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Isolation and culture techniques of foetal calf chondrocytes

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Large quantities of differentiated mammalian chondrocytes from normal hyaline cartilage were isolated after digestion of foetal bovine tracheas with collagenase. Incubation of the newly isolated cells for 1 day in the presence of dextran sulphate inhibited formation of cell aggregates during subsequent subculture in the absence of dextran sulphate. After incubation with dextran sulphate, the cells were plated in Ham's F12 medium with or without foetal calf serum on hydrophilic or hydrophobic Petri dishes. Chondrocytes cultured on hydrophilic substrates in the presence of serum attached to the substrate and showed cytoplasmic spreading. The cells did not attach to hydrophobic substrates in the presence of serum, but remained in suspension as single cells. In the absence of serum the chondrocytes attached to either substrate, but did not show any cytoplasmic spreading. By using labelling with [^{35}S]sulphate and [^{3}H]-thymidine it was shown that glycosaminoglycan synthesis did not require the presence of serum, whereas DNA synthesis required serum factors. Extracellular glycosaminoglycans were recovered in two pools: the medium pool and the pericellular pool, the latter being isolated by proteolytic digestion. The kinetics of these pools differed, depending on the presence or absence of serum and the type of substrate used. The turnover of the pericellular pool was studied in a pulse–chase experiment. At the end of the chase (72h), only 60% of the material in the pericellular pool had been metabolized.

In recent years a number of details of the cartilage proteoglycan structure have been revealed (for references see Hascall & Heinegård, 1979). The progress made provides the necessary information for investigation of the different steps involved in the biosynthesis of proteoglycans.

Earlier investigations have shown that the synthesis of sulphated glycosaminoglycans by chondrocytes depends on the environment to which the cells adapt (Coon & Cahn, 1966; Abbott & Holtzer, 1966; Nevo *et al.*, 1972; Srivastava *et al.*, 1974; Müller *et al.*, 1977; Wiebkin & Muir, 1977; Malemud, 1978; Madsen & Lohmander, 1979). In the living tissue the cell is surrounded by a matrix, which is destroyed and removed during cell isolation. The relationship between the isolated cell and its microenvironment, then, is most probably disturbed. Therefore the environmental characteristics of the culture system become critical. There are three environmental parameters to consider: the culture medium, the cell density and the substrate.

Depending on the physical properties of the substrate, the cells might attach to it and eventually form a monolayer, or alternatively remain in suspension in the culture medium. Cell–substrate interactions affect DNA synthesis and may also change the pattern of the cell products, e.g. collagen (Müller *et al.*, 1977; von der Mark & von der Mark, 1977) or proteoglycans (Srivastava *et al.*, 1974; Madsen & Lohmander, 1979) synthesized.

In the present paper a procedure for isolating tracheal chondrocytes from bovine foetuses is described. The production of sulphated glycosaminoglycans was studied in primary cultures of these chondrocytes, maintained (*a*) at different cell densities, (*b*) in medium that either contained serum or was serum-free, or (*c*) on substrates with different adhesion properties.

Materials

All chemicals used were of analytical grade. The following enzymes and chemicals were used for cell cultures: collagenase CLS (Worthington Biochemical Corp., Freehold, NJ, U.S.A.), trypsin (type III, 2× crystallized) (Sigma Chemical Co., St. Louis, MO, U.S.A.), hyaluronidase (type I) (Sigma), papain (2× crystallized, in a suspension containing 25mg of protein/ml) (Sigma), Ham's F12 medium (GIBCO, Grand Island, NY, U.S.A.), Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] (Sigma),

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foetal calf serum (Flow Laboratories, Rockville, MD, U.S.A.), dextran sulphate (Pharmacia Fine Chemicals, Uppsala, Sweden), carrier-free [^{35}S]-sulphate (The Radiochemical Centre, Amersham, Bucks., U.K.), penicillin (KABI, Stockholm, Sweden), streptomycin (Glaxo, Greenford, Middx., U.K.) and Instagel (Packard Instrument Co., La Grange, IL, U.S.A.). Two types of Petri dishes were used: (1) hydrophobic Falcon Standard Petri dishes no. 1008 (35mm inside diam.) and no. 1007 (60mm inside diam.), and (2) hydrophilic Falcon Tissue Culture Petri dishes no. 3001 (35mm inside diam.) and no. 3003 (60mm inside diam.) (Falcon Plastics, Oxnard, CA, U.S.A.). Calf foetuses 7–9 months old were obtained from a local abattoir within 1–2h after slaughter.

