Biochem. J. (1993) 290, 563–570 (Printed in Great Britain)

UDP-sugar metabolism in Swarm rat chondrosarcoma chondrocytes

Christine SWEENEY,* David MACKINTOSH† and Roger M. MASON‡
Department of Biochemistry, Charing Cross and Westminster Medical School, Fulham Palace Road, London W6 8RF, U.K.

UDP-sugars and adenine nucleotides were extracted from freshly isolated chondrocytes and primary cell cultures and analysed by anion-exchange h.p.l.c. The pool sizes of UDP-*N*-acetylglucosamine, UDP-*N*-acetylgalactosamine, UDP-glucose-galactose, UDP-glucuronate and UDP-xylose were 2.9, 1.2, 2.5, 0.6 and 0.03 nmol/10^6 freshly isolated chondrocytes. When chondrocytes were maintained in Dulbecco's modified Eagle medium supplemented with 15% foetal-bovine serum, synthesis of [^{35}S]proteoglycan and [^{3}H]protein decreased over the first 48 h in culture, as did the pools of UDP-glucuronate and ATP. In contrast, the size of the UDP-*N*-acetylhexosamine pools underwent little change during culture. [^{35}S]Proteoglycan and [^{3}H]protein syntheses were stimulated in cultures supplemented with serum or insulin compared with those maintained in medium alone, in agreement with previous results. However, the UDP-sugar pool sizes were the same in both supplemented and non-supplemented cultures. In cultures maintained in the presence of [1-^{3}H]glucose, the UDP-sugars were labelled to a constant ^{3}H specific radioactivity which was very similar to that of the labelling medium. UDP-*N*-acetylhexosamines were labelled to constant ^{3}H specific radioactivity with [6-^{3}H]glucosamine as a precursor, but only about 1 in 375 of these UDP-sugars was derived from the amino sugar in the presence of glucose. The half-life ($t_{\frac{1}{2}}$) for UDP-hexoses, UDP-glucuronate and UDP-*N*-acetylhexosamines was about 12, 12 and 50 min respectively.

INTRODUCTION

Proteoglycans are important components of cell membranes and extracellular matrices in many tissues. They represent the largest and most complex type of glycoconjugate. Their predominant carbohydrate components are glycosaminoglycan chains which are linked covalently to a core protein. Most proteoglycans also contain a number of O-linked and N-linked oligosaccharides with similar structures to those found in other glycoproteins. All the carbohydrate components are synthesized from sugar nucleotide precursors, the first of these, UDP-glucose, being discovered by Leloir [1].

In some proteoglycans the carbohydrate components account for 90% or more of the mass of the molecule. For example, Aggrecan, the large aggregating proteoglycan synthesized by chondrocytes, is composed of as many as 100 chondroitin sulphate chains, 80 keratan sulphate chains and a number of O-linked and N-linked oligosaccharides linked to a protein core of molecular mass about 350 kDa [2]. Aggrecan forms an important part of the extracellular matrix of cartilage, where it is entrapped within the collagen fibre network. Cartilage proteoglycans undergo metabolic turnover, with half-lives measured in days in younger animals and months in older animals [3,4]. Chondrocytes must therefore maintain the tissue structure by continual synthesis of new proteoglycans to replace those lost through turnover. Although the steps leading to the formation of UDP-sugars are well established [5], little is known about how closely the synthesis of UDP-sugars is linked to the demands of proteoglycan synthesis.

Much has been learned about the biosynthesis of cartilage proteoglycans by using a model system of cultured chondrocytes from the Swarm rat chondrosarcoma [2], but little is known about UDP-sugar metabolism in these cells. The aims of the present investigation were therefore to define the pool sizes of the various UDP-sugars involved in glycosaminoglycan synthesis, to study the kinetics of their synthesis and turnover and to establish whether factors such as foetal-calf serum and insulin, which stimulate proteoglycan synthesis in these chondrocytes, had any effect on UDP-sugar metabolism.

METHODS

Chondrocyte cultures

Primary confluent cultures of chondrocytes (4×10^6 cells per 35 mm dish or 12×10^6 cells per 60 mm dish) were established from the transplantable Swarm rat chondrosarcoma [6] maintained in our Department of Comparative Biology. Cultures were plated in Dulbecco's modified Eagle medium (DMEM) containing 100 units/ml penicillin and streptomycin, Hepes, Tes, Bes and 15% foetal-bovine serum and were maintained at 37 °C in a humidified atmosphere of air/CO_2 (19:1). The medium was renewed daily, and in most experiments the serum component was omitted after the first 24 h in culture and replaced with 100 ng of insulin/ml. $Na_2{}^{35}SO_4$ (350–600 mCi/mmol; 15 μCi/ml), [^{3}H]leucine (60 Ci/mmol; 6–15 μCi/ml) and [^{3}H]serine (18–24 Ci/mmol; 6–15 μCi/ml) were included in the medium in some experiments to label newly synthesized proteoglycans and proteins respectively. All radioisotopes were supplied by NEN DuPont (U.K.). Incorporation of radioactivity into macromolecules in the culture medium and cell layer was measured by Sephadex G-25 (PD-10 column) chromatography [7]. The cell layer was solubilized by a 4% CHAPS/4 M guanidine hydrochloride procedure in the presence of a cocktail of proteinase inhibitors [7].

