

# Synthesis of a Low Molecular Weight Collagen by Chondrocytes from the Presumptive Calcification Region of the Embryonic Chick Sterna: The Influence of Culture with Collagen Gels

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**ABSTRACT** The mature chick sternum is divisible almost equally into cephalic calcified and caudal cartilagenous regions. Isolation and culture of cells derived from embryonic precursors of these regions has revealed two discrete populations of cells with distinct morphological features and synthetic capabilities. Both cell populations grew well in culture within or upon collagen gels or upon plastic and maintained morphologies similar to those observed in the parent tissue. Polyacrylamide gel electrophoresis of radiolabeled proteins synthesized by the cells in culture demonstrated large differences in the types of collagens synthesized. Both chondrocyte populations synthesized type II and minor cartilage collagens but only chondrocytes isolated from the presumptive calcification region synthesized the previously identified, low molecular weight collagen, termed G collagen. Synthesis of G collagen was stimulated by culture within or upon collagen gels such that it represented an average of 65% of the total collagen synthesized by presumptive calcification region chondrocytes after 7 d of culture within collagen gels. Light and scanning electron microscopy demonstrated that the two chondrocyte types exhibited distinct morphological features and accumulated different extracellular matrices in culture.

Type II collagen has been recognized as the major collagenous component of cartilagenous tissues for a number of years (19). However, several additional, apparently cartilage specific, collagenous molecules have recently been isolated. These include the  $3\alpha$ ,  $2\alpha$ ,  $1\alpha$  chains described by Burgeson and Hollister (5) and a group of possibly related, low molecular weight, collagenous peptides obtained by pepsin digestion of cartilagenous tissues (1, 22, 23, 25, 31, 32, 34). We have also reported the identification and partial characterization of three further collagenous polypeptides, the G, H, and J chains, synthesized by chondrocytes in culture (11, 12). The H and J chains occur as disulphide-linked aggregates which give rise to chains of 69,000 and 84,000 mol wt, respectively, upon reduction. The major product of mild chymotrypsin digestion of these collagens is a 53,000-mol-wt peptide which exhibits characteristics similar to some of the previously mentioned collagenous peptides that have been isolated from cartilage by pepsin digestion. G collagen has a number of characteristics that distinguish it from other cartilage collagens. The component

chains have a molecular weight of 59,000 which is reduced to 45,000 by chymotrypsin digestion of the native molecule. It is cleaved to discrete products by mammalian collagenase digestion and digestion with cyanogen bromide, chymotrypsin, or V8 protease, which give rise to distinct peptide fingerprints. The synthesis of G collagen was markedly stimulated by the culture of chondrocytes within collagen gels.

It has long been recognized that two major types of cartilage and chondrocytes are found in the embryonic chick; hypertrophic cartilage, which contains large cells and is always associated with ossification, and small-celled cartilage, which does not ossify (9). We have found that 18-d embryonic chick sterna, a popular source of chondrocytes for culture, contain almost equal proportions of both types of cartilage. In this communication we compare the behavior in culture on plastic and within or upon collagen gels of chondrocytes isolated from both regions of 18-d embryonic sterna. We found that G collagen is specifically synthesized by hypertrophic chondrocytes derived from the presumptive calcification region

but is not produced by the smaller chondrocytes isolated from the permanent cartilagenous zone. Furthermore its synthesis was greatly enhanced by culture of chondrocytes from the presumptive calcification region within collagen gels. Like the type II collagen, the H and J collagen chains and  $3\alpha$ ,  $2\alpha$ ,  $1\alpha$  species are synthesized by both species of chondrocytes.

## MATERIALS AND METHODS

### Cell and Explant Culture

**MEDIUM AND SUBSTRATA:** Chick embryo chondrocytes were cultured on plastic or within collagen gels as described previously (11). Collagen was prepared from rat-tail tendons by extraction with 0.1 M acetic acid (26), supplemented with penicillin (100 U/ml) and streptomycin (0.1 mg/ml) and dialyzed against distilled water before use. The tissue culture medium used throughout was Dulbecco's modified Eagle's medium (DME)<sup>1</sup> supplemented with penicillin (100 U/ml) and streptomycin (0.1 mg/ml), ascorbate (50  $\mu$ g/ml), nonessential amino acids, and 16% (vol/vol) fetal calf serum, except in studies of the type of collagen synthesis only where 24 h before incubation with isotope the cells were returned to DME without the nonessential amino acid supplement.

**CELLS:** Chondrocytes were prepared from sternal cartilages dissected from 18-d-old chick embryos. The sterna were cleaned of surrounding perichondrium and divided into presumptive calcification regions (cephalic half) and permanent cartilagenous regions (caudal half). Any remaining perichondrial fibroblasts were released by incubation of the tissue with bacterial collagenase (type 1A, Sigma Chemical Co., St. Louis, MO) (10 mg/ml) and trypsin (3 mg/ml) in DME for 30 min at 37°C. Sternal tissue washed free of contaminating fibroblasts was then chopped and digested with bacterial collagenase (10 mg/ml) and trypsin (3 mg/ml) for 90 min at 37°C.

Released chondrocytes were washed with DME and plated on plastic ( $7 \times 10^6$  cells/cm<sup>2</sup>) or within collagen gels ( $5 \times 10^2$  cells/ml) as previously described (11). Chondrocytes were also cultured upon collagen gels. Type I collagen gels were prepared as described previously (11), allowed to stand at room temperature for 1 h, and plated with chondrocytes at a density of  $7 \times 10^4$  cells/cm<sup>2</sup>.

**GROWTH KINETICS:** The number of cells per culture dish was determined by DNA estimation. After various times of incubation cultures were frozen and thawed twice, DNA was precipitated with cold trichloroacetic acid (TCA) and hydrolyzed in 5% TCA at 90°C for 15 min followed by rehydrolysis in 0.5 M HClO<sub>4</sub> for 15 min at 70°C. After centrifugation diphenylamine/acetaldehyde was added to the supernatant solution and the DNA content determined from absorbance at 600 nm (6).

**EXPLANT CULTURE:** Sterna were dissected from ten 18-d-old chick embryos and divided into presumptive calcification zones and permanent cartilagenous zones as described. Cartilage from each region was then finely sliced and incubated in 5% CO<sub>2</sub>/95% air at 37°C in DME.

### Light Microscopy

Presumptive calcifying and permanent cartilagenous regions of 18-d embryonic chick sterna and chondrocyte cultures upon or within collagen gels were fixed in cetylpyridinium chloride formalin (10). After routine processing the regions of sterna were sectioned transversely and the cultures were sectioned at right angles to the culture surface. Sections were then stained with Alcian blue/periodic acid Schiff (pH 2.5) to visualize the proteoglycan rich cartilagenous matrix.

### Scanning Electron Microscopy

Chondrocyte cultures upon or within collagen gels were fixed by immersion in 2% glutaraldehyde in 0.1 M sodium cacodylate for 2 h; postfixed in 1% OsO<sub>4</sub> in 0.1 M sodium cacodylate buffer for 2–3 h at 4°C; stained *en bloc* with uranyl acetate, and dehydrated through a graded series of alcohols (13). After critical-point drying, samples were sputter coated with gold and viewed in an ISI DS 130 scanning electron microscope.