Culture media

F12 medium was Ham's F12 medium buffered with 14mM-$NaHCO_3$ and containing penicillin (100i.u./ml) and streptomycin (100μg/ml), pH7.4.

F12 FCS medium was F12 medium supplemented with 10% (v/v) foetal calf serum, pH7.4.

F12 HAD medium was F12 medium supplemented with 20ml of 50× essential amino acids for Eagle's minimum essential medium (Flow)/l and dextran sulphate (20μg/ml) and buffered with 10mM-Hepes, pH7.4.

Methods

Cell isolation procedure

Tracheas were removed from bovine foetuses and stripped from their mucosal layer under aseptic conditions. After removal of the perichondrium by incubation with collagenase (2mg/ml) in 40mM-Hepes/NaOH buffer, pH7.5, containing 4.8mM-$CaCl_2$, dextran sulphate (20μg/ml), penicillin (100i.u./ml) and streptomycin (100μg/ml), for 1h at 37°C, the 'purified' cartilage was rinsed with F12 medium and cut into small pieces. Chondrocytes were subsequently isolated by collagenase (20mg/g of cartilage) digestion for 10–16h in F12 medium containing the above buffer mixture. The cells were pelleted and washed in fresh medium. Viability was tested by Trypan Blue exclusion. The cells were suspended in F12 HAD medium at a concentration of 0.5×10^6cells/ml and transferred to a 500ml spinner culture flask. Preincubation was then performed at 37°C for 1–2 days with continuous stirring at 60rev./min.

Culture techniques

Cells were collected by centrifugation, washed and suspended in F12 medium or F12 FCS medium. Cells were cultured at 0.5×10^6cells/ml, corresponding to 1.0×10^5cells/cm^2, unless otherwise stated. Two types of substrates were used: Falcon Standard Petri dishes (hydrophobic) or Falcon Tissue Culture Petri dishes (hydrophilic). The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air.

The cultures were harvested as follows. The medium was centrifuged at 170*g* for 5min to remove cells still in suspension. The supernatant was decanted and saved. The Petri dish was washed once with fresh medium (3ml), the wash was centrifuged and the supernatant was pooled with the previous supernatant. The cell pellet was suspended in a collagenase-containing buffer (Ham's F12 medium with 2mg of collagenase and 2mg of trypsin added per ml), and transferred back to the Petri dish and incubated for 30min at 37°C on a shaker. The cell viability after digestion exceeded 95% as judged by Trypan Blue exclusion. The cells were pelleted and washed once as described above, and the supernatants were pooled. Cells, digest and medium were kept frozen until analysed. Continuous-pulse experiments were performed by adding [^{35}S]sulphate (2.5μCi/ml) or [^{3}H]thymidine (1μCi/ml) to the medium after the cells had been plated on Petri dishes. Pulse–chase experiments were performed as follows. Cultures were labelled for 3 days with carrier-free [^{35}S]sulphate (0.2μCi/ml). The chase was then initiated by adding 0.6ml of 0.22M-Na_2SO_4 in F12 medium also containing 6μCi of [^{3}H]-glucosamine to the 6ml cultures. Cells were harvested as above and then incubated for 15min at 37°C with trypsin (2mg/ml) to solubilize cell-associated material.