UDP-sugar analysis

UDP-sugars were labelled by incubating the cells with medium containing either D-[1-^{3}H]glucose (15.5 Ci/mmol; 50 μCi/ml) or D-[6-^{3}H]glucosamine (30 Ci/mmol; 25 μCi/ml), both from NEN

Abbreviation used: DMEM, Dulbecco's modified Eagle medium.

* Present address: Department of Clinical Biochemistry, King's College Hospital, Denmark Hill, London SE5 9RS, U.K.
† Present address: School of Life Science, Kingston University, Penrhyn Road, Kingston upon Thames, Surrey, U.K.
‡ To whom correspondence should be addressed.

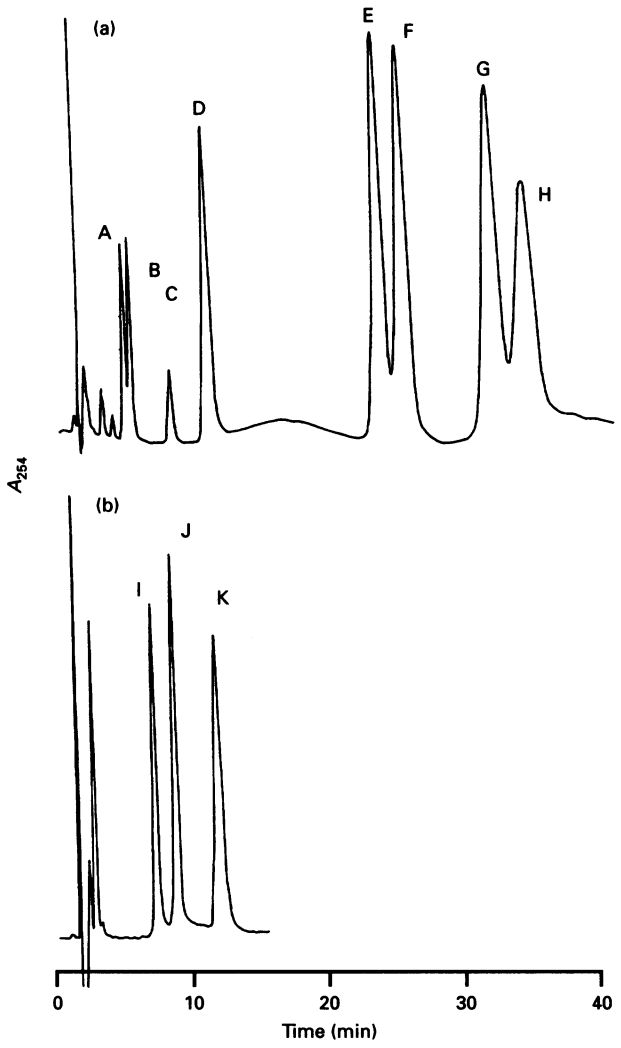

Figure 1 H.p.l.c. separation of standard UDP-sugars and nucleotides

UDP-sugars and nucleotides were separated by h.p.l.c. on a Partisphere SAX column eluted with either (**a**) 30 mM KH_2PO_4, pH 3.5, or (**b**) 200 mM KH_2PO_4, pH 4.0. Standards (1.44 nmol) were eluted with the following retention times (min): run **a**, A, AMP (4.73); B, UMP (5.20); C, CMP-NeuNAc (8.07); D, GMP (10.73); E, UDP-GlcNAc (23.75); F, UDP-GalNAc (25.57); G, UDP-glucose/-galactose (31.72); H, UDP-xylose (33.99); run **b**; I, UDP (7.15); J, UDP-GlcUA (8.70); K, ADP (11.89).