### Determination of Rates of Collagen Synthesis

The incorporation of [5-<sup>3</sup>H]proline into peptidyl-hydroxy [<sup>3</sup>H]proline was used to determine the rate of collagen synthesis by cells maintained in culture for 7 d. Since cultures upon or within collagen gels contained a considerable

volume of unreplaced medium within the gel itself, initial adjustment for the corresponding dilution of isotope was made by the addition of an equivalent volume of medium to cultures on plastic. The radioactivity (counts per minute per milliliter) of the medium was monitored after 30 min and any difference between cultures was compensated by the addition of unlabeled medium. After incubation for 2, 4, or 6 h with [5-<sup>3</sup>H]proline (10  $\mu$ Ci/ml) at 37°C, the chondrocytes and matrix in radiolabeled medium was scraped from the dishes and homogenized. Peptides were precipitated by addition of trichloroacetic acid to 12.5% (wt/vol) in the presence of proline (1 mg/ml) and kept at 4°C overnight. Precipitates were washed with 5% (wt/vol) trichloroacetic acid containing proline (1 mg/ml) until no radioactivity was released from the pellet and then hydrolyzed in 6 M HCl at 110°C for 18 h. Total incorporation of [<sup>3</sup>H]proline was determined by scintillation counting and the synthesis of hydroxy [<sup>3</sup>H]proline was determined by a specific radiochemical assay (15).

### Isolation of Newly Synthesized Collagen

Cells maintained in culture on plastic, upon collagen gels or within collagen gels for up to 2 wk were labeled at selected times for 24 h with [<sup>3</sup>H]proline (20  $\mu$ Ci/ml). Cells plus matrix were then scraped from the dishes, separated from the medium by centrifugation, and washed three times with DME.

Cartilage slices incubated in DME for 1 h were labeled with [5-<sup>3</sup>H]proline (20  $\mu$ Ci/ml) for a further 24 h. Medium and slices were separated and slices washed three times with DME. Proteinase inhibitors were added to the medium and washings of cell and explant cultures to the following concentrations: phenylmethylsulfonyl fluoride (2 mM), EDTA (25 mM), and 6 aminohexanoic acid (50 mM). Medium was cooled at 4°C and radiolabeled proteins precipitated by the addition of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> to 30% saturation were recovered by centrifugation at 30,000 g for 30 min at 4°C, then redissolved in 0.1 M Tris/HCl buffer (pH 7.4) containing 0.4 M NaCl and proteinase inhibitors before extensive dialysis against this solution. In some experiments samples of radiolabeled culture medium proteins, after dialysis against buffer without proteinase inhibitors, were digested with chymotrypsin (300  $\mu$ g/ml) at 20°C for 6 h.

The matrix-containing fraction of radiolabeled cultures were digested with pepsin (1 mg/ml) in 0.5 M acetic acid (1.0 ml) for 16 h at 4°C. The incubation mixture was centrifuged at 30,000 g for 30 min at 4°C and the residue extracted twice more with pepsin in 0.5 M acetic acid. Collagens present in the pooled supernatant solution were precipitated by the addition of NaCl to 2.0 M, redissolved in 0.5 M acetic acid, and dialyzed extensively against acetic acid before electrophoretic analysis. Radiolabeled proteins not released by pepsin-acetic acid digestion were extracted with 0.1 M Tris/HCl buffer (pH 7.4) containing 0.4 M NaCl at 4°C for 18 h. This extraction procedure was repeated twice more and pooled extracts dialyzed extensively against extraction buffer.

Residual matrix resisting extraction was suspended in distilled H<sub>2</sub>O and dialyzed sequentially against H<sub>2</sub>O containing proline (1 mg/ml) followed by distilled H<sub>2</sub>O at 4°C. This dialysis cycle was repeated 4 or 5 times over a 48-h period. Suspended residual matrix was hydrolyzed in 6 M HCl at 110°C for 18 h after removal from dialysis. Radioactivity present in material resisting extraction was determined by scintillation counting.

### Electrophoretic Analyses

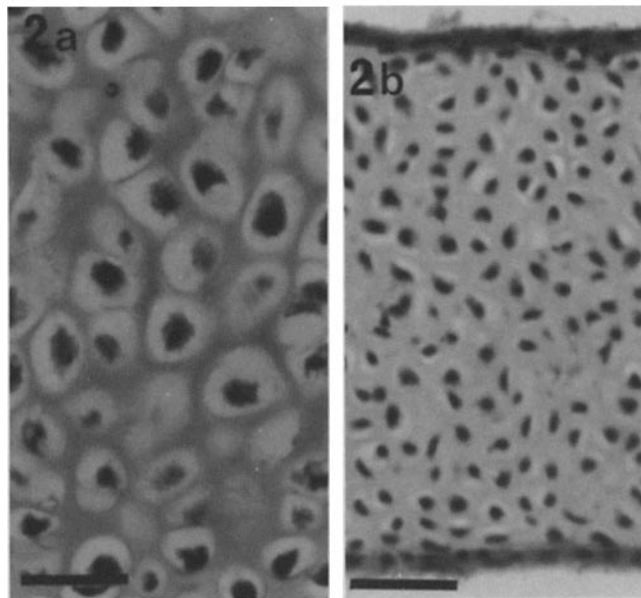
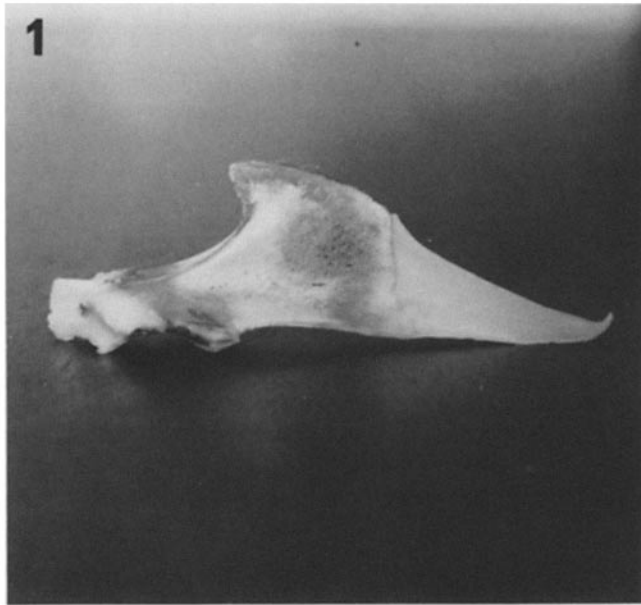
Radiolabeled proteins were resolved after denaturation by PAGE according to the method of Laemmli (17) using a separating gel of 8% and a stacking gel of 3%. All gels were run under reducing conditions. The procedure of Bonner and Laskey (2) was used to impregnate the gel with 2,5-diphenyloxazole, and gels dried onto filter paper were exposed to x-ray film (Agfa Gevaert Currex RP2) at -70°C. To determine the ratio of radioactivity incorporated into specific proteins, strips corresponding to exposed bands on the fluorograms were cut from the dried gels. Attached paper was removed by rehydration in H<sub>2</sub>O and the strip of gel dissolved by incubation in a solution (0.3 ml) of H<sub>2</sub>O<sub>2</sub> (100 vol) containing 0.3% NH<sub>4</sub>OH in a humidified atmosphere for 16 h at 37°C. Scintillation fluor was added and radioactivity determined by scintillation counting.

