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The Biosynthesis *in vitro* of Chondroitin Sulphate in Neonatal Rat Epiphysial Cartilage

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1. A system is described, which was used to incubate neonatal rat epiphysial cartilage *in vitro* with [U-^{14}C]glucose and [^{35}S]sulphate. 2. The acid glycosaminoglycans of neonatal rat epiphyses were extracted and fractionated on cetylpyridinium chloride–cellulose columns. The major components were chondroitin 4-sulphate (65%), chondroitin 6-sulphate (15%), hyaluronic acid (4%) and keratan sulphate (2%). 3. The acid-soluble nucleotides and intermediates of glycosaminoglycan synthesis were separated on a Dowex 1 (formate) system. The tissue contents and cellular concentrations of these metabolites were determined. 4. The rates of synthesis of UDP-glucuronic acid and UDP-*N*-acetylhexosamine from [U-^{14}C]glucose were found to be 0.79 ± 0.16 and 3.2 ± 0.08 nmol/min per g wet wt. respectively. 5. The incorporation of [U-^{14}C]glucose into the uronic acid and hexosamine moieties of the polymers was also measured and the turnover rates of the glycosaminoglycans were calculated. It was found that chondroitin sulphate was turning over in about 70h and hyaluronic acid in about 120h. 6. The relative rates of synthesis of the sulphated glycosaminoglycans were calculated from [^{35}S]sulphate incorporation and were found to be in good agreement with those obtained from [U-^{14}C]glucose labelling.

In a series of investigations of the biosynthesis of glycosaminoglycans in neonatal rat skin (Hardingham & Phelps, 1968, 1970*a*,*b*) the time-course of the incorporation of [U-^{14}C]glucose and [^{35}S]sulphate into the intermediates of polymer synthesis and into the glycosaminoglycans produced was followed.

Neonatal rat skin contains a heterogeneous population of cell types and produces a wide spectrum of acidic polysaccharides, though hyaluronic acid predominates. For this reason the present work was done with epiphysial cartilage, which is known to contain predominantly a homogeneous cell type, and produces a greatly restricted pattern of sugar polymers, of which the chondroitin sulphates comprise over 80% of the tissue content. A further complication in the experiments with neonatal rat skin was the interpretation of measurements *in vivo* of glucose disappearance after injection into animals. We have developed a system in which isolated epiphyses are incubated *in vitro* under stringently controlled conditions.

Comparison between the content of the sugar nucleotides and the flux of radioisotope through the intermediates of glycosaminoglycan biosynthesis in these two tissues would be expected to yield valuable information on the way in which a cell controls the pattern of the acidic polysaccharides that it produces.

Materials and Methods

Materials

NAD, NADP, nucleotide sugars, hexose monophosphate, fructose 1,6-diphosphate, glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and phosphoglucose isomerase (EC 5.3.1.9) were supplied by Boehringer und Soehne G.m.b.H., Mannheim, Germany. All other nucleotides, serum albumin (fraction V) and twice-crystallized papain (EC 3.4.4.10) were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Shark skin chondroitin sulphate and bovine testicular hyaluronidase (EC 3.2.1.35; 300 units/mg) were supplied by Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. Glucosamine 6-phosphate was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Heparin (150i.u./mg) was supplied by Evans Medical Ltd., Speke, Liverpool, U.K. 2,5-Bis-(5-t-butylbenzoxazol-2-yl)thiophen was obtained from Thorn Electronics Ltd., Tolworth, Surrey, U.K. D-[U-^{14}C]-Glucose (specific radioactivity 196mCi/mmol), [U-^{14}C]sucrose (specific radioactivity 9.6mCi/mmol), [^{3}H]water (specific radioactivity 1.0mCi/ml) and carrier-free [^{35}S]sulphate were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Ethanol (A.R. grade) was obtained from James

Burrough Ltd., London S.E.11, U.K. Dowex 1 (Cl^- form) was supplied by Dow Chemical International S.A., Midland, Mich., U.S.A. Whatman CC41 microgranular cellulose and Whatman 3MM chromatography paper were supplied by H. Reeve Angel and Co., London E.C.4, U.K. Silica gel G was obtained from E. Merck A.-G., Darmstadt, Germany. Polyethyleneimine-cellulose was supplied by Macherey, Nagel and Co., Düren, Germany. Oxoid cellulose acetate strips (12.0cm×1.5cm) were supplied by Oxo Ltd., London S.E.1, U.K. Alcian Blue was obtained from G. T. Gurr Ltd., London S.W.6, U.K. Sephadex G-50 (fine grade) and DEAE-Sephadex (A-50) was obtained from Pharmacia, Uppsala, Sweden. Gelatin (Bacto) was obtained from Difco Laboratories, Detroit, Mich., U.S.A. Visking tubing was supplied by H.M.C. Ltd., London W.1, U.K. It was boiled for 3h in several changes of water before use. Hyaluronic acid was purified from bovine synovial fluid and human umbilical cords by papain digestion and cetylpyridinium chloride fractionation. All other reagents were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

General methods

Centrifugation. Centrifugation was done in an MSE High-Speed 18 centrifuge with an 8×50ml head (type 69181).

Measurement of extinction. Extinctions were read on a Hilger H700 spectrophotometer.

Measurement of radioactivity. Water-based samples of up to 0.3ml in volume were counted for radioactivity in low-background glass bottles containing 8ml of scintillation fluid [toluene 2-methoxyethanol (3:2, v/v) containing 80g of naphthalene and 4g of 2,5-bis-(5-t-butylbenzoxazol-2-yl)thiophen/l] in a Nuclear–Chicago mark 1 liquid-scintillation system. An external standard was used to correct for quenching in the samples. Simultaneous measurement of ^{14}C and ^{3}H radioactivity was achieved by suitable discrimination in the above machine.

Incubation procedure

Apparatus. The apparatus consisted of two glass-jacketed containers (8.0cm×2.5cm), one of which is shown in Fig. 1. The first vessel was used to store epiphyses during dissection. When sufficient epiphyses had been collected they were transferred by using the nylon supports to the second vessel, which contained the radioactively labelled incubation mixture. The O_2+CO_2 was introduced by stainless-steel tubes, and both oxygenated the media and mixed the contents of the vessels.

Isolation and incubation in vitro of neonatal rat cartilage. Wistar rats (3 days old) were killed by decapitation, and the proximal and distal epiphyses of the femur and humerus were quickly dissected out. They were stored in the first incubation vessel at 37°C with 10ml of Krebs improved Ringer 1 medium (Krebs, 1950), containing 1mM-glutamine, and aerated with O_2+CO_2 (95:5). When the epiphyses of two litters (approx. 20 rats) had been isolated, they were transferred to the second container and incubated with the same volume of Ringer solution containing 5.8×10^6c.p.m. of [U-^{14}C]glucose or 3.0×10^6c.p.m. of [^{35}S]sulphate. After the incubation in the radioactively labelled medium for a suitable time, the epiphyses were removed, washed in a jet of 0.9% NaCl, blotted dry between filter paper and frozen in liquid N_2. The cartilage was then stored at −26°C. This procedure was repeated until 10g of cartilage had been incubated.

Tissue-space experiments. Epiphysial cartilage was dissected out of the animals and stored in the Ringer solution in the apparatus described. When about 2g of cartilage had been isolated, it was transferred to the second vessel containing about 1×10^6c.p.m. of [^{3}H]water and 1×10^5c.p.m. of [U-^{14}C]sucrose in 10ml of Ringer solution. At various times, samples of the epiphysial cartilage were removed, blotted dry and weighed. They were then homogenized in three times their weight of 0.75M-$HClO_4$ and left for 12h at 4°C. The homogenate was neutralized with 6M-KOH, centrifuged at 5000g for 10min and the supernatants were counted for radioactivity.

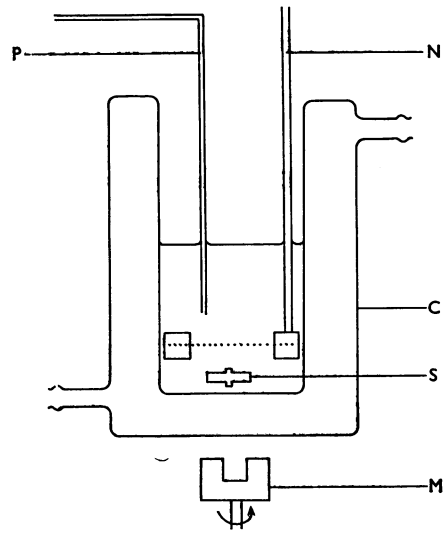


Fig. 1. *Incubation apparatus*

C, Water-jacketed container; M, rotating magnet; S, stirrer; N, nylon support with handle; P, stainless-steel tube.