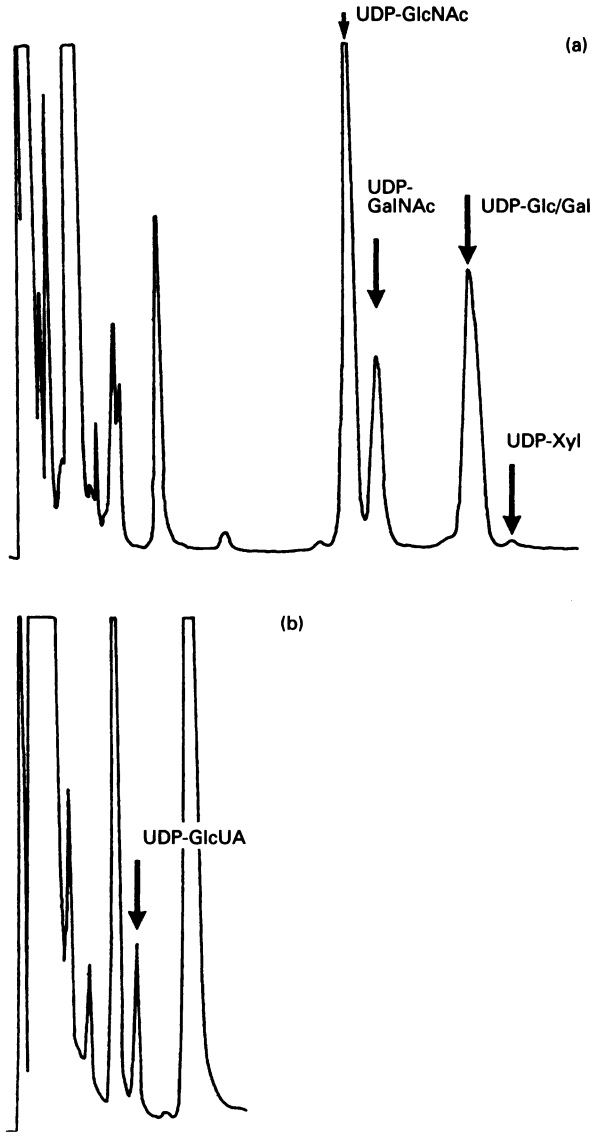

Figure 2 H.p.l.c. analysis of UDP-sugar pools in Swarm chondrocytes

Freshly isolated chondrocytes were extracted with boiling water and analysed for UDP-sugars by separating them on a Partisphere SAX column eluted with either (**a**) 30 mM KH_2PO_4 or (**b**) 200 mM KH_2PO_4. Arrows indicate the elution of UDP-*N*-acetylglucosamine (UDP-GlcNAc), UDP-*N*-acetylgalactosamine (UDP-GalNAc), UDP-glucose and UDP-galactose (UDP-Glc/Gal), UDP-xylose (UDP-Xyl) and UDP-glucuronate (UDP-GlcUA).

DuPont (U.K.). UDP-sugars were released from the cells by a modification of the method of Singh et al. [8]. The culture dishes were placed on ice, the medium was removed and the cell layer was washed once rapidly with cold phosphate-buffered saline. The cell layer was then scraped gently into cold phosphate-buffered saline (2 ml) and the plate was washed with a further 1 ml of cold phosphate-buffered saline, which was combined with the cells. After centrifuging (bench centrifuge, 1000 rev./min, 4 °C, 5 min) the supernatant was removed and the cell pellet resuspended in 2 ml of cold distilled water. The cell suspension was immediately boiled for 2 min, after which it was frozen at −20 °C. The extracts were thawed subsequently, centrifuged, and the supernatant was filtered through a 0.2 μm-pore filter before analysis of UDP-sugars by h.p.l.c.

Nucleotides (ADP, ATP) were extracted with $HClO_4$, by adapting previously reported methods [9,10]. The cell layers were placed on ice and washed rapidly with cold phosphate-buffered saline as above. Then 2 ml of ice-cold 0.3 M $HClO_4$ was added to the cell layer, which was left for 5 min before scraping and collecting it. The culture dish was washed with a further 1 ml of 0.3 M $HClO_4$, which was added to the initial extract. The extract was centrifuged (bench centrifuge, 2500 rev./min, 4 °C, 10 min), and the supernatant collected, neutralized with 8 M KOH/1 M K_3PO_4 (90 μl), and filtered through a 0.2 μM-pore filter before storage at −20 °C. Extraction with $HClO_4$ yielded about 8.2 nmol of ATP/10^6 fresh chondrocytes and an ATP/ADP ratio of 9.0, compared with 3.4 nmol of ATP and a ratio of 0.9 for boiling-water extracts. The latter procedure was used for UDP-sugar extraction, since UDP-glucuronate extracted by $HClO_4$ does not remain stable on storage [11].

Analysis of UDP-sugars and nucleotides

Solvents and chemicals used for the analysis were of the highest

grade available (AristaR or h.p.l.c. grade) and were obtained from BDH, and Anachem. Standard nucleotide sugars and nucleotides were purchased from Sigma Chemical Co.

Portions (200 μl) of cell extracts were loaded on to a Partisphere SAX column (125 mm × 4.7 mm internal diam.) connected to a Spectrophysics 8700 h.p.l.c. delivery system. The A_{254} of the column effluent was monitored with a Spectrophysics LC871 detector coupled to an integrator (Spectrophysics 4370). Fractions (0.5 ml) were collected for counting of ^{3}H radioactivity. Two isocratic runs were used to separate all the UDP-sugars of interest. In the first, the column was eluted with 30 mM KH_2PO_4, pH 3.5 (1 ml/min, 20 °C). This resolved UDP-*N*-acetylglucosamine, UDP-*N*-acetylgalactosamine, UDP-glucose/-galactose and UDP-xylose (Figure 1). In the second, the column was eluted with 200 mM KH_2PO_4, pH 4.0 (1 ml/min, 20 °C). This resolved UDP-glucuronate from UDP, CDP, ADP and GDP (Figure 1). ATP was resolved from 3′-phosphoadenosine 5′-phosphosulphate and UTP/CTP with 400 mM KH_2PO_4, pH 4.0 (1 ml/min, 20 °C). A range of standards (0.1–2.5 nmol) was run for each UDP-sugar and nucleotide and gave a linear response of concentration against peak area. UDP-sugars were identified in analyses of cell extracts by retention times and comparison with the data reported by Wice et al. [9] for peaks eluted with 30 mM KH_2PO_4. UDP-glucuronate was identified by its co-elution with trace amounts of [^{14}C]UDP-glucuronate. In some experiments the cell extract was treated with acid phosphatase (Sigma Type II, 0.04 unit/ml final concn.; 5 min, room temperature) to remove terminal phosphates from nucleotides [9]. This did not affect UDP-glucuronate or any other nucleotide sugar. This treatment was also used to remove unknown [^{3}H]-glucose-labelled products which otherwise interfered with the analysis of [^{3}H]UDP-glucuronate.