## RESULTS

### Gross and Histological Appearance of Mature and Embryonic Sterna

The sterna of the mature chick contains distinct, clearly delineated regions of bone and residual cartilage (Fig. 1). Corresponding regions are clearly discernible in the cartilagenous 18-d embryonic chick sterna. The cephalic half, which is eventually replaced by calcified cartilage and subsequently by bone, contains hypertrophic chondrocytes sepa-

<sup>1</sup> Abbreviations used in this paper: DME, Dulbecco's modified Eagles medium; TCA, trichloroacetic acid.



FIGURES 1 and 2 Fig. 1: The sternum of the mature chick showing the obvious bony and cartilagenous regions.  $\times 0.5$ . Fig. 2: Histology of the 18-d embryonic chick sterna. (a) Transverse section through the presumptive calcification region. H and E stain. (b) Transverse section through the permanent cartilagenous region. H and E stain. Bars,  $30 \mu\text{m}$ .  $\times 465$ .

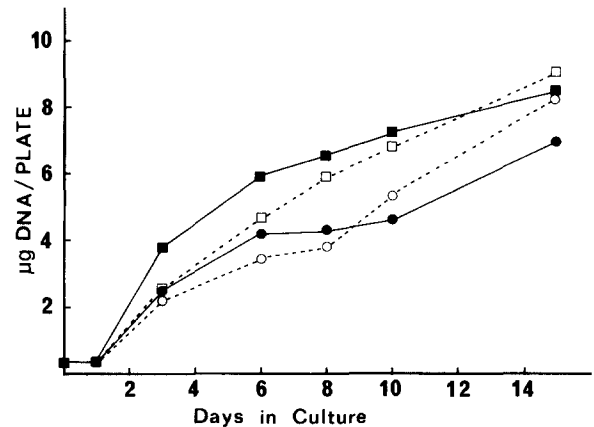


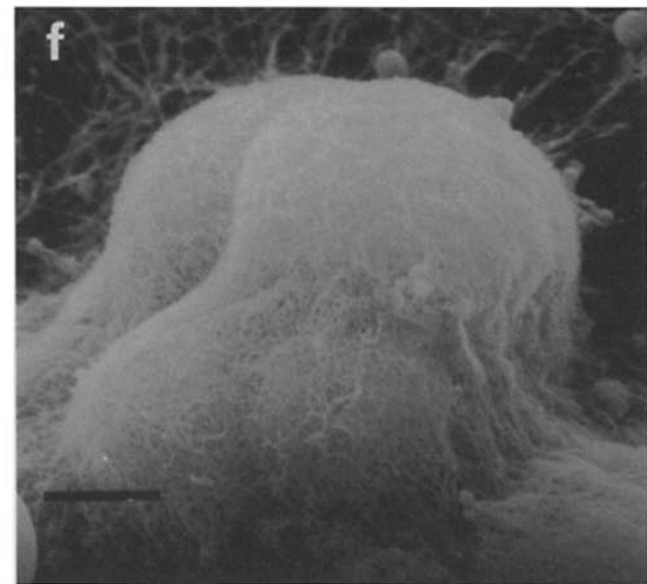
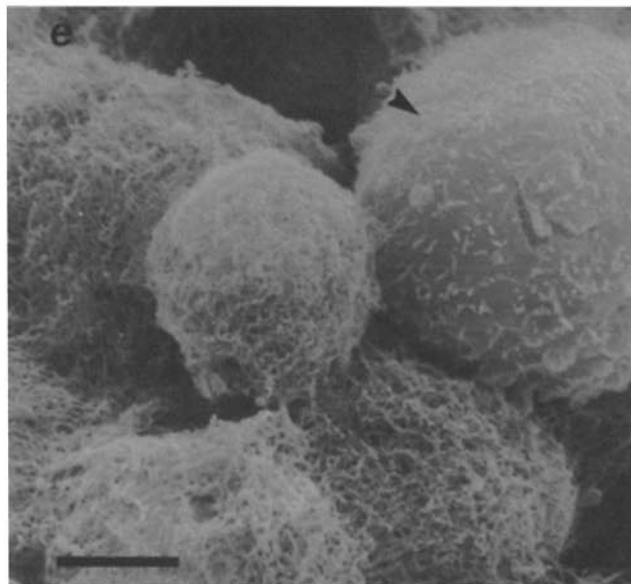
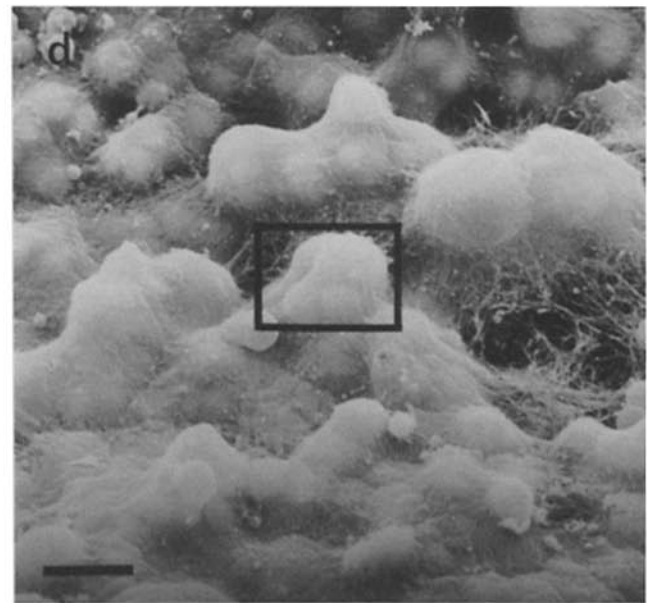
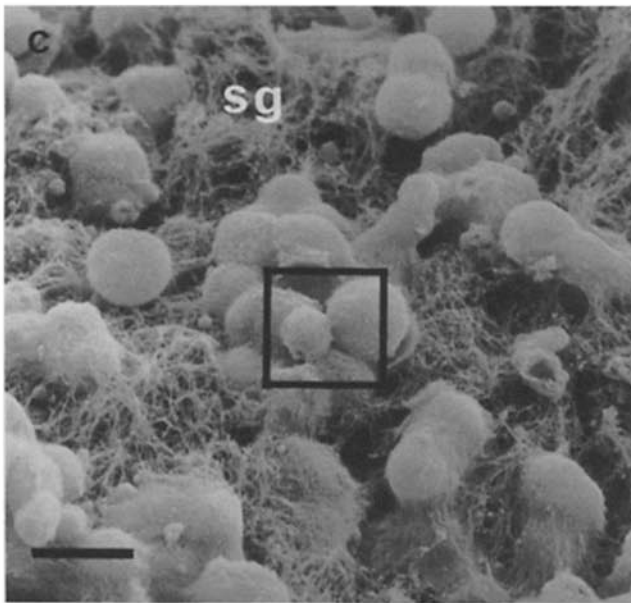
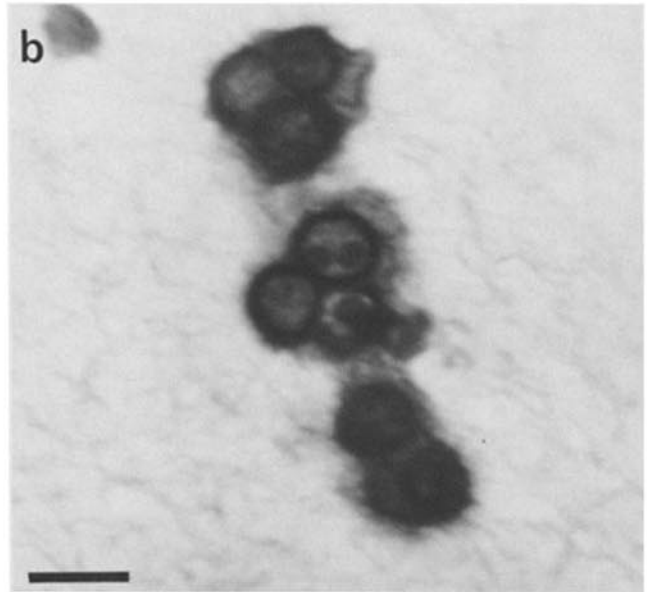
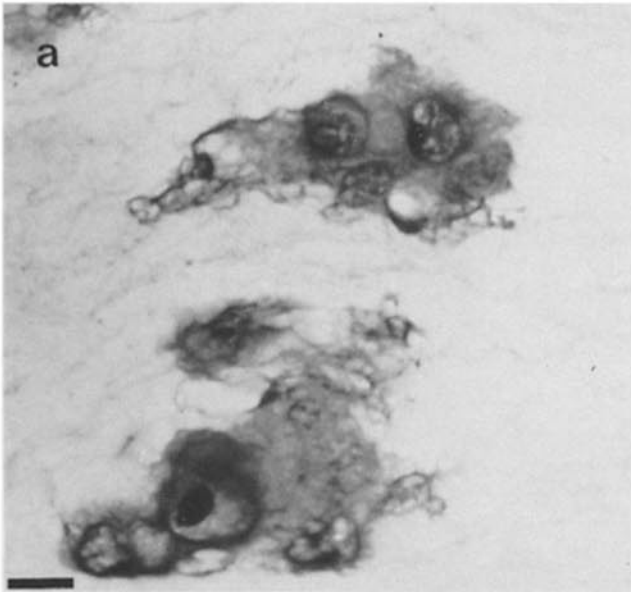
FIGURE 3 Growth curves of chondrocytes isolated from the permanent cartilagenous and presumptive calcification regions. ■ and □, chondrocytes isolated from the permanent cartilagenous region cultured within collagen gels and on plastic, respectively. ● and ○, chondrocytes isolated from the presumptive calcification region cultured within collagen gels and on plastic, respectively. Cells cultured upon collagen gels showed the same growth behavior as cells cultured within collagen gels. Points represent the average of analysis of duplicate cultures. The range of duplicates was on average 7% of the average of the duplicates.