Measurement of oxygen uptake in neonatal rat epiphysial cartilage. Epiphysial cartilage (0.5g) was incubated in 5ml of air-saturated Krebs improved Ringer 1 medium (containing 1mM-glutamine) in a water-jacketed container at 37°C. The uptake of oxygen from this medium was measured with an oxygen electrode as described by Kielley & Bronk (1958).

Separation and analysis of nucleotides and intermediates

Extraction of neonatal rat epiphysial cartilage with $HClO_4$. About 10g of epiphysial cartilage, incubated with [U-^{14}C]glucose or [^{35}S]sulphate for a fixed period of time, was powdered in portions of 1g in a stainless-steel percussion mortar at −40°C. The powder was extracted in three times its weight of 0.75M-$HClO_4$, then the homogenate was mixed in a cooled Omnimix apparatus (Ivan Sorvall Inc., Norwalk, Conn., U.S.A.) for 30s and centrifuged for 10min at 18000*g*. The pellet was extracted twice as described above, then finally frozen and kept at −26°C. The supernatants were combined, the pH was adjusted to pH6.8 with 6M-KOH and the solution was left at 4°C for 1h. The pH was re-checked before centrifugation at 15000*g* for 10min. The temperature throughout this extraction was kept below 4°C.

Separation of intermediates on Dowex 1 (formate form). The neutral $HClO_4$ extract of 10g of epiphysial cartilage was applied to a column (20.0cm×1.5cm) of Dowex 1 (X8; formate form; 200–400 mesh), which had been calibrated with known standards as described by Hardingham & Phelps (1968). The column was eluted as described by Hurlbert *et al.* (1954) at 4°C at a flow rate of 1.2ml/min; 5ml fractions were collected, their extinctions read at 260nm and 0.2ml samples of each fraction counted for radioactivity.

Analysis of the nucleotides and intermediates separated on Dowex 1 (formate form). Pooled fractions of nucleotides and intermediates were deionized and freeze-dried as described by Hardingham & Phelps (1968). Individual nucleotides were identified on the basis of their spectral ratios (Hurlbert *et al.*, 1954) and chromatographed on polyethyleneimine-cellulose with known standards as described by Randerath & Randerath (1965). The amount of each nucleotide was measured by using the molar extinction coefficient at 260nm (Dawson *et al.*, 1969). Glucose 6-phosphate, fructose 1,6-diphosphate, *N*-acetylglucosamine 6-phosphate and UDP-glucuronic acid were isolated, assayed and counted for radioactivity as described by Hardingham & Phelps (1968). UDP-hexose and UDP-*N*-acetylhexosamine overlapped in this elution scheme. No attempt was made to separate them at this stage. Instead the fractions were pooled and deionized, and the freeze-dried material was analysed for glucose, galactose, glucosamine, galactosamine and xylose by g.l.c. of the trimethylsilyl ethers (Clamp *et al.*, 1967). The rest of the material was hydrolysed in 0.01M-HCl for 30min at 100°C. The hydrolysate was then freeze-dried, dissolved in a small volume of water and passed down a column (5.0cm×1.5cm) of Dowex 1 (X8; formate form; 200–400 mesh) to remove UDP and P_i. The water eluate was freeze-dried before being deacetylated by heating in 1M-HCl in a sealed tube at 100°C for 2h. This hydrolysate was dried by vacuum desiccation over KOH and P_2O_5, dissolved in a small volume of water and applied to a column (5.0cm×1.5cm) of Zeo-Karb 225 (H^+ form; 14–52 mesh). The column was washed with a small volume of water. The hexoses were eluted in the water wash; this fraction was freeze-dried, counted for radioactivity and assayed by the anthrone method of Yemm & Willis (1954). The amino sugars were eluted with 0.5M-HCl, and the eluate was freeze-dried, counted for radioactivity and assayed for hexosamine by the method of Blix (1948). A sample of the hexose fraction was chromatographed against standards on a thin layer of cellulose phosphate, the solvent used being acetone–butan-1-ol–water (5:4:1, by vol.). The sugars were located with *p*-anisidine reagent (Hough *et al.*, 1950). The galactose/glucose radioactivity ratio was determined by removing the areas of the cellulose phosphate that contained the individual hexoses, placing them in low-background glass vials with 10ml of scintillation fluid and counting the radioactivity. Samples of the hexosamine were chromatographed on a thin layer of silica gel G in propan-1-ol–aq. NH_3 (sp.gr. 0.88) (13:7, v/v) (Marzullo & Lush, 1967). The *p*-anisidine reagent was used to locate the hexosamines. The ratio of radioactivity between the hexosamines was determined in the same manner as described for the hexose fraction.

Separation and analysis of cartilage components

Extraction of acid glycosaminoglycans. The residue after extraction with $HClO_4$ was washed with acetone until all traces of the acid had been removed and were then dried by vacuum desiccation. The dried powder was suspended in a solution of 0.1M-sodium phosphate buffer (pH6.5)–0.5mM-cysteine–0.5mM-EDTA containing 5mg of twice-crystallized papain/g of dry cartilage (Scott, 1960). The papain was preactivated by incubation in the buffer for 30min at 50°C, after which the cartilage powder was added and the mixture was incubated overnight at 50°C, and then cooled to 4°C. Any material left undigested was separated by centrifugation at 5000*g* for 10min, then washed and kept for treatment with alkali. The supernatant and washings were combined and solid trichloroacetic acid was added to give a final

concentration of 5% (w/v). After 2h at 4°C the precipitate was removed by centrifugation at 5000*g* for 10min and washed twice with 5% (w/v) trichloroacetic acid. The combined supernatants were adjusted to pH6.8 with NaOH and dialysed against tap water for 6h, then against several changes of water for a further 6h at 4°C. The 6M-NaOH was added, to a final concentration of 0.5M, and the resulting solution was shaken for 18h at 4°C. The residue left behind after papain digestion was added back at this point after being made completely soluble by the addition of 1M-NaOH (Hardingham & Phelps, 1970*a*). The extract was then adjusted to pH7.0 with 5M-HCl and dialysed for at least 48h at 4°C against many changes of water. The diffusate was then passed down a column (20.0cm × 2.0cm) of Zeo-Karb 225 (H^+ form; 14–52 mesh). The water eluate and washings were then freeze-dried; the resulting material was used for further analysis. Every care was taken throughout to ensure that there was no loss of uronic acid-containing material in the dialysis steps.

Cetylpyridinium chloride fractionation of acid glycosaminoglycans. The cetylpyridinium chloride complexes of the acid glycosaminoglycans were fractionated on a cellulose column according to their critical salt solubilities as described by Antonopoulos *et al.* (1961). A water-jacketed column (15cm × 1.5cm) of Whatman CC41 cellulose was prepared as described by Scott (1960). It was washed overnight with 1% (w/v) cetylpyridinium chloride. The glycosaminoglycan extract dissolved in 5mM-Na_2SO_4 (1.5ml) was added to the column as a succession of drops, interspersed with an equal number of drops of 1% cetylpyridinium chloride. Each drop was allowed to soak into the column before the next one was added. The column was then eluted with: 25ml of 1% cetylpyridinium chloride, 25ml of 0.3M-NaCl in 0.05% (w/v) cetylpyridinium chloride, 25ml of 0.25M-$MgCl_2$ in 0.05% cetylpyridinium chloride, 25ml of 0.5M-$MgCl_2$ in 0.05% cetylpyridinium chloride, 25ml of 0.75M-$MgCl_2$ in 0.05% cetylpyridinium chloride, 25ml of 1.25M-$MgCl_2$ in 0.05% cetylpyridinium chloride, and finally 15ml of 6M-HCl. The column was run at 25°C and at a flow rate of 4ml/h. The glycosaminoglycans in these eluates were isolated as follows (Scott, 1960). The 1% cetylpyridinium chloride fraction was concentrated by rotary evaporation at 40°C, after which 4–5vol. of ethanol plus several drops of saturated sodium acetate were added. The mixture was left for 12h at 4°C. The precipitate was collected by centrifugation at 5000*g* and washed tnree times with ethanol and dried. The other fractions were treated in the same way except that only 2–3vol. of ethanol was necessary to precipitate the glycosaminoglycans.