When the h.p.l.c. column showed any sign of decreased resolving power, it was cleaned by pumping the following solvents in sequence: 5-fold-concentrated buffer, distilled deionized water, 0.5 M H_3PO_4, distilled deionized water, 0.1 M-EDTA, distilled deionized water, methanol (h.p.l.c. grade).

Table 1 Levels of UDP-sugars in freshly isolated Swarm chondrocytes

Cell extracts were analysed as in Figure 2, and the results are expressed as nmol of UDP-sugar/10^6 cells. The range for duplicate cell extracts was $< \pm 8\%$.

UDP-sugar	Content (nmol/10^6 cells)	Ratio
UDP-*N*-acetylglucosamine	2.9	1.00
UDP-*N*-acetylgalactosamine	1.2	0.41
UDP-glucose/-galactose	2.5	0.86
UDP-glucuronate	0.6	0.21
UDP-xylose	0.03	0.01

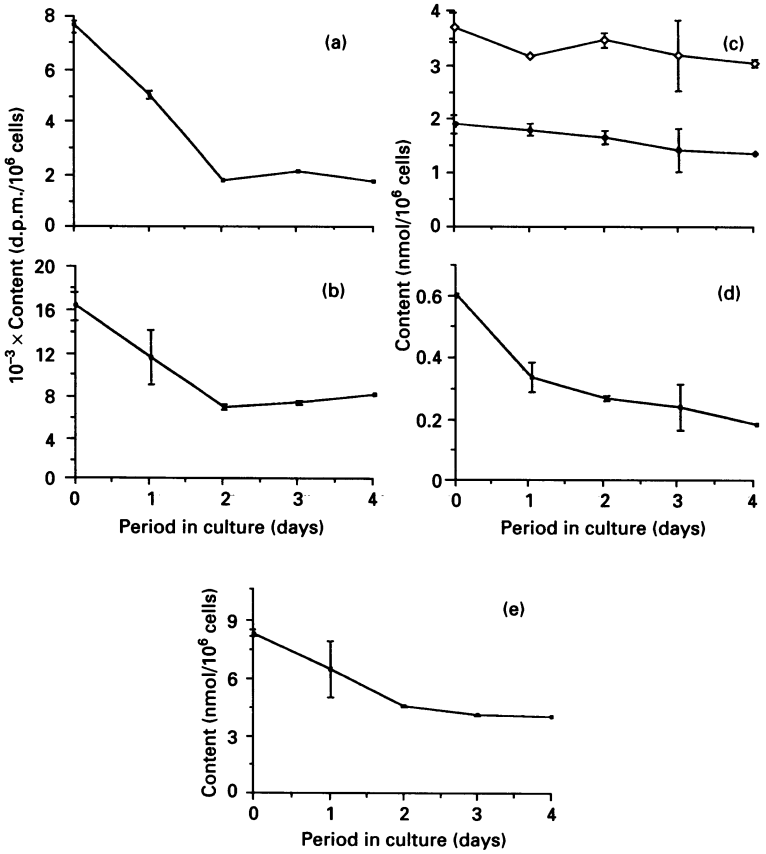

Figure 3 Effect of time in culture on UDP-sugar pools, ATP pool and [^{35}S]proteoglycan synthesis

Chondrocytes were isolated from the Swarm chondrosarcoma and either analysed immediately for UDP-sugar and ATP pools (day 0) or cultured in DMEM + 15% foetal-calf serum for up to 4 days. Cultures were labelled with [^{35}S]sulphate and [^{3}H]leucine for 1 h after 2 h (day 0) and on days 1–4 in culture and analysed for: (**a**) [^{35}S]proteoglycan; (**b**) [^{3}H]protein; (**c**) UDP-*N*-acetylhexosamines (◇) and UDP-hexoses (◆); (**d**) UDP-glucuronate; and (**e**) ATP. Results show the mean ± range for duplicate cultures at each time point.

