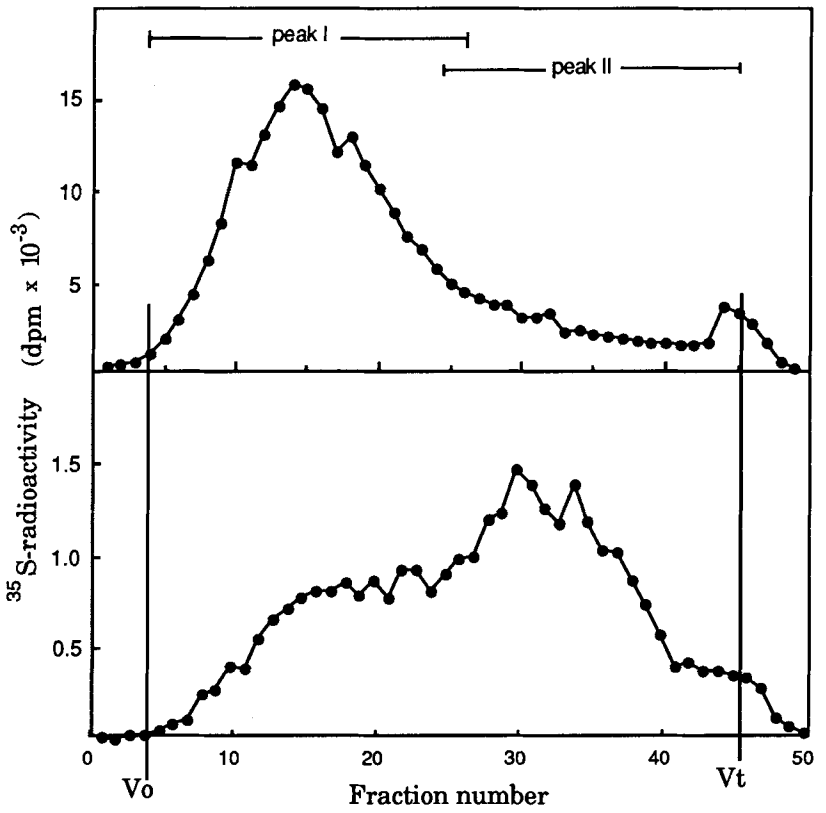


FIGURE 1. CL-2B Sepharose chromatogram of extracted $^{35}$S-labeled proteoglycans from cultured limb bud chondrocytes (top panel) and corresponding culture medium (lower panel).

## RESULTS

After digestion with chondroitinase ABC, more than 95% of the $^{35}S$-macromolecules present in the dialyzed extracts of limb bud and mandibular arch chondrocyte cultures, eluted as low molecular weight material following chromatography on Sepharose CL-6B, demonstrating that the label was mainly present in chondroitin sulphate (data not shown).