rated by large areas of matrix, whereas the caudal portion which remains cartilagenous into maturity contains smaller more closely packed cells (Fig. 2).

#### Growth of Chondrocytes in Culture

Chondrocytes were isolated separately from each half of the embryonic sterna and cultured on plastic or on collagen gels at  $1.5 \times 10^5$  cells/10-mm dish or within 0.3 ml collagen gels at  $5 \times 10^5$  cells/ml. Cultures were incubated at  $37^\circ\text{C}$  and the increase in cell numbers measured by determining the DNA content. The results of a typical growth experiment are shown in Fig. 3. Chondrocytes isolated from both regions divided slightly more quickly when cultured on or within collagen gels than when cultured on plastic. There was no distinguishable difference in growth behavior between chondrocytes cultured on collagen gels and chondrocytes cultured within collagen gels, however chondrocytes isolated from the permanent cartilagenous region reached a slightly higher saturation density than those isolated from the presumptive calcification region of the sterna (Fig. 3).

FIGURE 4 Light and scanning photomicrographs demonstrating the different morphological features of chondrocytes from the presumptive calcification (Calc) and permanent cartilagenous (Cart) regions of 18-d embryonic chick sternae after 7-d culture. (a) Photomicrograph of histological section of Calc chondrocyte cultured within collagen gels showing groups of loosely aggregated large cells surrounded by a fluffy proteoglycan-staining matrix embedded in the clearer collagen culture gel. PAS Alcian blue pH 2.5 stain. (b) Section of Cart chondrocytes cultured with collagen gels showing the more tightly compacted clusters of smaller nonvacuolated cells with dense pericellular halo of proteoglycan staining matrix. PAS Alcian blue pH 2.5 stain. (c) Low power scanning electron microscopic view of Calc chondrocytes grown upon collagen gels showing the pericellular matrix closely confined to surround individual cells or small groups exposing the intervening supporting collagen gel stromal fibers (SC). (d) Similar scanning view of Cart chondrocytes cultured on collagen gels showing that the cells are covered by a denser blanket of pericellular matrix forming a more confluent sheet obscuring the fibers of the underlying supporting collagen gel. (e) Higher power scanning view of Calc chondrocyte marked in Fig. 4c showing the loose crimped fibers forming discrete balls of individual cells and matrix. Some cells (arrowhead) were free of any surrounding matrix. (f) Similar high power scanning view of Cart chondrocytes showing a blanket of straighter, more orderly fibers enveloping groups of cells and obscuring the spherical outline of individual cells. Bars,  $30 \mu\text{m}$  (a and b);  $20 \mu\text{m}$  (c and d);  $5 \mu\text{m}$  (e and f).  $\times 285$  (a);  $\times 400$  (b);  $\times 620$  (c);  $\times 670$  (d);  $\times 3,200$  (e and f).



## Morphology of Cells Isolated from the Two Areas of *Sterna* in Culture on or within Collagen Gels

Both preparations of chondrocytes produced "typical" chondroid cells in culture. However, chondrocyte cultures from the permanent cartilagenous region contained a larger proportion of elongated cells than those from the presumptive calcification region at earlier times of culture and tended to form linear arrays similar to those described previously (11). Chondrocytes from the calcification region tended to form compact clumps of cells in culture.

Microscopy showed that chondrocytes isolated from the permanent cartilagenous region were smaller and became surrounded by a dense proteoglycan matrix (Fig. 4*b*) that had a fine fibrous appearance under scanning electron microscopy (Fig. 4*f*) after 1 wk in culture within or upon collagen gels. In parallel cultures, chondrocytes from the presumptive calcification region were larger and were surrounded by a distinct lacunae and a more diffuse extracellular proteoglycan matrix (Fig. 4*a*), that exhibited a distinct, more crinkled fibrous appearance under the scanning electron microscope (Fig. 4*e*).