Cetylpyridinium chloride fractionation of chondroitin sulphate isomers and dermatan sulphate. The glycosaminoglycans isolated in the fractions eluted between 0.25M- and 0.75M-$MgCl_2$ were subfractionated by the method described by Antonopoulos & Gardell (1963), by using the preferential solubility of the chondroitin 4-sulphate in organic solvents and of chondroitin 6-sulphate in acid solvents compared with dermatan sulphate, which is insoluble in both solvents. A jacketed column (15cm × 1.5cm) of Whatman CC41 cellulose was prepared as described by Antonopoulos & Gardell (1963) and washed overnight with 1% cetylpyridinium chloride. The sample was added and eluted with: 25ml of 0.3M-NaCl in 0.05% cetylpyridinium chloride, 25ml of 0.05% cetylpyridinium chloride, 25ml of propan-1-ol–methanol–acetic acid–water (80:40:3:77, by vol.) containing 0.4g of cetylpyridinium chloride/100ml, 35ml of 0.05% cetylpyridinium chloride, 25ml of 0.75M-$MgCl_2$ in 0.1M-acetic acid–0.05% cetylpyridinium chloride, 25ml of 0.05% cetylpyridinium chloride, 25ml of 0.75M-$MgCl_2$ in 0.05% cetylpyridinium chloride, and finally 20ml of 6M-HCl. The glycosaminoglycans were isolated from these fractions by evaporation to dryness in a rotary evaporator, then were dissolved in a small volume of water and precipitated by the addition of 4–5vol. of ethanol. After the mixture had stood for 12h at 4°C the precipitate was separated by centrifugation, then washed and dried.

Analysis of the fractionated acid glycosaminoglycans. Sulphate was measured by the turbidimetric method of Dodgson (1961) after release from the glycosaminoglycans by hydrolysis in 3M-HCl at 100°C for 6h. Hexosamines were released from the polymers by hydrolysis in 3.8M-HCl at 100°C in sealed tubes for 6h; the hydrolysates were then dried by vacuum desiccation over solid KOH and P_2O_5. The hexosamines were assayed by the method described by Blix (1948) and identified by t.l.c. on silica gel G plates in propan-1-ol–aq. NH_3 (sp.gr. 0.88) (13:7, v/v) (Marzullo & Lush, 1967). The hexosamines were located by spraying with *p*-anisidine reagent (Hough *et al.*, 1950). Uronic acid was assayed by the methods of Bitter & Muir (1962) and Brown (1946) as modified by Khym & Doherty (1952).

Measurement of the specific radioactivities of hexosamine and uronic acid in the fractionated acid glycosaminoglycans. Small samples of acid glycosaminoglycans fractionated by the method of Antonopoulos *et al.* (1961) were hydrolysed in 0.05M-HCl containing about 20mg of Zeo-Karb 225 (H^+ form; 14–52 mesh) in a sealed tube at 100°C for 24h. The uronic acid and hexosamine in this hydrolysate were separated on a column (5.0cm × 1.0cm) of Zeo-Karb 225 (H^+ form; 14–52 mesh) as described by Hardingham & Phelps (1970*b*). They were analysed and counted for radioactivity.

Electrophoresis of acid glycosaminoglycans. This was done in an EEL horizontal electrophoresis apparatus on cellulose acetate paper (12.0cm × 1.5cm),

with 0.2M-$ZnSO_4$ (pH4.8) as the electrolyte (Haruki & Kirk, 1967). The cellulose acetate strips were carefully wetted from one side and excess of electrolyte was removed by blotting before they were placed in the electrophoresis tank. Whatman 3MM chromatography paper was used for the conducting wicks. The whole system was allowed to equilibrate for at least 1h before the glycosaminoglycan sample was applied. The strips were run at 0.4mA/cm width for 6h. After being dried for 5min in an oven at 100°C, the papers were stained with 1% (w/v) Alcian Blue in 10% (v/v) acetic acid for 1min. The strips were washed in 10% acetic acid until all the excess of stain had been removed and were finally placed in running water for several hours before drying between paper.

Hyaluronidase digestion of acid glycosaminoglycans. Commercial bovine testicular hyaluronidase was purified on DEAE-Sephadex A-50 as described by Soru & Ioneso-Stoian (1963). The glycosaminoglycans were digested with this purified enzyme in 0.1M-sodium citrate–phosphate buffer, pH5.5 (Mathews & Inouye, 1961), for 24h at 37°C. After digestion, trichloroacetic acid was added to give a final concentration of 5% (w/v). This mixture was left for 2h at 4°C before the precipitate was removed by centrifugation at 5000*g* for 10min. The supernatant was dialysed against tap water for 6h and then several changes of water for 12h at 4°C. The extent of digestion by bovine hyaluronidase was found by comparing the hexosamine content before and after digestion.

Gel filtration of acid glycosaminoglycans. A column (60cm×1.5cm) of Sephadex G-50 was prepared according to the manufacturer's instructions. The included and excluded volumes were determined by using Methylene Blue and Blue Dextran respectively. A small sample of the glycosaminoglycan was applied carefully to the top of the gel and allowed to soak in before the buffer (0.2M-sodium phosphate, pH7.4) was added; 2ml fractions were collected. Each was assayed for uronic acid (Bitter & Muir, 1962) and hexosamine (Blix, 1948) after hydrolysis. The separation was done at 4°C.

Other assays

Fructose was assayed by the method of Kulka (1956). Hexose monophosphates were measured enzymically by the method of Hohorst (1963). Glycogen was extracted as described by Carrol *et al.* (1956) and measured as hexose by the anthrone method of Yemm & Willis (1954).

Results

An incubation system was developed for studies *in vitro* in which the physiological state of the epiphyses was maintained after removal from the animal, as judged by the following criteria.

(*a*) The morphology of the epiphyses was still identical with that of freshly excised material after 2h of incubation.

(*b*) In preliminary experiments the change in the wet weight of the incubated tissue was determined in the presence of various concentrations of bovine serum albumin. There was an increase in weight with 2.5% (w/v) and a decrease with 7.5% (w/v) albumin (Fig. 2). The colloid osmotic support of the suspending medium was changed and the [U-^{14}C]sucrose- and [^{3}H]water-penetrable spaces were observed with time. Stable values were obtained by using 5% (w/v) defatted bovine serum albumin. Under these conditions the total exchangeable [^{3}H]-water space was stable for at least 3h and averaged 86.5±3.0% of the wet weight of the tissue compared with a water space, determined by drying in an oven at 103°C for 24h, of 86.0±0.9%. The sucrose-penetrable space was also stable for 3h, and averaged 71.0±3.9%. These values for the [^{3}H]water and [U-^{14}C]sucrose spaces were obtained from 12 experiments, and the extracellular distribution of sucrose was used to calculate the concentration of metabolites in the cell water discussed below.

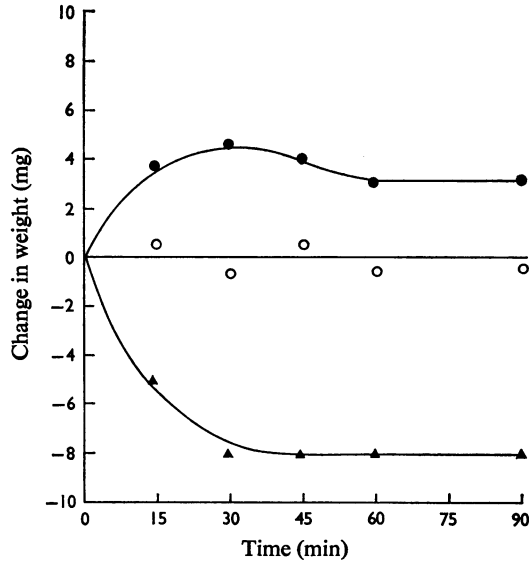


Fig. 2. *Change in weight of cartilage during incubation*

Epiphysial cartilage (1.2g) was incubated in Krebs improved Ringer 1 medium, containing different concentrations of serum albumin as colloid osmotic support: ●, 2.5% (w/v) serum albumin; ○, 5.0% serum albumin; ▲, 7.5% serum albumin.