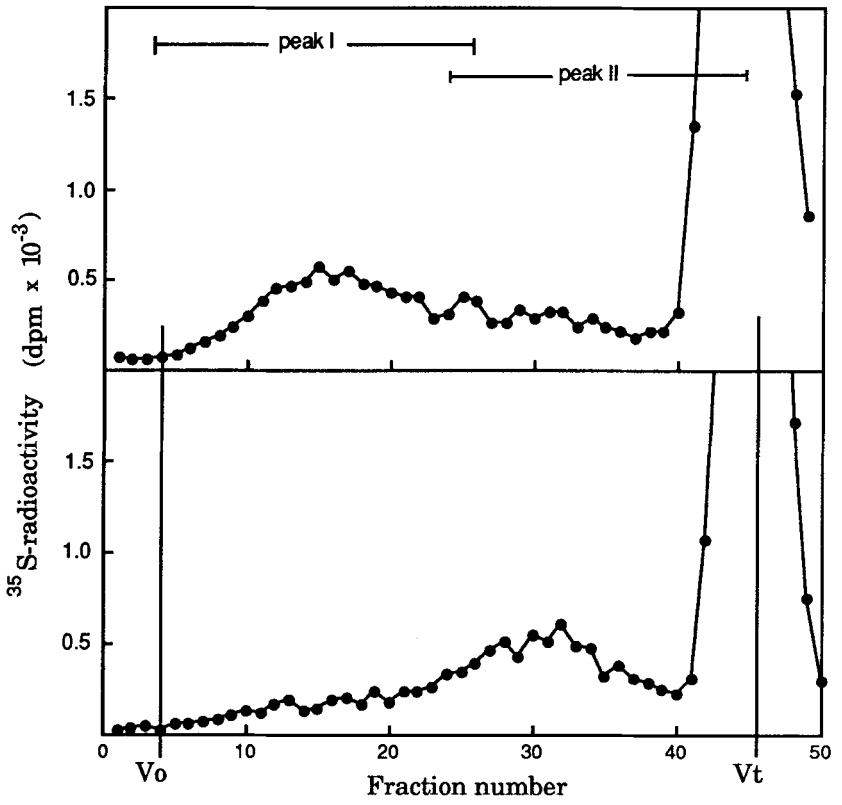


FIGURE 2. CL-2B Sepharose chromatogram of extracted $^{35}S$-labeled proteoglycans from cultured mandibular arch chondrocytes (top panel) and corresponding culture medium (lower panel).The large peak in the $V_t$ position consists of free sulphate.

When dialyzed chondrocyte extracts were analyzed by gel chromatography on Sepharose CL-2B, the $^{35}S$-labeled material was separated into two peaks (I and II; $K_{av}$ about 0.3 and 0.7) in which peak I was dominant in the cell culture and peak II was dominant in the medium (Figures 1 and 2). Due to the low total incorporation in mandibular arch cultures, the free sulphate peak is relative large (Figure 2).

Elevated ambient concentrations of D-glucose (44.4 mmol/l) or β-hydroxybutyric acid (32.0 mmol/l) had no effect on either the $K_{av}$ values (Figures 3 and 4) or the relative proportions of the two peaks (Figure 5) in the limb bud or mandibular arch cell cultures. In particular, there were no significant differences between the amount of $^{35}S$ incorporation in peak I in different culture conditions in mandibular arch medium (Figure 5).

TABLE 1. Combined $K_{av}$ values on Sepharose CL-6B of proteoglycans isolated from cell and medium fractions of limb bud (LB, n = 10) and mandibular arch (MA, n = 9) chondrocytes. Mean ± SEM of values from all types of culture conditions (see text).

| | CULTURE | | MEDIUM | |
|---|---|---|---|---|
| | **peak I** | **peak II** | **peak I** | **peak II** |
| LB | 0.26 ± 0.01 | 0.62 ± 0.01 | 0.28 ± 0.01 | 0.67 ± 0.01 |
| MA | 0.29 ± 0.01 | 0.66 ± 0.02 | 0.27 ± 0.01 | 0.69 ± 0.02 |

Combined data for limb bud and mandibular arch chondrocytes from all three types of culture conditions yielded $K_{av}$ values of approximately 0.28 and 0.66, respectively, for the two different proteoglycans (Table 1). Incorporation of $^{35}S$ into the larger proteoglycan (peak I) was significantly greater in the limb bud cells than

in the mandibular arch cells (Table 2). In contrast, there was no statistically significant difference between the distribution of the label between the two proteoglycans in the medium from limb bud and mandibular arch chondrocyte cultures (Table 2). The total sulphate incorporation of limb bud chondrocytes was more than tenfold greater than that of mandibular arch cells. Furthermore, the ratio of medium-to-chondrocyte radioactivity was markedly different in the two types of culture. In limb bud cultures the ratio was 1:6, whereas in mandibular arch culture the ratio was approximately 1:1 (Table 2).

TABLE 2. The amount of $^{35}S$-radioactivity in peak I (expressed as a percentage of the total $^{35}S$-incorporation in eluted macromolecules) and total $^{35}S$-radioactivity (d.p.m.per 6 culture dishes) from cultured chondrocytes and media. In the last column the ratio between total $^{35}S$-incorporation in the culture and in the media is shown. Mean ± SEM of pooled values from all types of conditions per LB (n = 10) or MA (n = 9).

| | CULTURE | | MEDIUM | | Ratio |
|---|---|---|---|---|---|
| | peak I% | $^{35}S \cdot 10^3$ | peak I% | $^{35}S \cdot 10^3$ | M:C |
| LB | 80 ± 2 | 125.6 ± 9.6 | 29 ± 2 | 21.9 ± 9.6 | 1:5.7 |
| MA | 53 ± 3*** | 9.6 ± 0.4*** | 25 ± 5 | 11.2 ± 0.6** | 1:0.9 |

** = $p < 0.01$

*** = $p < 0.001$

Gel chromatography on Sepharose CL-6B of alkali-released polysaccharide chains from proteoglycans in limb bud cell extracts and media showed a single peak. This suggest that there was no significant difference in polysaccharide chain length between the proteoglycans in the cells and media (data not shown). The $K_{av}$ value was about 0.36 in all peaks, indicating a molecular weight of about 35000 according to the calibration of Wasteson (18).