Determination of synthesis of glycosaminoglycans, collagen and DNA by cells cultured in different environments

The glycosaminoglycans synthesized by the cells were quantified as the amounts of hexosamines and [^{35}S]sulphate that could be precipitated with cetylpyridinium chloride from papain digests of the fractions obtained as described above. The following procedure was used. To all samples 0.1vol. of a papain digestion solution (0.5M-sodium acetate/40mM-EDTA/50mM-cysteine hydrochloride/0.1M-Na_2SO_4, pH5.0, containing 50μl of suspension of crystalline papain/ml) was added, and digestion with papain was performed at 65°C for 5h. The glycosaminoglycans in the samples were then precipitated with 1ml of aq. 1% (w/v) cetylpyridinium chloride as described by Antonopoulos *et al.* (1964). The precipitates were hydrolysed in 6M-HCl for 8h at 100°C and samples were taken for hexosamine determinations (Antonopoulos *et al.*, 1964). Other samples were mixed with Instagel (2ml of sample plus 3ml of Instagel) and ^{35}S radioactivity was determined. Samples for collagen determination were freeze-dried and then hydrolysed in 6M-HCl at

100°C for 24h. Hydroxyproline was determined by the method of Stegeman & Stalder (1967).

DNA synthesis in the cell cultures was determined as incorporation of [^{3}H]thymidine. Cells were recovered, precipitated and washed with cold 5% (w/v) trichloroacetic acid. After hydrolysis of the precipitate in 6% trichloroacetic acid (1ml) for 15min at 90°C a 500μl sample of the clear supernatant was added to Instagel (5ml) for ^{3}H determination.

DNA was determined as follows. The cell pellet was lysed in 1M-NaOH at 37°C for 1h. DNA was precipitated and washed with cold 2M-$HClO_4$ and hydrolysed in 1.5M-$HClO_4$ (1.2ml) for 30min at 70°C. Two 500μl samples of the clear supernatant were assayed for DNA by the method of Richards (1974).

Results and discussion

Cell isolation procedure

Tracheas were obtained from bovine foetuses during the last trimester of gestation. At this age the cartilage proteoglycans are similar to those of the adult animal (S. Inerot & D. Heinegård, unpublished work). About 10g of cartilage was obtained from one trachea. Collagenase, at a concentration of 2mg/ml (250–400 units/ml), was required for the liberation of cells from the matrix. Trypsin was not as efficient as collagenase and had no additional effect on the rate of cartilage digestion, when used together with collagenase. In an attempt to shorten the time of the digestion, the concentration of collagenase was raised or hyaluronidase, at two different concentrations, was added to the digestion solution (Table 1). There was no effect on the time course of the digestion, although the viability of the cells was lowered at high concentrations of either collagenase or hyaluronidase. In conclusion, we found that the cell viability was less affected by the duration of the digestion than by using higher concentrations of the enzymes. The decrease in pH during the extended digestion was kept within tolerable limits (pH 7.4–7.2) by incubating no more than 0.1g of cartilage/ml of Earle's balanced salt solution buffered with Hepes (Shipman, 1969).

Preliminary experiments showed that the cells aggregated during the isolation procedure. Therefore dextran sulphate, which inhibits cell–substrate adhesion (Bremerskov, 1973), was tried and indeed inhibited cell aggregation completely, when used at a concentration of 20μg/ml in the digestion buffer.

The time required for the solubilization of the tissue could vary from 10 to 16h, depending on the age of the foetus and the size of the cartilage pieces. Attempts to isolate cells from older bovine cartilage were not successful. Both yield (2×10^6cells/g) and viability (50%) of cells isolated from calf or adult tracheal cartilage were insufficient for use in primary cultures.

Preincubation

The removal of cell-surface components by proteolysis during the digestion influences cell–cell interactions and substrate adhesion. Freshly isolated cells that were kept in a spinner flask rapidly formed aggregates when removed during the first day and subcultured (Kuroda, 1964), whereas cells preincubated for 1 day or more in the presence of dextran sulphate (Bremerskov, 1973; Kuroda, 1974) remained as single cells during the subculture. No differences were observed in the quantities of glycosaminoglycans or their distribution between medium and cells with regard to the time spent in incubation with dextran sulphate. Isolated chondrocytes were always incubated in the presence of dextran sulphate for at least 1 day before being used in an experiment.