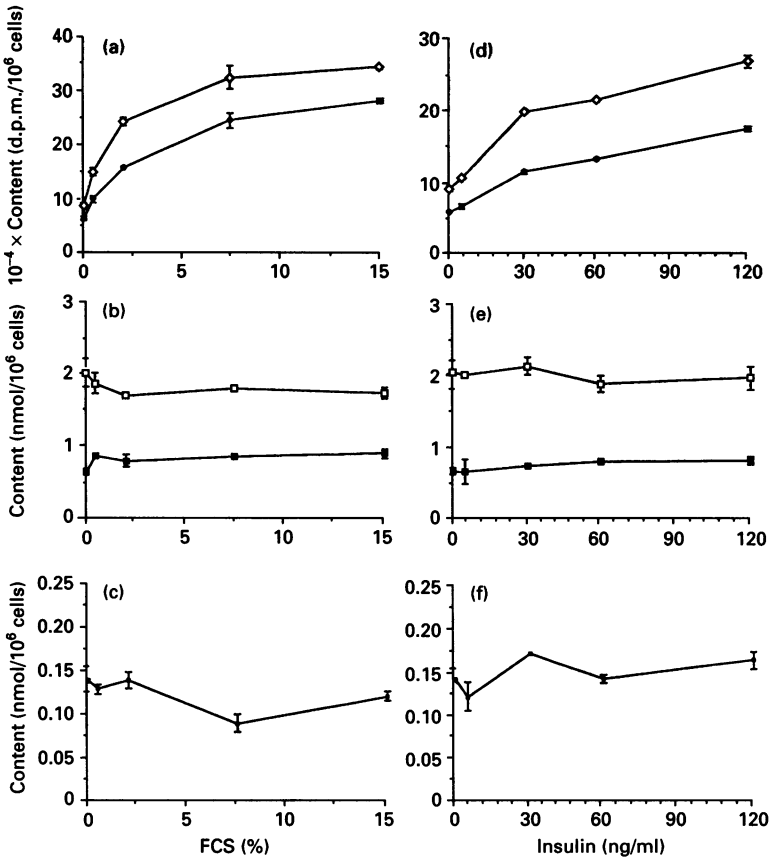

Figure 4 Effect of foetal-calf serum and insulin on UDP-sugar pools and [^{35}S]proteoglycan synthesis

Chondrocyte cultures were maintained with various concentrations of either foetal-calf serum (FCS) (**a–c**) or insulin (**d–f**) from day 1 and labelled with [^{35}S]sulphate and [^{3}H]glucosamine on day 3, after which they were analysed for [^{35}S]proteoglycans (◇) and ^{3}H-labelled macromolecules (◆) (**a**, **d**); parallel cultures were analysed for UDP-*N*-acetylhexosamines (◇) and UDP-hexoses (◆) (**b** and **e**), or UDP-glucuronate (**c** and **f**). Results show the mean ± range for duplicate cultures for each point.

RESULTS

Preliminary experiments established that the maximum release of UDP-sugars from chondrocytes was obtained by boiling the cell layer for 2 min. Boiling UDP-sugars in distilled water had no effect on their subsequent recovery when analysed by h.p.l.c. However, when known amounts of [^{14}C]UDP-glucuronate and UDP-xylose were added to cell layers before boiling to release endogenous UDP-sugars, recoveries of 83 ± 4% and 74 ± 5% respectively were obtained in subsequent h.p.l.c. analyses. Extracts based on boiling gave more consistent analyses for UDP-glucuronate than did those with $HClO_4$, even though the latter were quickly neutralized.

Portions of cell extracts corresponding to the products from 10^6 chondrocytes were analysed for UDP-sugar content. A typical analysis is shown for freshly isolated cells (Figure 2 and Table 1). UDP-*N*-acetylglucosamine and UDP-*N*-acetylgalactosamine are well separated from one another, but UDP-glucose and UDP-galactose are co-eluted. Only trace amounts of UDP-xylose were detected in freshly isolated cells, even when the products from large cultures (25×10^6 chondrocytes) were analysed. UDP-glucuronate is clearly resolved, but is present in much lower amounts than the UDP-*N*-acetylhexosamines and UDP-hexoses.

We reported previously that the level of proteoglycan synthesis in Swarm chondrocytes falls during the first 2 days in culture. This was observed irrespective of whether cells were cultured in the presence of foetal-calf serum or insulin [12]. In the present experiments cultures were maintained in DMEM supplemented with 15% foetal-calf serum for 4 days. A similar decrease was observed in the synthesis of both [^{35}S]proteoglycans and [^{3}H]leucine-labelled macromolecules during the first 48 h, after which these activities remained constant (Figures 3a and 3b). ATP pools in the chondrocytes also decreased during this period (Figure 3e), but the ATP/ADP ratio (9.0) remained unchanged. The individual UDP-sugar pools differed from one another in their response to cell culture. The UDP-*N*-acetylhexosamine pool remained relatively constant in the monolayer culture, with both UDP-*N*-acetylglucosamine and UDP-*N*-acetylgalactosamine being maintained at 80–90% of the levels in freshly isolated cells (Figure 3c). The UDP-hexose pools were also relatively constant in culture, being maintained at about 80–95% of the fresh-cell levels (Figure 3c). However, the UDP-glucuronate pool size decreased rapidly in culture (Figure 3d). The decrease in cellular concentration of this sugar nucleotide was similar to the decreasing pattern seen for ATP, [^{35}S]proteoglycan synthesis and [^{3}H]protein synthesis.

Stevens and Hascall [13] showed that Swarm chondrocyte monolayers maintained in the presence of insulin synthesized larger chondroitin sulphate chains than those synthesized by cells maintained without insulin. They suggested that this could be the result of increased intracellular levels of sugar nucleotides in the