(*c*) Nutrient supply. The glucose utilization under the conditions of incubation in our experiments averaged $1.20 \pm 0.10 \mu$mol/h per g wet wt. and the oxygen utilization was shown to be $6.42 \pm 0.05 \mu$mol/h per g wet wt. Dickens & Weil-Malherbe (1936) reported for rat costal cartilage a glucose utilization of 0.94μmol/h per g wet wt. and an oxygen utilization of 4.25μmol/h per g wet wt.

(*d*) Nucleotide sugar and hexose phosphate content. Freshly excised epiphyses were frozen in liquid N_2, then extracted and chromatographed on the Dowex 1 (formate) system (Hardingham & Phelps, 1968) and the constituent metabolites were measured. A similar extraction was performed on tissue after exposure for different times in the incubation mixture. No significant changes were recorded between the treatments *in vivo* and *in vitro*, and in particular the values for ATP concentration in cell water were within 11% of each other. The hexose monophosphate concentrations were 55.3 ± 11.9 and $75.5 \pm 7.0 \mu$mol/g wet wt. for the incubation *in vivo* and *in vitro*.

(*e*) Care was taken during dissection and isolation to preserve the temperature of the epiphyses at 37°C (Hardingham & Muir 1970), and they were exposed to no deviation in temperature thereafter till the end of the experiment, when they were frozen in liquid N_2.

(*f*) Absence of an anaerobic centre. The equation derived by Hill (1928) enables the theoretical anaerobic centre to be calculated. The maximum depth r (cm) to which O_2 will diffuse is given by: $r = C \cdot k \cdot p / Q \cdot 760$ where C depends on the shape of the tissue, k is Krogh's constant of diffusion of O_2, p is the partial pressure of O_2 and Q the rate of O_2 uptake.

By assuming a dissected epiphysis is spherical, Harvey (1928) gives C the value of 6. Krogh's constant for connective tissue at 37°C was found by Krogh (1918) to be 0.0134μl of O_2 diffusing across 1 cm/min in a concentration gradient corresponding to a partial pressure gradient of $101 \text{ kN} \cdot \text{m}^{-2} \cdot \text{cm}^{-1}$ (1 atm/cm). The measured respiratory rate was 2.4μl of O_2/min per g wet wt. and p for $O_2 + CO_2$ (95:5) is $96.3 \text{ kN} \cdot \text{m}^{-2}$ (722 mmHg).

From these results, the maximum radius of diffusion of O_2 into neonatal rat epiphysis can be calculated to be 0.18 cm. The mean radius of the epiphyseal cartilage used was found to be 0.098 ± 0.012 cm. Therefore there is no anaerobic centre in the tissue under the conditions described.

It is concluded from the above experiments that the system definitely preserves the physiological integrity of the isolated epiphyses *in vitro*.

Nature of the acidic polysaccharides produced by the epiphysial cartilage

The extraction procedure described above released

Table 1. *Cetylpyridinium chloride fractionation of acid glycosaminoglycans from 10 g of neonatal rat epiphysial cartilage*

For experimental details see the text. Values are given as means ±S.D. of four to six experiments.

Fraction	Content of uronic acid (%)	Molar ratios			Identification of hexosamine by t.l.c.	Digestion by hyaluronidase (%)	Glycosaminoglycan present
		Uronic acid / Hexosamine	Carbazole / Orcinol	Sulphate / Uronic acid			
1% cetylpyridinium chloride	10.0 ± 1.0	0.83	0.85	0.94	Glucosamine	80	Keratan sulphate
					Galactosamine		Chondroitin sulphate
0.3 M-NaCl	3.6 ± 0.5	1.00	1.05	0.07	Glucosamine	100	Hyaluronic acid
0.25 M-$MgCl_2$	23.0 ± 1.7	1.07	0.96	0.87	Galactosamine	100	Chondroitin sulphate
0.5 M-$MgCl_2$	53.3 ± 3.0	0.98	0.98	0.89	Galactosamine	100	
0.75 M-$MgCl_2$	6.0 ± 1.4	1.33	1.28	0.83	Galactosamine	100	
1.25 M-$MgCl_2$	0	—	—	—	—	—	
6 M-HCl	0	—	—	—	—	—	

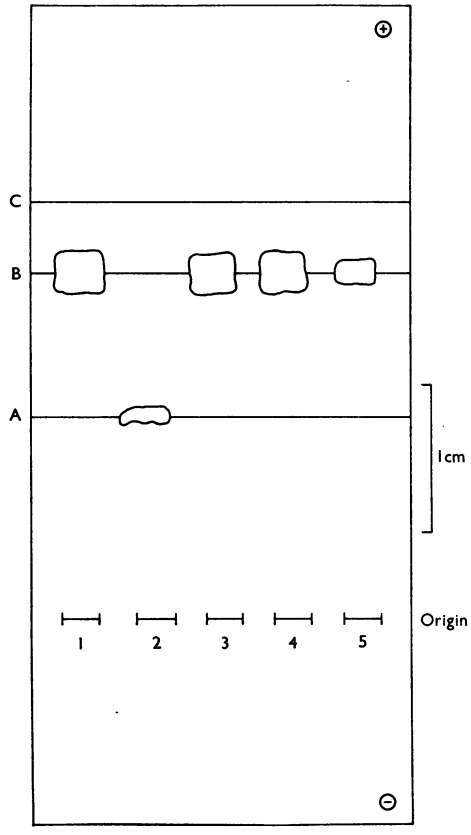


Fig. 3. *Electrophoresis of fractionated glycosaminoglycans*

Cetylpyridinium chloride-fractionated glycosaminoglycans of epiphysial cartilage were subjected to electrophoresis on cellulose acetate paper in 0.2M-$ZnSO_4$ (pH 4.8, 0.4mA/cm width, for 6h). A, Mobility of hyaluronic acid; B, mobility of shark chondroitin sulphate; C, mobility of heparin; 1, 1% cetylpyridinium chloride fraction; 2, 0.3M-NaCl fraction; 3, 0.25M-$MgCl_2$ fraction; 4, 0.5M-$MgCl_2$ fraction; 5, 0.75M-$MgCl_2$ fraction.

3384±268nmol of uronic acid from the acid glycosaminoglycans per g wet wt. of tissue. Electrophoresis of the whole extracts in 0.2M-$ZnSO_4$ showed one major component, which corresponded in the mobility to standard chondroitin sulphate. Fractionation with cetylpyridinium chloride-impregnated cellulose columns (Antonopoulos *et al.*, 1961) enabled further separation of the glycosaminoglycan extract (Table 1).

The 1%-cetylpyridinium chloride fraction consistently contained a significant amount of uronic acid-containing polymer. Electrophoresis of this fraction (Fig. 3) indicated a major component with the same mobility as standard chondroitin sulphate. G.l.c. of the trimethylsilyl derivatives of fractions digested with methanolic HCl revealed the presence of *N*-acetylgalactosamine, *N*-acetylglucosamine, mannose and galactose in the molar proportions 4.7:1.0:1.2:1.1. The presence of so much mannose strongly suggests the presence of glycoprotein material, and the occurrence of an equimolar ratio of galactose and *N*-acetylglucosamine indicates that a small amount of keratan sulphate is present. However, the *N*-acetylgalactosamine occurs with an exact equivalent of uronic acid, and the ratio of sulphate to uronic acid is 0.94, suggesting that in this cetylpyridinium chloride fraction a small amount of chondroitin sulphate is found. It seems unlikely that this material differs in size from the bulk material that is fractionated between 0.25M- and 0.75M-$MgCl_2$ since they have an identical elution profile on Sephadex G-50 (Fig. 4). This may reflect incomplete precipitation on the columns, though every care was taken to prevent this. The 0.3M-NaCl fraction was identified as hyaluronic acid by its analysis, electrophoretic behaviour and digestion with hyaluronidase.

The fractions eluted between 0.25M- and 0.75M-$MgCl_2$ contained one component on electrophoresis in 0.2M-$ZnSO_4$ whose mobility matched that of chondroitin sulphate. The analysis showed equimolar amounts of galactosamine, sulphate and uronic acid. The component was digested by bovine testicular hyaluronidase and was identified as chondroitin sulphate.