Primary culture of isolated chondrocytes

The composition of the medium, the type of the substrate and the cell density were varied as follows. (1) Cultures were maintained either in Ham's F12 medium with no supplements or in Ham's F12 medium supplemented with 10% (v/v) foetal calf serum. (2) Cells were plated either on hydrophilic plastic Petri dishes for use in tissue culture (hydrophilic substrate) or on standard hydrophobic Petri dishes (hydrophobic substrate). (3) Cells were plated

Table 1. *Digestion of cartilage with use of different combinations of enzymes to liberate the cells*
For experimental details see the text.

Expt.	Digestion solution (enzyme concentration)	Yield (10^{-6} × no. of cells/g of cartilage)	Viability (%)
1	Collagenase (2mg/ml)	43.3	95
2	Collagenase (2mg/ml)	36.8	97
3	Collagenase (2mg/ml)	20.8	94
4	Collagenase (4mg/ml)	25.4	80
5	Collagenase (2mg/ml) + hyaluronidase (2mg/ml)	22.2	96
6	Collagenase (2mg/ml) + hyaluronidase (5mg/ml)	19.2	83

at cell densities of 2.0×10^5, 1.0×10^5 and 0.5×10^5 cells/cm^2. In all cases 0.2 ml of medium/cm^2 was used. Therefore the concentrations of cells were 1.0×10^6, 0.5×10^6 and 0.25×10^6 per ml respectively.

Cultures maintained on hydrophilic substrates in F12 FCS medium showed the characteristics of traditional monolayer cultures of chondrocytes. A large proportion of the newly plated cells attached to the dish, although a number of cells remained in suspension as 'primary floaters'. The cells that attached to the dish often showed excessive cytoplasmic spreading in areas with low cell density, whereas cells in areas with high cell densities remained round or polygonal. Whether this heterogeneity reflects differences in the biosynthesis of macromolecules is uncertain.

The chondrocytes in cultures maintained on hydrophobic substrates in F12 FCS medium did not attach and remained in suspension. In contrast with monolayer cultures, the chondrocytes in suspension cultures were evenly distributed and all the cells had the same rounded shape.

The cells cultured in F12 medium attached very rapidly to both hydrophilic and hydrophobic substrates in the absence of serum. The cells did not spread their cytoplasm, but remained round and appeared similar to cells cultured in suspension.

Glycosaminoglycan synthesis

Glycosaminoglycan synthesis was studied with a continuous pulse of [^{35}S]sulphate under the different culture conditions described above. The cultures were harvested at days 0, 2, 4 and 6 as described in the Methods section, and the accumulation of [^{35}S]sulphate-labelled glycosaminoglycans and hexosamine was determined.

Monolayer cultures on hydrophilic substrates with F12 FCS medium. Extracellular glycosaminoglycan hexosamine was found in two pools with different kinetics (Fig. 1*a*). One pool was soluble and withdrawn with the culture medium: the medium pool. The other was closely associated with the cells and was recovered only after solubilization with proteolytic enzymes: the pericellular pool. Very small amounts of the glycosaminoglycans were associated with the cells subsequent to such treatment: the cellular pool (results not shown). The accumulation of [^{35}S]sulphate-labelled glycosaminoglycans in the medium increased with time for 6 days (Fig. 1*a*). In this type of culture a fairly large proportion of the newly synthesized extracellular glycosaminoglycans (about 50%) was retained in the cell layer, rather than secreted into the medium.

The incorporation of [^{35}S]sulphate per 10^6 cells in the medium pool as a function of plating cell density is shown in Fig. 2(*a*). The increased rate of synthesis with time of culture can be explained in two ways: either the cells increased their synthesis as they adapted to the environment *in vitro*, i.e. by releasing factors that stimulate glycosaminoglycan synthesis (Solursh & Meier, 1973), or the number of cells increased during the culture period. The decreased rate of synthesis seen at the highest cell concentration might be due to lack of nutritional components as the medium became exhausted at the end of the culture period. The amount of [^{35}S]-sulphate in the cellular pool (not shown in the Figure) was very low, being about 1% of the total [^{35}S]sulphate incorporated into polymers at day 6.