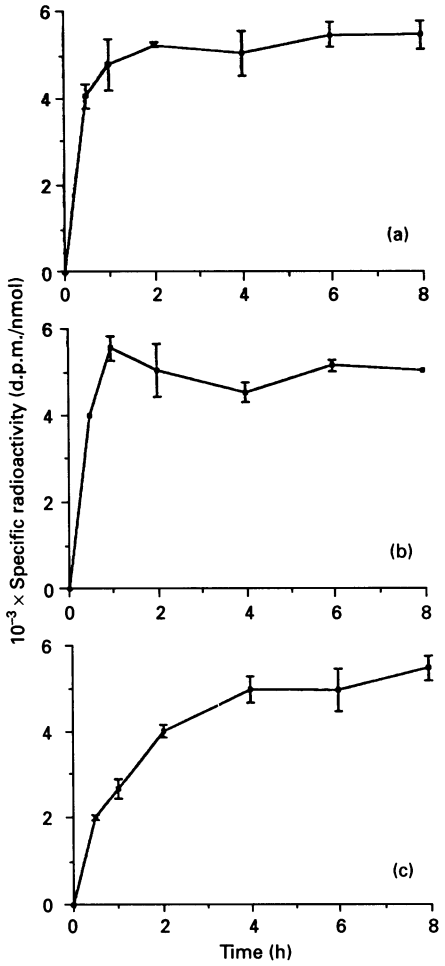

Figure 5 Equilibration of UDP-sugar pools with [1-^{3}H]glucose

Chondrocyte cultures were labelled on day 1 with [1-^{3}H]glucose for various times, after which they were analysed for UDP-hexoses (**a**), UDP-glucuronate (**b**) and UDP-*N*-acetylhexosamines (**c**), and the ^{3}H specific radioactivity of each sugar nucleotide was calculated. Results show the mean specific radioactivity per nmol of sugar nucleotide ± range for duplicate cultures.

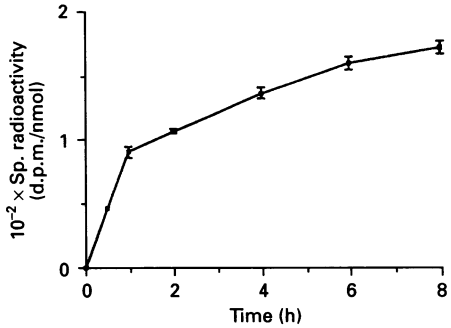

Figure 6 Equilibration of the UDP-*N*-acetylhexosamine pool with [6-^{3}H]glucosamine

Except for the use of [6-^{3}H]glucosamine as the precursor, details of the experiment are as described in Figure 5.

supplemented cultures. To investigate whether foetal-calf serum and insulin affected UDP-sugar pool sizes, chondrocytes were plated in the presence of 15% foetal-calf serum and changed to medium containing various concentrations of insulin or foetal-calf serum on day 1. They were maintained at these concentrations for a further 2 days, and on the last day of culture the cells were labelled with either [^{35}S]sulphate or [^{3}H]glucosamine to measure incorporation of these radioisotopes into macromolecules. Parallel cultures were extracted and analysed for UDP-sugars. Both foetal-calf serum and insulin stimulated the synthesis of labelled macromolecules in a dose-responsive fashion, as expected (Figures 4a and 4d). The maximum increase in [^{35}S]sulphate incorporation was about 3-fold. Incorporation of [^{3}H]serine into macromolecules was maximally stimulated 2-fold (results not shown). However, neither insulin nor foetal-calf serum had any effect on the pool sizes of UDP-hexose, UDP-*N*-acetylhexosamines or UDP-glucuronate (Figures 4b, 4c, 4e, 4f). Thus in Swarm chondrocytes the pool sizes of the UDP-sugar precursors of glycosaminoglycans are independent of the rate of [^{35}S]proteoglycan synthesis. Therefore any increase in demand for UDP-sugars when proteoglycan synthesis is stimulated must be accompanied by an increased rate in their synthesis and utilization while maintaining overall pool sizes constant.

The synthesis of UDP-sugars was investigated by using either [1-^{3}H]glucose or [6-^{3}H]glucosamine as precursors. Day-1 Swarm chondrocyte monolayer cultures were labelled for various times from 30 min to 8 h, after which the cells were extracted and analysed for UDP-sugars. Cell extracts were treated with acid phosphatase before analysis for UDP-[^{3}H]glucuronate. With [1-^{3}H]glucose both the UDP-hexose and UDP-glucuronate pools become labelled to a constant specific radioactivity within 1 h (Figures 5a and 5b). The half time ($t_{\frac{1}{2}}$) to reach this steady-state plateau is approx. 12 min for each of these UDP-sugars. In contrast, the pool of UDP-*N*-acetylhexosamine sugars took about 4 h to reach a constant specific radioactivity (Figure 5c). The $t_{\frac{1}{2}}$ to reach this steady state is approx. 50 min, assuming first-order kinetics. The calculated specific radioactivity of the labelling medium used for these experiments was 4400 d.p.m./nmol of glucose. It is noteworthy that the steady-state specific activities at 4 h of the UDP-*N*-acetylhexosamine, UDP-hexose and UDP-glucuronate pools were 4942 ± 297, 5007 ± 500 and 4499 ± 227 d.p.m./nmol of sugar nucleotide respectively. Thus there is no dilution of the [^{3}H]glucose by intracellular intermediates, and glucose in the medium must be the major source of precursor for the formation of UDP-sugars.

Glucosamine is phosphorylated to glucosamine 6-phosphate and by-passes the amidation step required in the synthesis of UDP-*N*-acetylhexosamine from glucose. When [6-^{3}H]glucosamine was used as the precursor, the time taken for the UDP-*N*-acetylhexosamine pool to reach a constant specific radioactivity was about 5–6 h (Figure 6). Again, assuming first order kinetics, the $t_{\frac{1}{2}}$ to reach the steady state was 55 min, which is very similar to that found for [^{3}H]glucose-labelled UDP-*N*-acetylhexosamine (see above).