### Rate of Collagen Synthesis

The rate of collagen synthesis was determined from the incorporation of [<sup>3</sup>H]proline into peptidyl hydroxyproline after chondrocytes were cultured for 7 d on plastic or upon or within collagen gels. Change in the culture regime (i.e., culture on plastic, upon collagen or within collagen gels) did not appear to affect the rate of collagen synthesis of chondrocytes from the presumptive calcification zone (Fig. 5). Similarly the proportion of the total nondiffusible radioactivity present as peptidyl-4-hydroxy [<sup>3</sup>H]proline (percent hydroxyl-

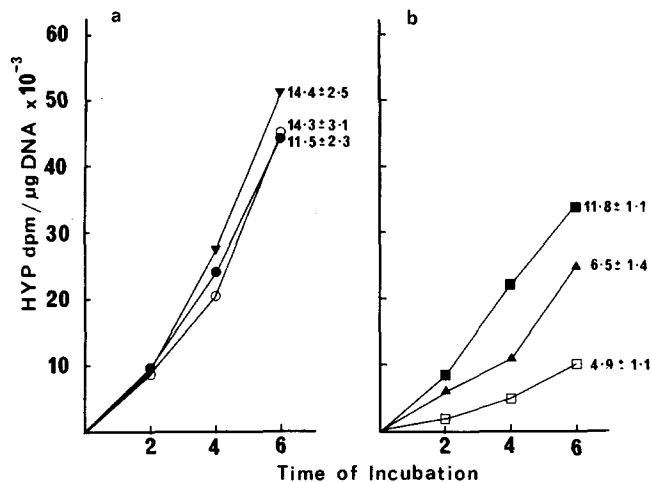


FIGURE 5 Rates of collagen synthesis by chondrocytes from the permanent cartilagenous and presumptive calcification zones after 7 d in culture. Cells cultured on plastic or within or upon collagen gels were incubated for 2, 4, or 6 h with [<sup>3</sup>H]proline, and the 4-hydroxy [<sup>3</sup>H]proline incorporated into trichloroacetic acid-precipitable material (medium plus cell layer) was taken as an index of collagen synthesis. (a) Chondrocytes from the presumptive calcification region. ▼, upon collagen gels; ●, within collagen gels; ○, on plastic. (b) Chondrocytes from the permanent cartilagenous region. ▲, upon collagen gels; ■, within collagen gels; □, on plastic. Points are averages from analysis of duplicate cultures. The range of duplicates was 11% (average from 18 points) of the average of the duplicates. Numbers on the right of each curve indicate the percent hydroxylation of proline for cells in each culture system (average of six samples analyzed with standard deviation).

ation of proline) was fairly constant at all three incubation times and under the three culture conditions for these cells.

However the culture regime had a marked effect on the rate of collagen synthesis by chondrocytes isolated from the permanent cartilagenous region. Chondrocytes from this region showed a similar rate of collagen synthesis to chondrocytes isolated from the presumptive calcification region when cultured within collagen gels, but parallel cultures on collagen or on plastic showed much lower rates of collagen synthesis (Fig. 5). This was accompanied by a marked decrease in the percent hydroxylation of proline, suggesting that cells from this region synthesized a decreasing proportion of collagenous proteins when cultured on collagen or on plastic.

### Collagen Types Detected

Cultures were labeled for 24 h with [<sup>3</sup>H]proline after various times in culture. <sup>3</sup>H proteins released into the medium were precipitated with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and those present in the matrix extracted with pepsin/acetic acid followed by 0.4 M NaCl/0.1 M Tris.

The major high molecular weight proteins precipitated from the medium from cultures of chondrocytes from the permanent cartilagenous zones migrated in the position of the pro  $\alpha 1$ , p N  $\alpha 1$ , p C  $\alpha 1$ , and  $\alpha 1$  chains (Fig. 6*a*). At earlier culture times small amounts of radiolabeled material with the mobility of the previously described H and J collagen polypeptides (12) were also seen. With prolonged culture fewer  $\alpha 1$  precursors were observed and, particularly from cultures on plastic, bands with the mobility of  $\alpha 2(I)$  chains appeared, indicating synthesis of type 1 collagen. Cultures of chondrocytes from the presumptive calcification region showed a similar pattern of radiolabeled proteins in the medium, but also produced a large amount of G collagen that was not produced by cells of the permanent cartilagenous zone (Fig. 6*a*).

A similar predominance of G collagen in the cell/matrix extracts of cultures of chondrocytes from the presumptive calcification region and its absence in parallel extracts of cultures from the permanent cartilagenous region is demonstrated in Figs. 6, *b* and *c*. Since pepsin digestion results in a decrease in the molecular weight of G collagen from 59,000 to 45,000 (11) the latter species is observed in both the pepsin and NaCl/Tris extracts of the matrix. Cultures of both types of chondrocytes, particularly cultures within collagen gels, also contained radiolabeled proteins with a mobility on PAGE similar to the 3 $\alpha$ , 2 $\alpha$ , 1 $\alpha$  species described by Burgeson and Hollister (5) (Fig. 6*c*).

Since 90% (93  $\pm$  7% for 90 samples analyzed) of the radiolabeled material synthesized by the cell cultures was recovered in the medium and extracts, we were able to determine G collagen as a percentage of total collagen synthesized after various times in culture. Regions of the polyacrylamide gel corresponding to bands on fluorograms were digested with H<sub>2</sub>O<sub>2</sub> and the proportion of radioactivity present in G collagen determined as a percentage of total radioactivity in collagen bands for each sample. To simplify measurements of G collagen in the culture medium, samples were first digested with chymotrypsin and G collagen determined as the 45,000-mol wt species.

The results of a typical experiment obtained with a single preparation of chondrocytes labeled after a number of different periods of culture is shown in Fig. 7. After brief periods of culture within collagen gels, G collagen represented only 8–12% of the total collagen synthesized by cultures of chondrocytes from the presumptive calcification region. However