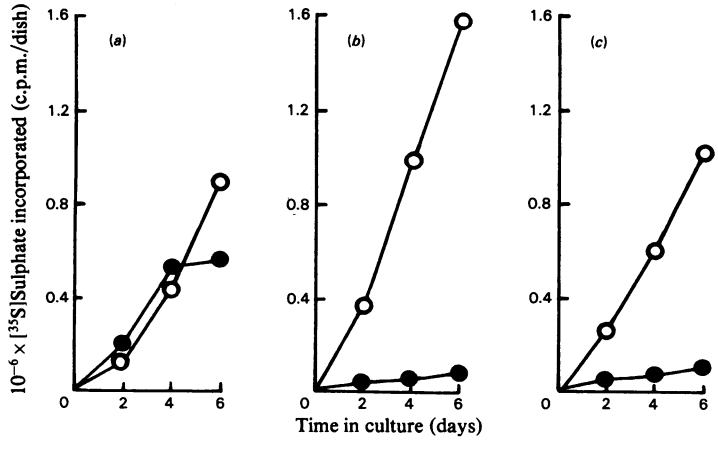

Fig. 1. *Incorporation of [^{35}S]sulphate into glycosaminoglycans in different types of cultures*
Experimental details are given in the text. (*a*) Cultures maintained in F12 FCS medium on hydrophilic substrates. (*b*) Cultures maintained in F12 FCS medium on hydrophobic substrates. (*c*) Cultures maintained in F12 medium on hydrophobic substrates. ○, Medium pool; ●, pericellular pool.

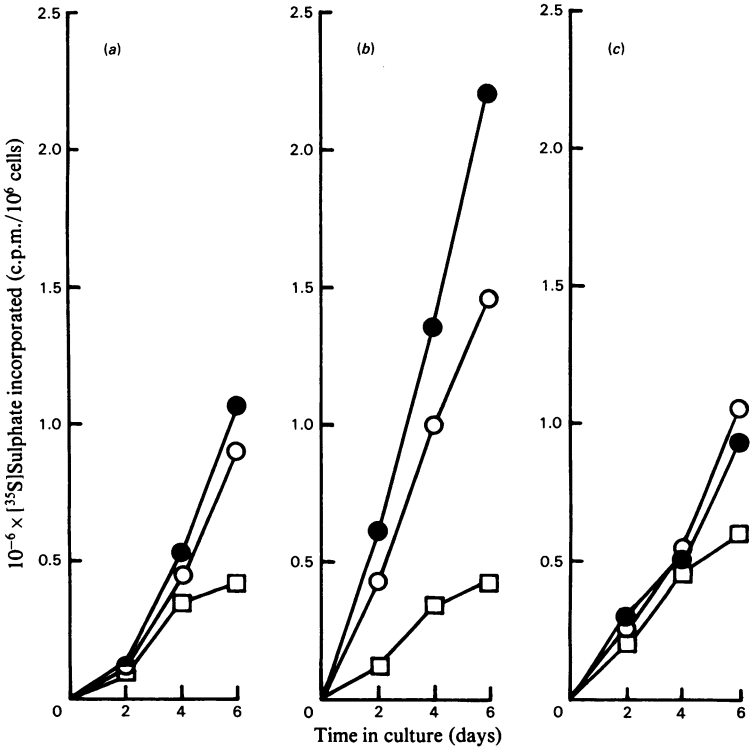

Fig. 2. *Incorporation of [^{35}S]sulphate into glycosaminoglycans in different types of cultures*
Experimental details are given in the text. The cultures are the same as those described in Fig. 1. (*a*) Cultures maintained in F12 FCS medium on hydrophilic substrates. (*b*) Cultures maintained in F12 FCS medium on hydrophobic substrates. (*c*) Cultures maintained in F12 medium on hydrophobic substrates. □, 1.0×10^6 cells/ml; ○, 0.5×10^6 cells/ml; ●, 0.25×10^6 cells/ml.