The specific radioactivity of the [^{3}H]glucosamine in the medium was 6.38×10^7 d.p.m./nmol, whereas after 8 h of labelling the specific radioactivity of the UDP[^{3}H]*N*-acetylhexosamine pool was 1.69×10^5 d.p.m./nmol. This implies that for every molecule of [^{3}H]glucosamine entering the pool, 377 molecules of unlabelled glucose also enter via the transamidation of fructose 6-phosphate to glucosamine phosphate. Thus when glucosamine is used as a metabolic precursor for glycosaminoglycans and glycoproteins it labels them by a vary minor pathway.

Trace amounts of radioactivity were sometimes detected in the UDP-hexose peak after labelling for 6–8 h with [6-^{3}H]-glucosamine. [^{3}H]Glucosamine could be converted into glucosamine 6-phosphate, then into fructose 6-phosphate by glucosamine-6-phosphate deaminase, and so via glucose 1-phosphate to UDP-glucose. It seems more likely that the ^{3}H radioactivity associated with the UDP-hexose pool is due to

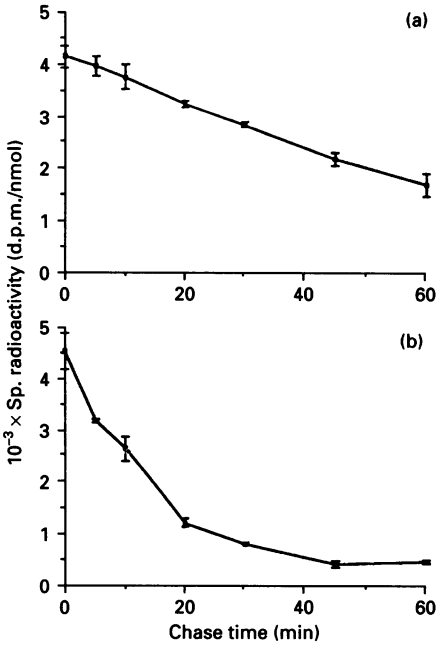

Figure 7 Turnover of UDP-sugar pools

Chondrocyte cultures were labelled on day 1 with [1-^{3}H]glucose for 4 h and then chased with unlabelled high-glucose DMEM for various times. Cultures were analysed for UDP-sugars during the chase period, and specific radioactivities were calculated; (**a**) UDP-*N*-acetylhexosamine pool; (**b**) UDP-hexose pool. Results show the mean ^{3}H specific radioactivity per nmol of sugar nucleotide, ±range for duplicate cultures.

minor contamination of the glucosamine with [^{3}H]glucose. The [^{3}H]glucosamine had a radiochemical purity of 98.5% as stated by the manufacturers.

The pool sizes of the UDP-sugars appear to be maintained in a relatively steady state, implying that in any particular conditions the rate of synthesis must be equivalent to the rate of utilization. To test this we measured the rate of disappearance of UDP-sugars in the chondrocytes after labelling cultures on day 1 with [^{3}H]glucose (50 μCi/ml) for 4 h, at which time all the UDP-sugar pools would have reached a constant specific radioactivity. After removing the labelling medium, the cell layer was washed rapidly three times with DMEM to dilute any free intracellular [^{3}H]glucose and the cultures were chased with unlabelled glucose by maintaining them in DMEM for various times ranging from 5 min to 1 h. The specific radioactivity of each UDP-sugar was determined (Figure 7). Semi-logarithmic plots of specific radioactivity against chase time were linear, indicating that the disappearance of UDP-sugars from each pool followed first-order kinetics (results not shown). The apparent half-life of the UDP-hexose and UDP-*N*-acetylhexosamine pools was 11 and 48 min respectively. These values are very close to the $t_{\frac{1}{2}}$ values estimated for these pools to reach a constant specific radioactivity during synthesis (12 and 50 min respectively). They indicate that the rate of synthesis of these UDP-sugars is precisely linked to their rate of utilization for synthesis of macromolecules. The UDP[^{3}H]glucuronate pool diminished at a rate ($t_{\frac{1}{2}}$ 25 min) intermediate between that for the UDP-hexose and UDP-*N*-acetylhexosamine pools. This is longer than the value determined for the $t_{\frac{1}{2}}$ for the UDP-glucuronate pool to reach constant specific radioactivity during its synthesis from [^{3}H]glucose (12 min). The discrepancy probably results from the UDP[^{3}H]hexose pool continuing to supply the UDP-glucuronate pool during the chase period. Thus the apparent half life for UDP[^{3}H]glucuronate would be over-estimated.