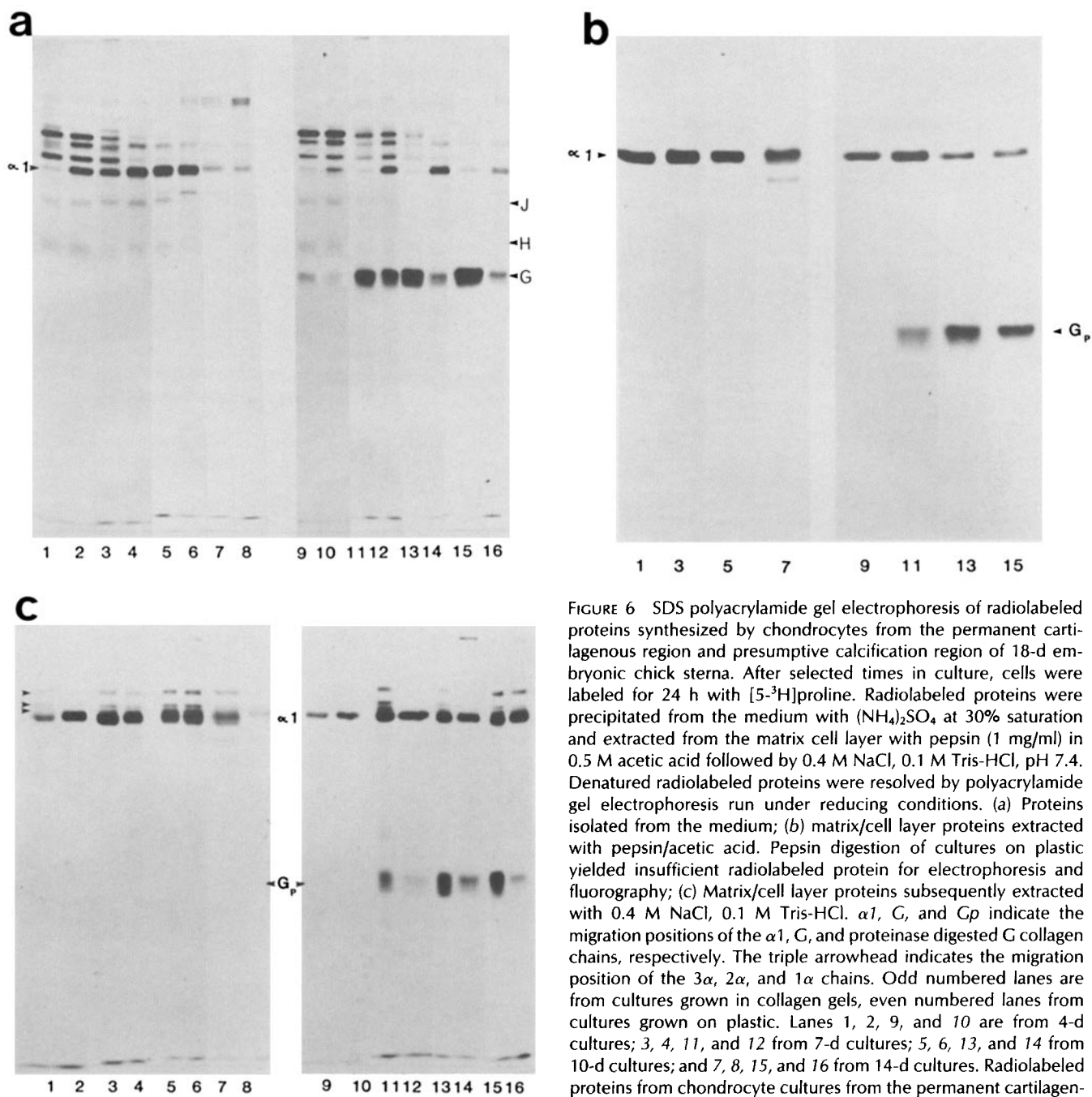


FIGURE 6 SDS polyacrylamide gel electrophoresis of radiolabeled proteins synthesized by chondrocytes from the permanent cartilagenous region and presumptive calcification region of 18-d embryonic chick sterna. After selected times in culture, cells were labeled for 24 h with [5-<sup>3</sup>H]proline. Radiolabeled proteins were precipitated from the medium with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> at 30% saturation and extracted from the matrix cell layer with pepsin (1 mg/ml) in 0.5 M acetic acid followed by 0.4 M NaCl, 0.1 M Tris-HCl, pH 7.4. Denatured radiolabeled proteins were resolved by polyacrylamide gel electrophoresis run under reducing conditions. (a) Proteins isolated from the medium; (b) matrix/cell layer proteins extracted with pepsin/acetic acid. Pepsin digestion of cultures on plastic yielded insufficient radiolabeled protein for electrophoresis and fluorography; (c) Matrix/cell layer proteins subsequently extracted with 0.4 M NaCl, 0.1 M Tris-HCl.  $\alpha 1$ , G, and G<sub>p</sub> indicate the migration positions of the  $\alpha 1$ , G, and proteinase digested G collagen chains, respectively. The triple arrowhead indicates the migration position of the 3 $\alpha$ , 2 $\alpha$ , and 1 $\alpha$  chains. Odd numbered lanes are from cultures grown in collagen gels, even numbered lanes from cultures grown on plastic. Lanes 1, 2, 9, and 10 are from 4-d cultures; 3, 4, 11, and 12 from 7-d cultures; 5, 6, 13, and 14 from 10-d cultures; and 7, 8, 15, and 16 from 14-d cultures. Radiolabeled proteins from chondrocyte cultures from the permanent cartilagenous region are shown in lanes 1–8, those from the presumptive

calcification region in lanes 9 to 16. All lanes contain the same amounts of radioactivity (10,000 cpm) except lanes 7, 8, and 16 in a, lanes 1, 7, 9, and 10 in c, which contained half this activity, and lane 8 in c, which contained 2,500 cpm. a–c show results obtained from three individual fluorograms, however, the sequence of tracks shown in each has been rearranged to simplify discussion.

after 1 wk of culture G collagen became the major collagenous species synthesized in culture within collagen gels. This high level of G collagen synthesis is maintained for ~7 more days of culture, after which time chondrocytes synthesized a much smaller proportion of G collagen, appeared more “fibroblastic” and began to synthesize significant quantities of Type 1 collagen as demonstrated by the appearance of the  $\alpha 2(1)$  band on PAGE. Chondrocytes from the presumptive calcification zone cultured on plastic synthesized a smaller proportion of G collagen and only after longer periods of culture. At no time were detectable quantities of G collagen found in the

medium or matrix extracts of cultures of chondrocytes from the permanent cartilagenous region.

Some variation in the amount of G collagen synthesized was observed between different batches of chondrocytes. Six separate preparations of chondrocytes grown for 1 wk within collagen gels synthesized an average of 65 ± 16% G collagen (Table I) and 30 ± 7% after 1 wk culture on plastic. Although considerable variation in the amount of G collagen found in the matrix/cell layer was observed between individual preparations of chondrocytes at all times in culture, cultures within collagen gels always contained a higher proportion of G



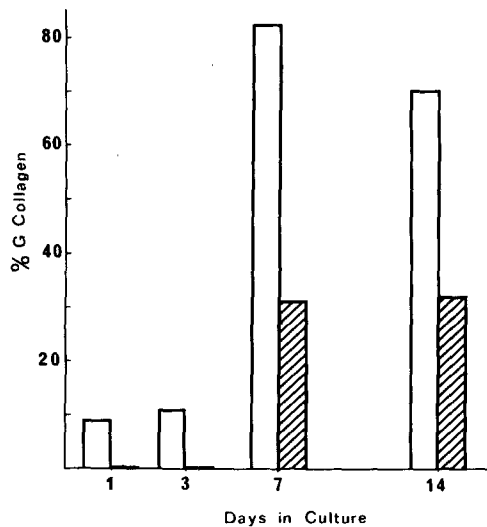


FIGURE 7 The effect of culture duration, within collagen gels and on plastic, on the synthesis of G collagen by chondrocytes isolated from the presumptive calcification region. After selected times in culture, cells were incubated with [5-<sup>3</sup>H] proline for 24 h. Radiolabeled proteins isolated from the medium or extracted from the matrix/cell layer were resolved by PAGE as shown in Fig. 6. Bands revealed by fluorography were cut from dried gels and the radioactivity present determined by scintillation counting after digestion with H<sub>2</sub>O<sub>2</sub>. The total radioactivity present in G collagen is expressed as a percentage of the total radioactivity present in 3 $\alpha$ , 2 $\alpha$ , 1 $\alpha$ ,  $\alpha$ 1, and G collagen bands in the three extracts. The results shown were obtained from a single preparation of chondrocytes. Chondrocytes isolated from the permanent cartilagenous region synthesized no detectable G collagen after the culture times examined. □, cultures within collagen gels; ▨, cultures upon plastic.