Suspension cultures on hydrophobic substrates with F12 FCS medium. The accumulation of the medium pool was linear with time at 0.25×10^6 and 0.5×10^6 cells/ml, whereas it levelled off between days 4 and 6 at 1.0×10^6 cells/ml (Figs. 1*b* and 2*b*). At a calculated cell concentration of 0.5×10^6 cells/ml the medium contained about 15 μg of glycosaminoglycan hexosamine/ml or 1.5 μg of glycosaminoglycan hexosamine/μg of DNA at day 6 (Fig. 3*a*). The amount of DNA increased slowly during the culture period. Notably, the rate of synthesis of glycosaminoglycans was inversely related to the cell concentration (Fig. 2*b*). The kinetics of glycosaminoglycan accumulation in the pericellular pool were different from that of monolayer cultures with serum (Fig. 1*b*). The proportion of the extracellular glycosaminoglycans recovered in the pericellular pool was smaller (approx. 5% of the total) in this type of culture. The initial content of glycosaminoglycan hexosamine was 0.035 μg/μg of DNA and increased 3-fold during the culture period. The high starting value may be due to the build-up of this pool during the preincubation performed before seeding of the cells on Petri dishes.

Serum-free monolayer cultures on hydrophobic substrates. Even in the absence of serum the cells still synthesized glycosaminoglycans, although the chondrocytes had not been exposed to serum proteins at any time during the isolation procedure. The accumulation of glycosaminoglycans in the medium pool, measured as incorporation of [^{35}S]-sulphate (Fig. 1*c*) or glycosaminoglycan content (Fig. 3*b*), increased linearly with time. Only at the highest cell density (1.0×10^6 cells/ml) was there a somewhat decreased rate of glycosaminoglycan synthesis between days 4 and 6 (Fig. 2*c*). The amount of hexosamine synthesized per μg of DNA in serum-free cultures was about 85% of that in suspension cultures at day 6. Serum had only a moderately stimulatory effect on glycosaminoglycan synthesis. DNA initially decreased to 75% of that at the beginning of the culture period but remained constant after day 2 (Fig. 3*b*). The pericellular pool (Fig. 3*b*) also increased linearly.