Treatment of the fractions containing chondroitin sulphate eluted between 0.25M- and 0.75M-$MgCl_2$ by the method described by Antonopoulos & Gardell (1963) indicated that 79.5% of the polymer in this fraction was chondroitin 4-sulphate, 17.6% chondroitin 6-sulphate and 2.1% dermatan sulphate (Table 2).

Concentration of the intermediate metabolites

To obtain information about the biosynthesis of the acidic glycosaminoglycans, experiments were performed on neutralized perchloric acid extracts of epiphyses that had been previously incubated for various times with [U-^{14}C]glucose. In Fig. 5 a typical elution profile obtained with 9g of this tissue is shown, together with the concomitant radioactivity profile after incubation for 1h. Samples corresponding to individual metabolites were pooled, deionized and freeze-dried as described by Hardingham & Phelps (1968), and the concentrations of nucleotides observed in neonatal rat cartilage are given in Table 3. In preliminary work extensive degradation of the nucleotide phosphates was

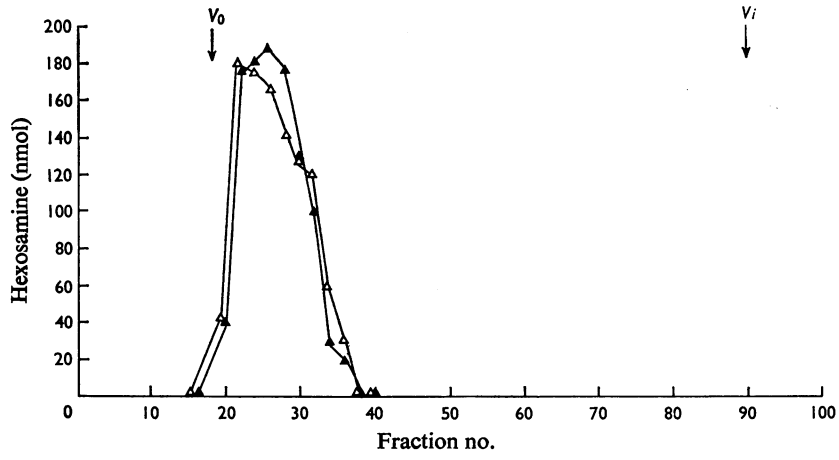


Fig. 4. *Gel filtration of fractionated glycosaminoglycans*

The glycosaminoglycans in the 1%-cetylpyridinium chloride fraction (△) and those in the fraction eluted between 0.25M- and 0.75M-$MgCl_2$ (▲) were subjected to gel filtration on a Sephadex G-50 column (60cm × 1.5cm). Fractions (2ml) were collected and after hydrolysis assayed for hexosamine. The total included volume (V_t) and the excluded volume (V_0) are indicated.

Table 2. *Fractionation of isomeric chondroitin sulphates and dermatan sulphate by the method of Antonopoulos & Gardell* (1963)

For experimental details see the text. Values are given as means ±S.D. of four to six experiments.

Fraction	Content of uronic acid (%)	Tissue content (%)	Molar ratios: Carbazole / Orcinol	Molar ratios: Uronic acid / Hexosamine	Isomer
0.4% Cetylpyridinium chloride–propan-1-ol–methanol–acetic acid–water (80:40:3:77, by vol.)	79.5±2.6	65.4±2.1	1.05	1.04	Chondroitin 4-sulphate
0.75M-$MgCl_2$–0.05% cetylpyridinium chloride–0.1M-acetic acid	17.6±2.9	14.5±2.3	0.86	1.18	Chondroitin 6-sulphate
0.75M-$MgCl_2$–0.05% cetylpyridinium chloride	2.1±0.7	1.7±0.6	—	—	Dermatan sulphate

observed, and it was attributed to the presence of a phosphatase. By raising the concentration of the perchloric acid in the extraction step to 0.75M, this degradation could be prevented and reproducible elution profiles obtained. From the total amount of each metabolite analysed, and from a knowledge of the cellular water space, the concentration of metabolites could be expressed as shown in Table 3.

Time-course of incorporation of [U-^{14}C]glucose into the intermediates of glycosaminoglycan synthesis

The specific radioactivity of each of the postulated precursors on the pathway to glycosaminoglycan biosynthesis as a function of time of incubation with radioactive glucose was also examined. The resulting specific radioactivity was plotted against

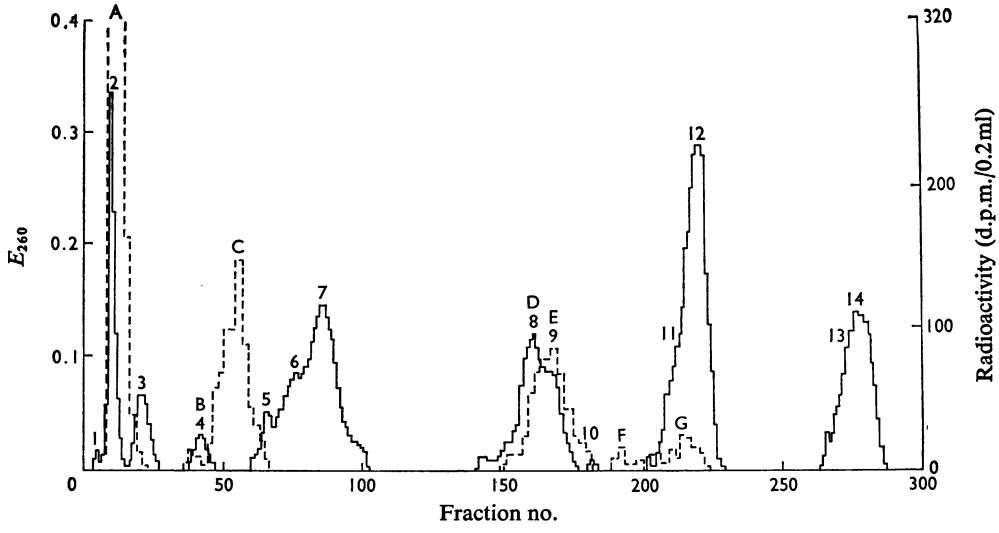


Fig. 5. *Incorporation of radioactive glucose into nucleotides and biosynthetic intermediates*

The nucleotide (E_{260}, ——) and radioactivity (---) profiles of the acid-soluble extract of 9g (wet wt.) of neonatal rat epiphysial cartilage that had been incubated for 1h with [U-^{14}C]glucose are shown. The extract was applied to a Dowex (X8; formate form; 200–400 mesh) column (20cm×1.5cm) and eluted with an extended gradient formed with a 200ml sealed mixing vessel and a reservoir that contained the following concentrations of formic acid and ammonium formate: fractions 1–30, 1M-formic acid; fractions 31–130, 4M-formic acid; fractions 131–180, 4M-formic acid–0.25M-ammonium formate; fractions 181–210, 4M-formic acid–0.4M-ammonium formate; fractions 211–end, 4M-formic acid–0.8M-ammonium formate. Fractions (5ml) were collected. Nucleotide peaks: 1, CMP; 2, NAD; 3, AMP; 4, NADP; 5, GMP; 6, UMP; 7, ADP; 8, UDP-*N*-acetylhexosamine; 9, UDP-hexose; 10, GDP; 11, UDP-glucuronic acid; 12, ATP; 13, GTP; 14, UTP. Radioactive peaks; A, glutamate and lactate; B, *N*-acetylhexosamine 6-phosphate; C, hexose monophosphate; D, UDP-*N*-acetylhexosamine; E, UDP-hexose; F, fructose 1,6-diphosphate; G, UDP-glucuronic acid. The identification of the components in the various fractions is described in the Materials and Methods section.

time for the following purified intermediates: hexose monophosphate, fructose 1,6-diphosphate, *N*-acetylglucosamine 6-phosphate, UDP-*N*-acetylhexosamine, UDP-hexose and UDP-glucuronic acid (Fig. 6). These results were used to calculate the turnover times of the intermediates as described by Zilversmit *et al.* (1942), and from the tissue contents of the intermediates their rates of synthesis were calculated (Table 4).