TABLE I  
G Collagen Synthesis and Distribution in Cultures of Presumptive Calcification Region Chondrocytes

	Culture Type	
	Collagen	Plastic
%		
G collagen synthesized* in matrix <sup>‡</sup>	65 ± 16 35 ± 20 n = 6 <sup>§</sup>	30 ± 7 28 ± 17

Chondrocytes isolated from the presumptive calcification region of chick sterna were incubated with medium containing (5-<sup>3</sup>H)proline for 24 h after 7-d culture within collagen gels or on plastic.

The ratios of G to type II collagen have not been adjusted for differences in the respective imino acid contents because we are currently unsure which species of G collagen is present in the matrix. Schmid and Linsenmayer (29) have shown that the 45,000-mol wt species contains ~10% more imino acid and the 59,000-mol wt species ~10% less imino acid than type II collagen. Consideration of these differences would result in either a decrease or increase, respectively, of ~10% in ratios shown.

\* The relative incorporation of radioactivity into G collagen is expressed as a percentage of the incorporation into total collagen (i.e., 3 $\alpha$ 2 $\alpha$ 1 $\alpha$ ,  $\alpha$ 1, and G).

<sup>‡</sup> The radioactivity associated with G collagen extracted from the cell/matrix layer is expressed as a percentage of the total radioactivity incorporated into G collagen.

<sup>§</sup> Results expressed are averages with standard deviation obtained from six preparations of chondrocytes.

collagen in the cell/matrix layer than concurrent cultures on plastic (35 ± 20% cf. 28 ± 17%). Results obtained with chondrocytes cultured within collagen gels were indistinguishable from those obtained with chondrocytes cultured upon collagen gels.

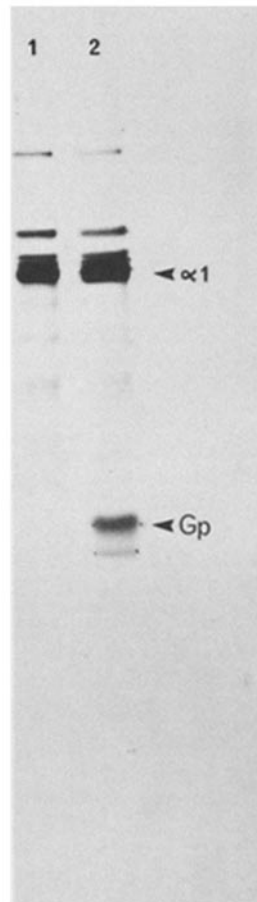


FIGURE 8 SDS PAGE of radiolabeled proteins synthesized by slices of sterna in culture. The presumptive calcifying regions and permanent cartilagenous regions of ten 18-d embryonic sterna were finely sliced and incubated in medium containing [5-<sup>3</sup>H] proline. Radiolabeled proteins were isolated from the medium by (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitation and extracted from the matrix with pepsin followed by 0.4 M NaCl/0.1 M Tris pH 7.4. More than 80% of the radiolabeled proteins obtained were found in the NaCl/Tris extract. The results of PAGE of these proteins is shown. Lanes 1 and 2 show radiolabeled proteins extracted from the permanent cartilagenous and presumptive calcification regions, respectively.  $\alpha$ 1 and Gp indicate the migration positions of the  $\alpha$ 1 and proteinase digested G collagen chains, respectively. Both lanes contain the same amount of radioactivity.

### Type of Collagen Synthesized by Slices of Sternal Cartilage in Culture

A number of sterna from 18-d embryonic chicks were divided into presumptive calcification and permanent cartilagenous regions, sliced and cultured for 24 h in the presence of [<sup>3</sup>H]proline. Greater than 90% (95.0 ± 3.4 for 10 preparations) of the radiolabeled polypeptides were recovered from the sterna using the techniques described for the cell cultures: of this more than 80% was found associated with the tissue. A fluorogram obtained from PAGE of denatured radiolabeled protein extracted from the cultured pieces of sterna is shown in Fig. 8. In addition to  $\alpha$ 1 chains and proteins migrating in the position of 3 $\alpha$ , 2 $\alpha$ , and 1 $\alpha$  chains, pieces of sterna from the presumptive calcifying region synthesized significant amounts of G collagen (seen as the 45,000-mol wt species in these extracts). G collagen was determined to represent 30% of the total collagen synthesized by slices of the presumptive calcification region over the 24-h period. Sternal segments from the permanent cartilagenous region synthesize only type II collagen and the minor cartilage collagen chains.

### DISCUSSION

The embryonic chick sternum contains a well delineated cephalic region that becomes fully ossified by an endochondral calcification process and a caudal region that remains cartilagenous at least until maturity. In this study we have shown that chondrocytes isolated from each region exhibit a number of distinct morphological and biochemical characteristics when cultured on plastic or upon or within collagen gels.

Light and scanning electron microscopy have shown that both populations of chondrocytes maintain an appearance in culture closely resembling that *in vivo* (Fig. 4). Chondrocytes from the caudal region of the sternum are smaller than those cells from the cephalic region which resemble the hypertrophic cells of developing chick tibia (14, 20). Culture of chondrocytes on collagen gels has also facilitated an examination of their accumulated matrix by scanning electron microscopy, which in support of light microscopy, has shown that the two populations of chondrocytes accrete distinctly different extracellular matrices in culture (Fig. 4).

Both populations of chondrocytes showed very similar cell proliferation when cultured on plastic or within or upon collagen gels. It is significant that very little fibronectin could be removed by gelatin-agarose affinity chromatography (data not shown), from the batches of serum used in the preparation of medium for these cultures, since previous studies (11) had shown that the presence of significant amounts of fibronectin caused chondrocytes, isolated from whole sterna, to reach a higher saturation density when cultured on plastic than when cultured in collagen gels, or when cultured on plastic in the absence of fibronectin.

It is perhaps surprising that chondrocytes isolated from the presumptive calcification region divide in culture to reach a saturation density only slightly lower than that of cultures of chondrocytes from the permanent cartilagenous region as intuitively as it would be expected that chondrocytes present in the presumptive calcification zone of the 18d-embryonic chick sterna would have passed through the stage of cell division and would have become committed to a course of development that results in the synthesis of a calcified matrix and eventually chondrocyte lysis. Similar cell division was, however, noted in chondrocytes isolated from the hypertrophic zone of the developing 12-d chick embryo tibiotarsus (16).