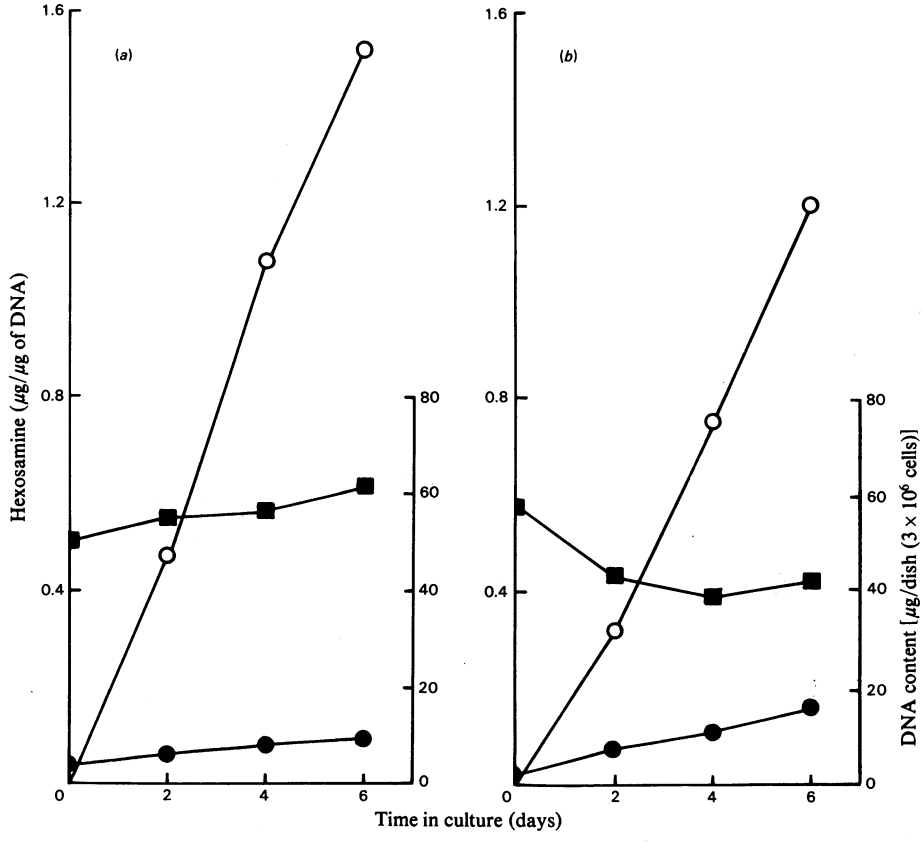

Fig. 3. *Accumulation of hexosamine relative to DNA content in different types of cultures*
Experimental details are given in the text. (*a*) Cultures maintained in F12 FCS medium on hydrophobic substrates. (*b*) Cultures maintained in F12 medium on hydrophobic substrates. O, Hexosamine in medium pool; ●, hexosamine in pericellular pool; ■, DNA content.

The proportion of glycosaminoglycans in the pericellular pool in serum-free cultures was lower compared with that in monolayer cultures in the presence of F12 FCS medium. It is likely that less material was trapped between the single cells of the serum-free cultures than in areas with high cell density of the monolayer cultures.

Turnover of the pericellular and cellular pools

A pulse–chase experiment was initiated by adding a large excess of unlabelled sulphate rather than a complete change of medium, which might induce shedding of the cell-associated glycosaminoglycans. The efficiency of the chase was acceptable, since the amount of [^{35}S]sulphate-labelled glycosaminoglycans in the medium pool remained constant during the time period studied (Fig. 4*a*). The incorporation into glycosaminoglycan of [^{3}H]glucosamine, added during the chase, increased linearly (Figs. 4*a* and 4*b*). Therefore the chase procedure did not inhibit glycosaminoglycan synthesis. The time-dependent disappearance of [^{35}S]sulphate from the pericellular pool is shown in Fig. 4(*b*). About 60% of the [^{35}S]sulphate present at the beginning of the chase had been released by 72 h. The rate of turnover, even during the first 4 h, was too low to allow for a substantial portion of the glycosaminoglycans obtained in the medium pool to pass through the pericellular pool. Therefore it is less likely that the pericellular pool represents an intermediate site in the export of proteoglycans from the cells. Mikuni-Takagaki & Toole (1979) also found a low turnover of the cell-associated glycosaminoglycans in a similar experiment with chick-embryo chondrocytes pulse-labelled for 6 h with [^{14}C]acetate.

About 65% of the intracellular [^{35}S]sulphate present at the begining of the chase was released from the cells during 24 h (Fig. 4*c*). The slow release of [^{35}S]sulphate measured at the time intervals used in this experiment cannot be attributed to the

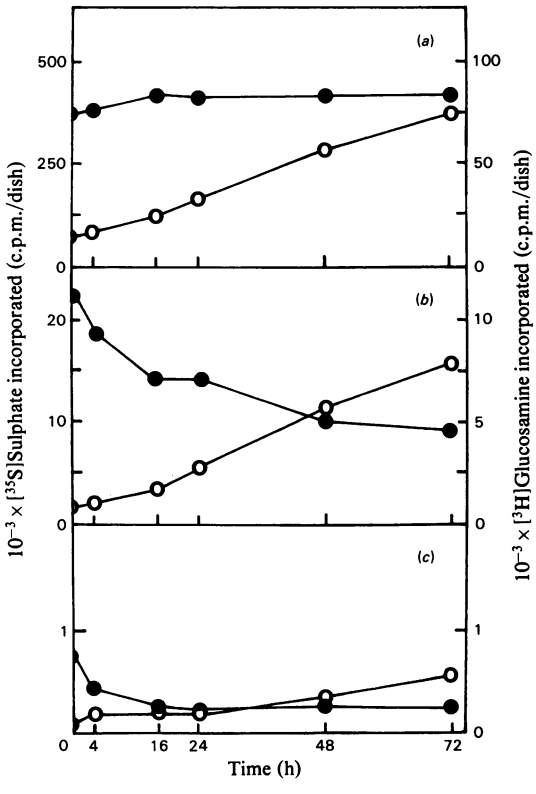

Fig. 4. *Incorporation of* [^{35}S]*sulphate and* [^{3}H]*glucosamine into glycosaminoglycans in pulse–chase experiments*

Experimental details are given in the text. (*a*) Medium pool. (*b*) Pericellular pool. (*c*) Cell pool. ●, [^{35}S]Sulphate; ○, [^{3}H]glucosamine.

turnover of the major pool of glycosaminoglycans destined for export. A considerable portion of the glycosaminoglycans for export was probably released already during the digestion with trypsin. The experiment shown in Fig. 4 was done with F12 FCS medium, but similar results were obtained with serum-free monolayer cultures.