To treat the results by this method, it was assumed that there was a direct product–precursor relationship between the following metabolites: (i) hexose monophosphate and glucose; (ii) fructose 1,6-diphosphate and hexose monophosphate; (iii) UDP-glucose and hexose monophosphate; (iv) UDP-glucuronic acid and UDP-glucose; (v) *N*-acetylglucosamine 6-phosphate and hexose monophosphate; (vi) UDP-*N*-acetylglucosamine and *N*-acetylglucosamine 6-phosphate.

A direct product–precursor relationship implies a conversion of a precursor into product without the involvement of a stable intermediate. The latter two statements need further explanation. In the hexosamine pathway from fructose 6-phosphate to UDP-*N*-acetylglucosamine there are five intermediates. Only two, *N*-acetylglucosamine 6-phosphate and UDP-*N*-acetylglucosamine, were easily isolated and counted for radioactivity. Neither glucosamine 6-phosphate nor *N*-acetylglucosamine 1-phosphate was separated. Glucosamine 6-phosphate was not identified on the Dowex 1 (formate) system which suggested that the pool size was very small in this tissue. Hence there would be little effect on the flow of radioactive label from fructose 6-phosphate to *N*-acetylglucosamine 6-phosphate. *N*-Acetylglucosamine 1-phosphate was not isolated, and again it appears that the pool size is very small. It is assumed in the present paper that the steady-state concentration of reactant and product of the *N*-acetylglucosamine phosphomutase (EC 2.7.5.2) differs little from

Table 3. *Cellular concentrations of nucleotides and intermediates of glycosaminoglycan biosynthesis in neonatal rat epiphysial cartilage*

For experimental details see the text. Values are given as means ± S.D. of four to six experiments.

Intermediate	Tissue content (nmol/g wet wt.)	Cellular concn. (mM)
CMP	12.1 ± 0.5	0.071 ± 0.002
NAD	94.7 ± 2.8	0.56 ± 0.012
AMP	42.3 ± 8.0	0.25 ± 0.05
NADP	40.8 ± 3.1	0.24 ± 0.02
Hexose monophosphate	75.0 ± 7.0	0.44 ± 0.04
N-Acetylglucosamine 6-phosphate	5.0 ± 2.0	0.029 ± 0.01
GMP	43.0 ± 7.7	0.25 ± 0.05
UMP	115.0 ± 7.0	0.68 ± 0.04
ADP	219.5 ± 12.5	1.29 ± 0.074
UDP-*N*-acetylhexosamine	31.0 ± 0.75	0.18 ± 0.004
UDP-hexose	18.4 ± 1.2	0.11 ± 0.007
GDP	15.1 ± 0.6	0.09 ± 0.004
UDP-glucuronic acid	15.8 ± 1.45	0.09 ± 0.009
Fructose 1,6-diphosphate	63.0 ± 4.3	0.37 ± 0.023
ATP	334.0 ± 15.0	1.96 ± 0.088
GTP	145.0 ± 14.7	0.85 ± 0.086
UTP	59.0 ± 0.6	0.35 ± 0.004

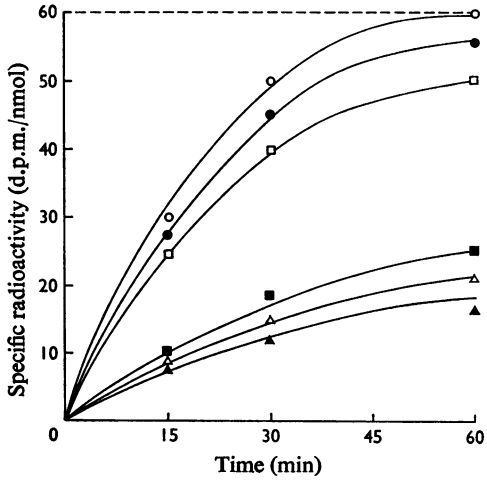


Fig. 6. *Specific radioactivities of intermediates of glycosaminoglycan biosynthesis in neonatal rat epiphysial cartilage*

The results are plotted against time of incubation with [U-^{14}C]glucose. ○, Hexose monophosphate; ●, *N*-acetylhexosamine 6-phosphate; □, UDP-hexose; ■, UDP-*N*-acetylhexosamine; △, fructose 1,6-diphosphate; ▲, UDP-glucuronic acid; ----, glucose. A large excess of glucose was present throughout the experiments to maintain a constant specific radioactivity of the glucose in the incubation media.

the free equilibrium concentrations. Reissig (1956) found that the equilibrium for the enzyme extracted from *Neurospora crassa* was obtained with a mixture of 14% *N*-acetylglucosamine 1-phosphate and 86% *N*-acetylglucosamine 6-phosphate. Therefore the time-specific radioactivity curve for *N*-acetylglucosamine 1-phosphate would be very close to that for *N*-acetylglucosamine 6-phosphate. The error in the calculated rate of synthesis of UDP-*N*-acetylhexosamine is unlikely to be great as a result of these assumptions.

Incorporation of [U-^{14}C]glucose and [^{35}S]sulphate into acid glycosaminoglycans of neonatal rat epiphysial cartilage

The specific and percentage radioactivities of the components of the fractionated polymers were measured after incubation with [U-^{14}C]glucose (Table 5). The ratios of the specific radioactivities of hexosamine and uronic acid in the various fractions (Table 6) and in the two precursors of polymer biosynthesis, UDP-*N*-acetylhexosamine and UDP-glucuronic acid, were similar (Fig. 6). These precursors are incorporated in equimolar amounts. Thus for hyaluronic acid, radioactivity is incorporated from UDP-*N*-acetylglucosamine and UDP-glucuronic acid as shown by Glaser & Brown (1955). For sulphated glycosaminoglycans, the radioactivity is derived from UDP-*N*-acetylglucosamine or UDP-*N*-acetylgalactosamine and UDP-galactose (Silbert,

Table 4. *Turnover rates of intermediates in the biosynthesis of glycosaminoglycan in neonatal rat epiphysial cartilage*

For experimental details see the text. Values are given as means ±S.D. of four to six experiments.

Intermediate	Turnover time (min)	Pool size (nmol/g wet wt.)	Turnover rate (nmol/min per g wet wt.)
Hexose monophosphate	3.75	75.0±7.00	20.0 ±1.8
Fructose 1,6-diphosphate	13.60	63.0±4.30	4.6 ±0.31
N-Acetylglucosamine 6-phosphate	1.30	5.0±2.00	3.8 ±1.5
UDP-*N*-acetylhexosamine	9.60	31.0±0.75	3.2 ±0.08
UDP-hexose	1.40	18.4±1.20	13.1 ±0.86
UDP-glucuronic acid	20.0	15.8±1.45	0.79±0.16

Table 5. *Incorporation of [^{35}S]sulphate and [U-^{14}C]glucose into glycosaminoglycans of neonatal epiphysial cartilage*

For experimental details see the text. Values are given as means ±S.D. of four to six experiments.

Fraction	Percentage of total radioactivity incorporated from		Proportional rate of synthesis from	
	^{14}C labelling	^{35}S labelling	^{14}C labelling	^{35}S labelling
Cetylpyridinium chloride (1%)	10.2±1.0	10.0±1.0	1.57	1.43
0.3M-NaCl	2.2±0.5	1.0±0.5	0.34	—
0.25M-$MgCl_2$	22.0±0.9	20.0±1.0	3.40	2.85
0.5M-$MgCl_2$	55.2±2.0	61.0±1.0	8.49	8.71
0.75M-$MgCl_2$	6.5±1.0	7.0±1.0	1.00	1.00

Table 6. *Specific radioactivity of monosaccharides isolated from neonatal rat epiphysial cartilage after incubation with [U-^{14}C]glucose in vitro*

For experimental details see the text. Values are given as means ±S.D. of four to six experiments.