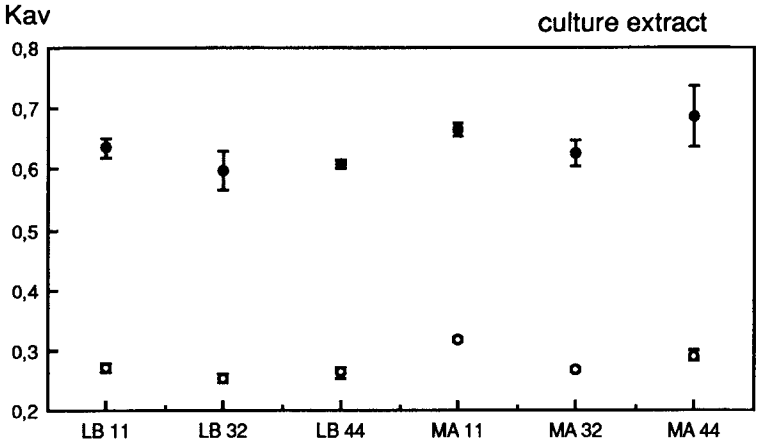


FIGURE 3. $K_{av}$ values for the small (peak II, upper part of diagram) and large (peak I, lower part of diagram) proteoglycan isolated from limb bud (LB) or mandibular arch (MA) chondrocytes, cultured in 11.1 mmol/l D-glucose (LB 11, MA 11), or 32 mmol/l β-hydroxybutyric acid (LB 32, MA 32), or 44 mmol/l D-glucose (LB 44, MA 44). Mean ± SEM, $3 \leq n \leq 4$.

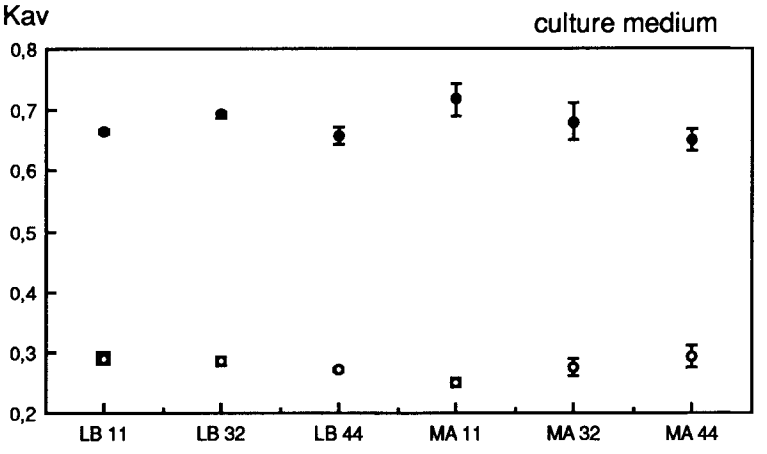


FIGURE 4. $K_{av}$ values for the small (peak II, upper part of diagram) and large (peak I, lower part of diagram) proteoglycan in medium from limb bud (LB) or mandibular arch (MA) chondrocytes, cultured in 11.1 mmol/l D-glucose (LB 11, MA 11), or 32 mmol/l β-hydroxybutyric acid (LB 32, MA 32), or 44 mmol/l D-glucose (LB 44, MA 44). Mean ± SEM, $3 \leq n \leq 4$.

## DISCUSSION

The present results show, that chondrocytes from limb buds and mandibular arches both produced two major types of proteoglycans. The larger of these (peak I, $K_{av}$ 0.28) was predominant in the limb bud extracts, whereas in the mandibular arch cells the peaks were evenly distributed. In the culture medium, the smaller proteoglycan (peak II, $K_{av}$ 0.66) was predominant, and this pattern was similar in media from both types of chondrocyte cultures. The production of proteoglycans, as reflected in $^{35}S$-sulphate incorporation into extracted macromolecules, was markedly lower in mandibular arch cultures compared to limb bud cultures. Furthermore, the different ratios between radioactive proteoglycans in medium and matrix showed that 85 % of the proteoglycans in the limb bud cultures remained in the matrix, in contrast to only about 50 % of the mandibular arch proteoglycans.