Collagen synthesis

No collagen was detected in the medium in either suspension culture (with F12 FCS medium) or serum-free culture. The pericellular pool contained small amounts of hydroxyproline (0.25 μg/μg of DNA) at the beginning of the culture period, but it did not increase with time (6 days). It is possible that the pool of collagen acquired during the pre-incubation was maintained at the same value during the whole culture period or that the colour measured was unspecific. Since primary cultures were used, the cells might have retained a pool of ascorbic acid sufficient for a limited synthesis of collagen.

DNA synthesis

Incorporation of [^{3}H]thymidine by the cells was determined at days 0, 2, 4 and 6 as described above. Comparatively large differences were observed with different culture conditions (Fig. 5). Cells in culture that contained serum synthesized DNA (Figs. 5*a* and 5*b*), but at a lower rate when the cell density was higher. Cultures maintained on hydrophilic substrates in the presence of serum (monolayer cultures) and with a low starting density showed a linear increase in the accumulation of [^{3}H]thymidine during the time studied. At high cell density the

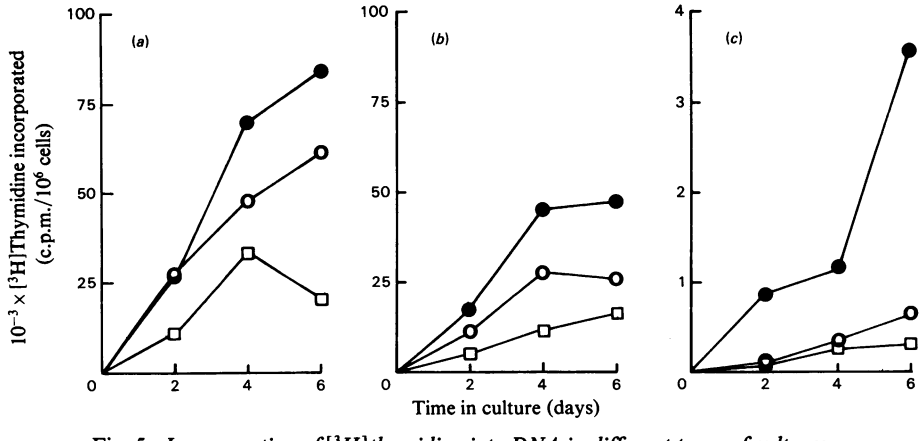

Fig. 5. *Incorporation of* [^{3}H]*thymidine into DNA in different types of cultures*

Experimental details are given in the text. (*a*) Cultures maintained in F12 FCS medium on hydrophilic substrates. (*b*) Cultures maintained in F12 FCS medium on hydrophobic substrates. (*c*) Cultures maintained in F12 medium on hydrophobic substrates. □, 2.0 × 10^{5} cells/cm^{2}; ○, 1.0 × 10^{5} cells/cm^{2}; ●, 0.5 × 10^{5} cells/cm^{2}.

rate of incorporation was lower, and incorporation levelled off at the end of the culture period when the cells became confluent (Fig. 5*a*). Cells maintained on hydrophobic substrates in the presence of serum incorporated less radioactivity and reached a maximal value after 4 days in cultures at all cell densities (Fig. 5*b*). In serum-free cultures there was negligible synthesis of DNA at all densities (Fig. 5*c*). The small amount of label incorporated was probably the result of DNA repair. It appears that the DNA synthesis in chondrocytes was more susceptible to changes in the environment, e.g. the presence of serum in the culture medium, than was the glycosaminoglycan synthesis.

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