Fraction	Specific radioactivity (d.p.m./μmol)		Recovery of radioactivity (%)
	Hexosamine	Uronic acid	
Cetylpyridinium chloride (1%)	1200	1125	92
0.3M-NaCl	800	500	79
0.25M-$MgCl_2$	1800	1200	89
0.5M-$MgCl_2$	1960	1500	86
0.75M-$MgCl_2$	1700	1100	80

1964; Perlman *et al.*, 1964; Markovitz *et al.*, 1959). Since the specific radioactivities of UDP-*N*-acetylglucosamine and UDP-*N*-acetylgalactosamine were the same (Table 7) the incorporation of radioactivity into sulphated glycosaminoglycans would be independent of which hexosamine was incorporated into polymer. The percentage radioactivities of the glycosaminoglycan fractions incubated for 60min in [^{35}S]sulphate are shown in Table 5. It appears that a very small amount of labelled sulphate appears in the hyaluronic acid fraction. As this fraction was so small it was not possible to determine whether the presence of sulphate was due to a contaminant or to a sulphated form of hyaluronic acid. The large amounts of [^{35}S]sulphate employed in these incubations precluded any detailed investigation of the sulphated intermediates. UDP-*N*-acetylgalactosamine 4-sulphate was eluted from the Dowex 1

Table 7. *Specific radioactivities of UDP-glucose, UDP-galactose, UDP-N-acetylglucosamine and UDP-N-acetylgalactosamine*

For experimental details see the text. Values are given as means ±S.D. of four to six experiments.

Intermediate	Tissue content (nmol/g wet wt.)	Cellular concn. (mM)	Ratio of specific radioactivity
UDP-glucose	14.89±0.99	0.089±0.006	1.08
UDP-galactose	3.44±0.23	0.021±0.001	1.00
UDP-*N*-acetylglucosamine	21.88±0.53	0.128±0.003	1.14
UDP-*N*-acetylgalactosamine	9.08±0.22	0.053±0.001	1.00

Table 8. *Rates of synthesis and turnover times of glycosaminoglycans of neonatal rat epiphysial cartilage calculated from the flux through UDP-glucuronic acid*

For experimental details see the text. Values are given as means ±S.D. of four to six experiments.

Fraction	Tissue content (nmol of uronic acid/g wet wt.)	Rate of synthesis (nmol/h per g wet wt.)	Turnover time (h)
Cetylpyridinium chloride (1%)	352.9± 35.3	4.8±0.5	73.5±0.3
0.3M-NaCl	127.0± 17.6	1.0±0.3	127.0±5.3
0.25M-$MgCl_2$	811.7± 59.9	10.3±1.0	78.7±1.7
0.5M-$MgCl_2$	1880.9±105.9	25.9±1.0	72.6±1.2
0.75M-$MgCl_2$	211.7± 49.4	3.1±1.0	68.1±4.5

(formate form) system too near to the large sulphate peak to be measured separately.

Calculation of the proportional rates of synthesis of acid glycosaminoglycans from ^{14}C and ^{35}S labelling

From the percentage incorporation of radioactivity from [^{35}S]sulphate and [^{14}C]hexosamine it was possible to obtain a proportional rate of synthesis of the polymers. For [^{14}C]hexosamine, it has been shown that both the UDP derivatives of *N*-acetylglucosamine and *N*-acetylgalactosamine have similar specific radioactivity (Table 7). In the calculation of the proportional rate of synthesis it was assumed that the incorporation of radioactivity from the [^{14}C]hexosamine pool was proportional to the rates of polymer synthesis. For [^{35}S]sulphate labelling, the rate of sulphation was uniform throughout the polymer fraction. As shown in Table 5 these proportional rates of synthesis calculated for both ^{14}C and ^{35}S labelling are in agreement with each other.

Calculation of the turnover rates of the acid glycosaminoglycans from ^{14}C labelling

The flux of the ^{14}C label through the UDP-*N*-acetylhexosamine and UDP-glucuronic acid pools are shown in Table 4. The hexosamine and uronic acid moieties are incorporated in equimolar amounts into uronic acid-containing glycosaminoglycans. But the flux through the hexosamine pathway was higher than that through the uronic pathway, which suggests that the latter value was rate-limiting in polymer biosynthesis. Assuming that all the uronic acid in this tissue was destined for acid glycosaminoglycan biosynthesis, the flux through the UDP-glucuronic acid was divided among the uronic acid-containing glycosaminoglycan fractions according to the percentage of radioactivity found in these fractions. This gave an estimate of the absolute rates of synthesis and together with the tissue content of each of the fractions, an estimate of the turnover time was calculated (Table 8). It appears that the hyaluronic acid found in the 0.3M-NaCl fraction was turning over much more slowly than the chondroitin sulphate found in the 1%-cetylpyridinium chloride fractions and in the fractions eluted between 0.25M- and 0.75M-$MgCl_2$.

Discussion

This paper describes a system *in vitro* whereby neonatal rat epiphysial cartilage is maintained in a physiological state for up to 3h. Cartilage of different species has been successfully incubated *in vitro*, for example calf costal cartilage (Klein & Hilz, 1968), bovine nasal cartilage (Kresse & Buddecke, 1968) and pig laryngeal cartilage (Hardingham & Muir, 1970). In the present work a colloid osmotic support

was necessary to preserve the cellular and intercellular spaces, because these were used to calculate the cellular and intercellular concentrations of the metabolites in neonatal rat epiphysial cartilage.

Hardingham & Muir (1970) showed the importance of not subjecting the explanted tissue to any thermal shock, as this caused abnormal sulphate labelling of the chondroitin 4-sulphate in pig laryngeal cartilage. This significant observation might be used to discriminate between the sulphation and the polymerization reactions.

A necessary prerequisite of the present studies is a knowledge of the quantitative glycosaminoglycan content of neonatal rat epiphysial cartilage. We have established that the predominant polymers are chondroitin 4-sulphate and chondroitin 6-sulphate with a small amount of hyaluronic acid and keratan sulphate. The proportions of the various glycosaminoglycans in neonatal rat epiphysial cartilage is compared with that of cartilage from other sources (Table 9). Meyer *et al.* (1958), with human articular cartilage, and Balazs (1969) with bovine epiphysial cartilage, showed that the keratan sulphate content increased and the chondroitin sulphate content decreased with age, and that chondroitin 4-sulphate was replaced by chondroitin 6-sulphate in older animals.

Previous work on the biosynthesis of glycosaminoglycans has been with cell-free systems from chick embryonic cartilage and has involved the use of the immediate nucleotide donors of polymer synthesis. The present paper reports the flux of radioactive label from glucose through all the precursors of glycosaminoglycan biosynthesis.

No attempt has been made in the present work to investigate the turnover rate of sulphate intermediates of polymer synthesis. The system is suitable for studies *in vitro* of the steady-state incorporation from [^{35}S]sulphate, but the Dowex 1 (formate) elution profile is dominated by the large sulphate peak, which is eluted close to the presumed position of elution of UDP-*N*-acetylgalactosamine 4-sulphate (Hardingham & Phelps, 1970*b*). Pulse-labelling experiments are needed to elucidate this pathway.

The perchloric acid-soluble nucleotides of neonatal rat epiphysial cartilage showed a similar distribution to that of rat brain (Mandel & Harth, 1960), rat lens (Klethi & Mandel, 1968) and neonatal rat skin (Hardingham, 1968; Hardingham & Phelps, 1968). The high concentrations of the nucleotide triphosphates, especially ATP, show how well the system sustains this tissue *in vitro*. The relatively high concentration of GTP, which is known to be required in peptide-bond synthesis (Slapikoff *et al.*, 1963) reflects at least in part the fact that neonatal rat cartilage is synthesizing considerable amounts of protein.

The cellular concentrations of the intermediates and the fluxes between the metabolite pools of intermediates of glycosaminoglycan biosynthesis in neonatal rat epiphysial cartilage are shown in Scheme 1. The fluxes are assumed to be unidirectional. The overall turnover rates of the intermediates in cartilage are about twice that observed in neonatal rat skin *in vivo* (Hardingham & Phelps, 1968, 1970*b*). This is consistent with the fact that cartilage consists of a denser and more homogeneous cell population. The low rate of fructose 1,6-diphosphate synthesis and the high rate of synthesis of UDP-hexose are notable. The former probably reflects the slow rate of glycolysis in this tissue.