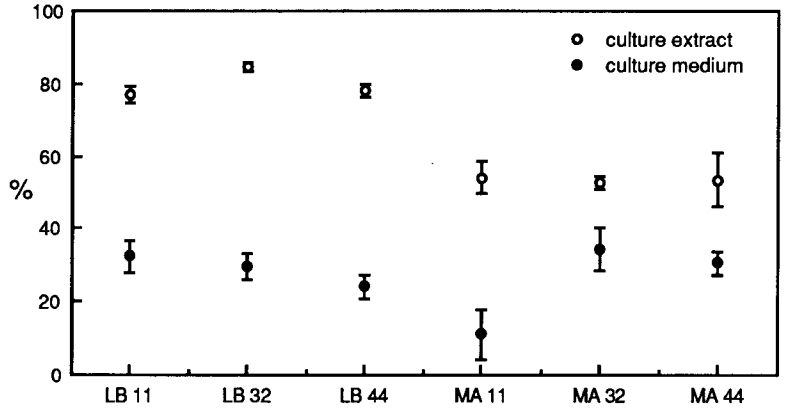


FIGURE 5. Amount of $^{35}S$-radioactivity in peak I expressed as a percentage of total $^{35}S$-incorporation in eluted macromolecules from cultured limb bud (LB) and mandibular arch (MA) chondrocytes (top row of values), and the corresponding culture media (lower row of values). Culture conditions, cf. Legends to Figures 3 and 4. Mean $\pm$ SEM, $3 \leq n \leq 4$.

This suggests differences in the metabolism of the proteoglycans in the two cell types. However, there were no detectable differences in $K_{av}$ values, or relative proportions of proteoglycans of each cell type when cultured in different concentrations of D-glucose or β-hydroxybutyric acid. Likewise, the $K_{av}$ and relative proportions of the proteoglycans did not change in the corresponding media. The polysaccharide chain length was also similar in the proteoglycans of all samples.

In previous *in vitro* studies, we found that the total DNA content of mandibular arch cultures was about 50 % of that of limb bud cultures, implicating a greater rate of sulphate incorporation per cell in the latter chondrocytes.

Mandibular arch chondrocytes from embryos of rats of the local colony are more sensitive to increased ambient D-glucose and β-hydroxybutyric acid levels than limb bud chondrocytes (8). This corresponds to the micrognathia and normal limb development in the offspring of diabetic rats of this strain (13, 14). The critical period for induction of diabetes-related skeletal malformations in this rat strain has recently been determined to be gestational days 6-10 (15), which suggests that the chondrocytes isolated on day 12 in the present study may already have passed their period of maximal sensitivity to a diabetic environment.

Human endothelial cells exposed to high concentrations of D-glucose display signs of DNA damage (19). Connective tissues in diabetic rats has an increased rate of synthesis of fibronectin (20). Increased D-glucose levels have been shown to exert effects on the gene expression of fetal cells. Culture of umbilical cord vessel endothelium in high D-glucose concentration caused marked changes in mRNA levels of several matrix proteins, including collagen and fibronectin (21). In fetal rat brain, increased β-hydroxybutyric acid levels yield decreased production of purine and pyrimidine moities, indicating a direct inhibitory effect on the biosynthesis of RNA and DNA (22, 23). In contrast, the results of the present study suggest that embryonic production of cartilage-associated proteoglycans is not markedly affected by elevated ambient D-glucose and β-hydroxybutyric acid levels.

In the present study, we have demonstrated that both limb bud and mandibular arch chondrocytes produce two size populations of proteoglycans. The $K_{av}$ values

of these proteoglycans are similar in the two types of chondrocyte cultures and are not influenced by the culture conditions. The length of the chondroitin sulphate chains of the proteoglycans is similar in all chondrocyte cultures and media, regardless of origin of the cells and culture conditions.

In contrast, the limb bud and mandibular arch chondrocytes differ markedly in the total amount of proteoglycan produced and secreted to the culture medium. Furthermore, different ratios between radioactive proteoglycans in medium and matrix suggest markedly different efficiencies for matrix formation in the two types of cultures.

## ACKNOWLEDGEMENTS

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Correspondence to:
Dr. Johan Styrud
Department of Medical Cell Biology
Biomedicum
P.O. Box 571
S-751 23 Uppsala
Sweden