Chondrocytes from the presumptive calcifying region showed a very similar rate of collagen synthesis whether cultured on plastic or upon or within collagen gels. Chondrocytes from the permanent cartilagenous region, in contrast, exhibited a rate of synthesis that was closely dependent upon the culture substrate used (Fig. 5). Culture of these chondrocytes on plastic and to a lesser extent culture upon collagen gels promoted noncollagenous protein synthesis in favor of collagenous protein synthesis. However, the rate of collagen synthesis within collagen gels was similar to that obtained with chondrocytes isolated from the presumptive calcification region of the sternum. The relative increase in collagen synthesis observed with permanent cartilagenous region chondrocytes cultured within collagen gels is consistent with the apparent stabilization of phenotypic expression with this culture system. Chondrocytes cultured on plastic consistently exhibited a transition to type I collagen synthesis as demonstrated by the appearance of the  $\alpha 2$  (1) chain on PAGE at earlier culture times than similar cultures within collagen gels (Fig. 6a).

Both chondrocyte types synthesized type II collagen and its precursors as well as the  $3\alpha$ ,  $2\alpha$ ,  $1\alpha$  species and H and J collagens in culture. Recent electron microscope studies indicate that articular cartilages (21) contain a number of distinct matrices, a pericellular matrix confined by a pericellular capsule, and more distant from the cell, a territorial and an interterritorial matrix. Recent reports (7, 25) have described the localization, by immunohistochemistry, of collagenous

peptides, which we believe may be related to the H and J collagens, to the pericellular matrix or pericellular capsule. Preferential synthesis of the pericellular matrix during the first few days of cell culture may account for the higher proportion of H and J collagens observed at these times (Fig. 6a).

The ability to synthesize G collagen most clearly distinguished the two chondrocyte types. Chondrocytes isolated from the presumptive calcification region were found to synthesize large quantities of G collagen in culture, particularly when grown within collagen gels. This was detected in both the medium and matrix. However, chondrocytes from the permanent cartilagenous region produced no G collagen. Schmid and Conrad (27, 28) have recently shown that chondrocytes isolated from the hypertrophic zone of the 12-d embryonic chick tibiotarsus also synthesize a low molecular weight collagen, termed 59 K collagen and apparently identical to G collagen, in monolayer culture. However, in contrast to the present results 59 K collagen in their sample was present in smaller amounts (8–10% of the total collagen synthesized) and was found almost exclusively in the culture medium. This may have been due to difference in the source of chondrocytes or to the age of the cultures examined for, in some experiments, we also found that G collagen was confined to the culture medium and accounted for <15% of the total newly synthesized collagen in 3 to 5-d cultures of sternal chondrocytes on plastic. We also noted that G collagen tended to be localized in the medium of early cultures of sternal chondrocytes within collagen gels (Fig. 6a). The inclusion of lathyrogen in the culture medium used by Schmid and Conrad probably also influenced the distribution of G collagen observed.

The level of G collagen synthesis observed with presumptive calcification region chondrocytes cultured for 7 d on plastic was similar to that found when slices of the presumptive calcification region of the embryonic sterna were incubated with labeled proline *in vitro*. Much greater quantities of G collagen, however, were always observed when chondrocytes were cultured within collagen gels (Figs. 6 and 7). A stimulation of G collagen synthesis was found previously when chondrocytes isolated from whole sterna were cultured within collagen gels (11), however the data reported here show that G collagen can become the major collagenous species synthesized by presumptive calcification region chondrocytes cultured within collagen gels, and may represent 50–80% of the total collagen synthesized. Similar high levels of G collagen synthesis have recently been reported by Schmid and Linsenmayer (29) after long term secondary culture of chick growth plate chondrocytes on plastic.

The similarity in growth curves of chondrocytes isolated from the presumptive calcification and permanent cartilagenous regions suggest that enhanced G collagen synthesis noted in the presumptive calcification region chondrocytes is not due to an overgrowth by a specific cell type. The greater rate of total collagen synthesis observed in chondrocytes isolated from the presumptive calcification region suggests that the relative increase in G collagen synthesis is not due to the inhibition of synthesis of the other collagens. It is possible that a lower oxygen tension that may be experienced by chondrocytes embedded within collagen gels in culture may, either directly or via an influence on cell development, stimulate G collagen synthesis. Low oxygen tension has been shown in regions of hypertrophic chondrocytes of the epiphyseal plate (4) and has been suggested to influence chondrocyte



development and the calcification process (3, 30), however, since chondrocytes cultured upon collagen gels synthesized the same levels of G collagen as those cells cultured within collagen gels and presumably experience an O<sub>2</sub> tension similar to those chondrocytes cultured on plastic, it is unlikely that the stimulation of G collagen synthesis observed is solely due to change in O<sub>2</sub> tension.

The stimulation of G collagen synthesis induced by culture within collagen gels may be a direct response to an imposed "foreign" matrix, i.e., a type I collagen substrate, since in normal physiological situations the only type I collagen encountered by chondrocytes *in vivo* is that produced by invading osteoblasts at the final stages of endochondral ossification. The observation that G collagen does not become the major collagenous species synthesized by chondrocytes until after 6 or 7 d of culture within collagen gels, or long term secondary culture on plastic (29), suggests that the expression of G collagen synthesis in these culture conditions is associated with an acceleration or maintenance of normal chondrocyte development already initiated *in vivo*. Schmid and Conrad (28) suggest that 59 K collagen (G collagen) synthesis is associated with the latter stages in the endochondral development of chondrocytes, since its synthesis by segments of the embryonic chick tibiotarsus, incubated *in vitro*, is confined to hypertrophic chondrocytes near the calcification front. These authors have also shown that the more "immature" chondrocytes from zones of elongated or resting chondrocytes of the embryonic chick tibiotarsus can be induced to synthesize G collagen in cell culture by prior subculture (28).

Investigations of the influence of substrates on the behavior of cells in culture has shown that the growth of various different cell types on or within collagen gels promotes the expression of phenotypic characteristics observed *in vivo* (8, 26, 35) and decrease the tendency of some cells to change their phenotypic expression with extended time in culture (26). Solorsh et al. (33) have also shown that the culture of chick wing bud mesenchyme cells on or within collagen gels promotes their differentiation into cartilage. If the development of presumptive calcification region chondrocytes is also enhanced by culture upon or within collagen gels then we may expect these cultures to contain large numbers of cells close to the final stages of their development. Since these cultures produce predominantly G collagen we may also expect hypertrophic chondrocytes to produce a matrix containing predominantly G collagen just prior to their lysis *in vivo*. A recent report by Remington et al. (24) describing the identification of G collagen and a possible higher molecular weight form of this molecule in organ cultures of the hypertrophic region of rabbit costochondral growth plate confirms the association of hypertrophic chondrocytes with G collagen synthesis and demonstrates that its synthesis is not restricted to the chick.

Thus the synthesis of G collagen is confined to chondrocytes involved in the process of endochondral calcification and may be associated with either one or more of the events occurring in the later stages of endochondral development: calcification, vascular invasion, or woven bone formation. A closer examination of the relationship between G collagen synthesis and the onset of calcification in the embryonic chick sterna is in progress.

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