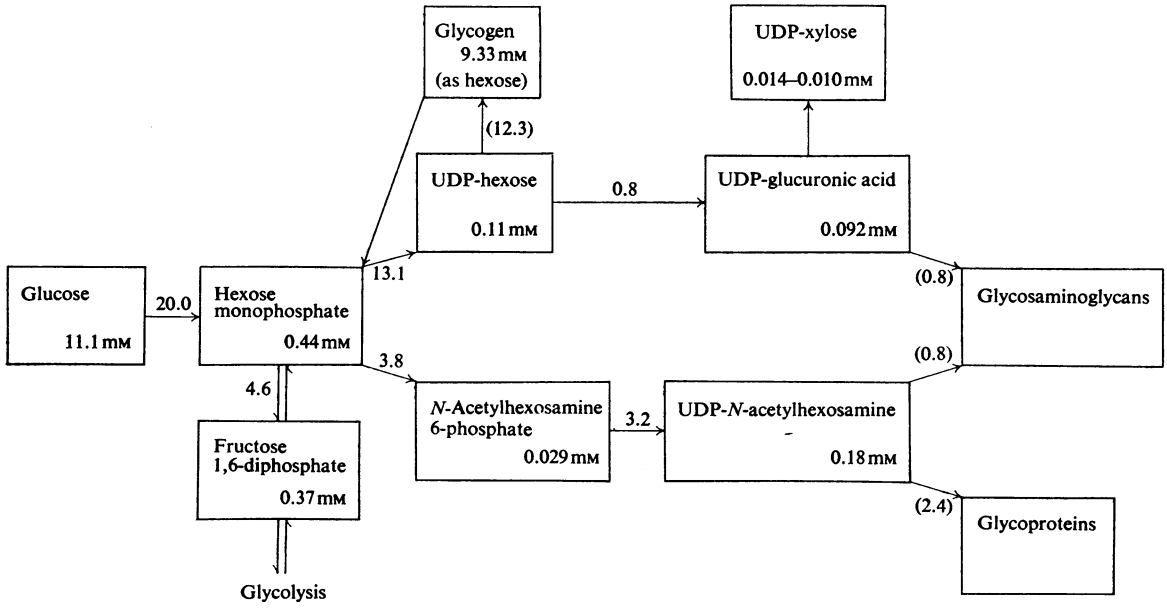
The efflux from the hexose monophosphate pool is slightly greater than the influx. This suggests that there is some recycling of the label. This could be caused by reversal of glycolysis, or by recycling of labelled hexose through glycogen or by a combination of both. Sims & Landau (1965) suggest that for muscle there may be within one cell two hexose monophosphate pools, one committed to UDP-glucose synthesis and the other to glycolysis, between which rapid equilibrium does not take place.

The rate of synthesis of UDP-hexose in rat cartilage is very high compared with the rate of synthesis of *N*-acetylglucosamine 6-phosphate and fructose 1,6-diphosphate. It appears that most of the UDP-hexose is destined for glycogen synthesis as only a tenth is converted into UDP-glucuronic acid. Eeg-Larsen (1956) showed that epiphysial cartilage synthesized very large amounts of glycogen until the third week, after which the relative rate of

Table 9. *Proportions of glycosaminoglycans in cartilage from various sources*

Source of cartilage	Chondroitin 4-sulphate	Chondroitin 6-sulphate	Hyaluronic acid	Keratan sulphate	Reference
Neonatal rat epiphyses	65	15	4	2	Present work
Neonatal pig epiphyses	60	40		Very small	Castellani *et al.* (1962)
Chick epiphyses (13 days old)	30+22*	48			Dorfman (1962)
Puppy epiphyses	+	+		–	Rokosová-Čmuchalová & Bentley (1968)

* There was 30% of chondroitin 4-sulphate plus a further 22% of under-sulphated chondroitin 4-sulphate.



Scheme 1. *Diagrammatic representation of the net rates of synthesis and pool sizes of the intermediates of glycosaminoglycan biosynthesis*

The net rates of synthesis observed are expressed as nmol/min per g wet wt. of tissue; the values in parentheses are calculated rates of synthesis. The concentrations of the intermediates in the cell water are also given.

synthesis appeared to decrease. Preliminary electron-microscopic studies of 3-day-old epiphysial cartilage shows very large masses of glycogen present in the cytoplasm of the chondrocyte (C. J. Handley, unpublished work).

UDP-*N*-acetylhexosamine, as well as being a precursor of glycosaminoglycan biosynthesis, is an intermediate of glycoprotein synthesis. The high rate of UDP-hexosamine synthesis measured reflects the rate of biosynthesis of glycoproteins in this tissue. Assuming that UDP-glucuronic acid is solely destined for glycosaminoglycan biosynthesis and is incorporated into these polymers at the same rate as it is synthesized from UDP-glucose, the biological half-lives of the acid glycosaminoglycans can be estimated. The rate of synthesis of UDP-xylose from UDP-glucuronic acid was not determined, as the amount of UDP-xylose was very low, but it was assumed to be small compared with the rate of incorporation of UDP-glucuronic acid into glycosaminoglycans. With these provisions, chondroitin sulphate turns over in about 70h and hyaluronic acid in about 120h. These results are similar to the values obtained by Hardingham & Phelps (1970*a*) for these glycosaminoglycans in neonatal rat skin. But these findings contrast with the results obtained for glycosaminoglycan synthesis in mature rabbit costal cartilage (Davidson & Small, 1963*a*,*b*) and mature rabbit skin (Schiller *et al.*, 1956) where it was shown that the turnover rate of hyaluronic acid was far greater than that of chondroitin sulphate. Junge-Hulsing & Wagner (1969) showed that the turnover times of sulphated glycosaminoglycans increased with age and varied from tissue to tissue. This might explain the discrepancy between the results obtained from neonatal and mature tissue.

Some information about the control of glycosaminoglycan biosynthesis was obtained. Thus the values of the specific radioactivities of UDP-glucose and UDP-galactose, UDP-*N*-acetylglucosamine and UDP-*N*-acetylgalactosamine are equal, which suggests that the epimerization steps are rapid and are therefore unlikely to be rate-limiting in polymer biosynthesis. The ratio of the epimers of UDP-hexose and UDP-*N*-acetylhexosamine are very similar to the free equilibrium ratio of the substances in the presence of the two epimerases, UDP-glucose 4-epimerase (EC 5.1.3.2), where the ratio of UDP-glucose to UDP-galactose is 75:25, and UDP-*N*-acetylglucosamine 4-epimerase (EC 5.1.3.7), where the ratio of UDP-*N*-acetylglucosamine to UDP-*N*-acetylgalactosamine is 67:33 (Leloir, 1951; Glaser, 1959). Further, these ratios are close to those observed in neonatal rat skin (Hardingham &

Phelps, 1968). Skin synthesizes mostly hyaluronic acid, which requires UDP-glucuronic acid and UDP-*N*-acetylglucosamine as precursors. Epiphysial cartilage, which produces predominantly chondroitin sulphate, requires UDP-glucuronic acid and UDP-*N*-acetylgalactosamine. This shows that the free equilibrium of reactants of the UDP-*N*-acetylglucosamine 4-epimerase is maintained under different conditions of UDP-*N*-acetylglucosamine and UDP-*N*-acetylgalactosamine demand. Investigation of the biosynthesis of glycosaminoglycans in cornea should reveal whether the equilibrium of UDP-glucose 4-epimerase is as firmly maintained in a tissue where the predominant polymer is keratan sulphate.

However, the enzyme converting UDP-glucose into UDP-glucuronic acid, UDP-glucose dehydrogenase (EC 1.1.1.22), was demonstrated by Neufeld & Hall (1965) to be inhibited by UDP-xylose. P. A. Gainey & C. F. Phelps (unpublished work) found the K_i of the enzyme from lamb nasal septa for UDP-xylose to be in the range 10–14 μM for a UDP-glucose concentration of 100 μM. It would appear from these results that the enzyme in 3-day-old rat epiphysial cartilage is about 50% inhibited, and indicates that this enzyme is susceptible to control. Similarly, the enzyme L-glutamine–D-fructose 6-phosphate aminotransferase (EC 2.6.1.16), which controls the first step in the synthesis of UDP-*N*-acetylhexosamine, has been shown by Kornfeld *et al.* (1964) to be subject to end-product inhibition by UDP-*N*-acetylglucosamine. Winterburn & Phelps (1971) showed that the inhibition by UDP-*N*-acetylglucosamine could be relieved by UTP; from their results, and the observed cellular concentrations of UDP-*N*-acetylglucosamine and UTP, the enzyme in neonatal cartilage appears to be 40% inhibited. The true value may be higher as glucose 6-phosphate has an added effect on the inhibition of this enzyme by UDP-*N*-acetylglucosamine.

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