DISCUSSION

Carbohydrate polymers and complex glycoconjugates are formed by the ordered, step-wise, addition of individual sugars from their nucleotide derivatives to an appropriate acceptor, for example an amino acid residue in a polypeptide chain, or a carbohydrate sequence in an incomplete polymer [14]. Chondrocytes actively synthesize proteoglycans and other glycoproteins, and those from Swarm rat chondrosarcoma, a tumour which assembles an extracellular matrix of macromolecules typical of hyaline cartilage, have been reported as making between 2 and 4 μg of proteoglyan/h per 10^6 cells [15]. Thus we expected that they would provide a useful model system for investigating UDP-sugar metabolism in chondrocytes. Although a great deal is known about the biosynthesis of cartilage proteoglycan by these chondrocytes [2,15], there is virtually no information about the generation and regulation of the UDP-sugars required to synthesize the glycosaminoglycan and oligosaccharide components of proteoglycans. UDP-sugars are synthesized in the cytosolic compartment and must be translocated across intracellular membranes into the Golgi [16–18], where glycosaminoglycan polymerization occurs. There are therefore two pools of UDP-sugars, but distinguishing one from another is technically difficult and no attempt was made to do so in this study. A number of factors may be involved in the regulation of UDP-sugar levels, including the availability of substrates for their synthesis, feedback-inhibition effects on specific enzymes involved in their synthesis, the energy status of the cell and the activity of the translocation systems and glycosyltransferase enzymes which utilize the sugar nucleotides. The cellular concentration of UDP-sugars has been estimated to be in the range 10^{-4}–10^{-5} M [19–21], whereas the K_m values for sugar nucleotides of the glycosyltransferases for glycosaminoglycan synthesis have been reported to be approx. 10^{-4} M [22]. However, these calculations do not take into account the compartmentation of the sugar nucleotides in the cell. Thus the Golgi concentration of sugar nucleotides could be higher than these estimates, providing saturating levels of substrates for the enzymes.

H.p.l.c. on a Partisphere SAX column provided a rapid method for analysing all the UDP-sugars of interest, except UDP-glucose and UDP-galactose, which are eluted as a single peak. Nevertheless, this is an improvement compared with separations using isotachophoresis, where UDP-*N*-acetylglucosamine and UDP-*N*-acetylgalactosamine were not resolved, nor UDP-hexose or UDP-xylose identified [23]. A previous h.p.l.c. method using a Partisil 10 SAX column failed to resolve UDP-glucuronate and UDP and NADP [9]. Despite the sensitivity of our analysis, we were barely able to detect UDP-xylose in freshly isolated chondrocytes, and the analysis shown should be regarded as an upper estimate of the concentration of this sugar nucleotide. We were unable to detect UDP-xylose at all in cultured cells. In mammalian cells the sole requirement for UDP-xylose is in proteoglycan synthesis, where it is the donor of the first sugar to be substituted on to the core protein to initiate glycosaminoglycan polymerization. Partially purified UDP-glucose dehydrogenase is inhibited specifically by UDP-xylose. The product of this enzyme is UDP-glucuronate, which in turn is decarboxylated to form UDP-xylose. Thus UDP-xylose could act as a feedback inhibitor of this pathway, regulating both its own availability and that of UDP-glucuronate for glycosaminoglycan synthesis. However, the studies testing this concept used isolated enzyme preparations with UDP-glucose/UDP-xylose ratios in the range 5–30 [24–26]. If one assumes a UDP-glucose/UDP-galactose ratio of 4:1 [1] for the UDP-hexose pool in the Swarm

chondrocytes, the ratio of UDP-glucose/UDP-xylose would, at best, be 65:1. Thus whether UDP-xylose has a physiological role as a feedback inhibitor of the pathway in the chondrosarcoma cells is very unclear. Moreover, proteoglycan synthesis in cultures maintained without foetal-calf serum or insulin is only about one-third of that in cultures supplemented with these factors, so the demand for UDP-xylose must be greatly decreased in the unsupplemented cultures. However, the size of the UDP-glucuronate pool is about the same in unsupplemented cultures as in those maintained with either 15% foetal-calf serum or 100 ng/ml insulin present. This argues against a feedback inhibition of the pathway in the unsupplemented cultures. It is noteworthy that UDP-xylose was not detectable in cultured fibroblasts, human colon cells and chick-embryo limb chondrocytes [9,21,23]. Bovine corneal epithelium, on the other hand, contains relatively high concentrations of UDP-xylose [20]. Balduini et al. [27] reported that the addition of UDP-xylose to cultures of corneal slices was accompanied by a decrease in chondroitin sulphate synthesis. However, no evidence was presented to show that the UDP-xylose actually entered cells.

Experiments *in vivo* suggest that in rat liver UDP-*N*-acetylglucosamine inhibits the amidation of fructose 6-phosphate by glutamine–fructose-6-phosphate aminotransferase [28]. This reaction is the committing step for UDP-*N*-acetylhexosamine synthesis, and its regulation has been studied in detail [29–31]. The transamidase, unlike other transamidases [32], cannot use ammonia as a substrate and has an obligatory requirement for glutamine [29]. In our experiments the high concentration (4 mM) of glutamine in the medium would ensure that the enzyme operates under saturating conditions. The size of the UDP-*N*-acetylhexosamine pool in the chondrosarcoma chondrocytes is remarkably constant, showing little or no change on culturing the cells, even though protein synthesis, proteoglycan synthesis and ATP levels all decline during the first 48 h of culture. Neither is it affected by the presence or absence of foetal-calf serum or insulin in the culture medium. The ratio of UDP-*N*-acetylglucosamine/UDP-*N*-acetylgalactosamine was about 2.5:1 under all conditions.

The UDP-hexose and UDP-glucuronate pool sizes were also unaffected by foetal-calf serum or insulin, even though these factors stimulated proteoglycan synthesis in the chondrocytes, in agreement with previous observations [13,33]. However, in contrast with the other UDP-sugars, the size of the UDP-glucuronate pool contracted greatly during the first 24–48 h of culture as compared with freshly isolated cells. This change paralleled the decreasing rates of [^{35}S]proteoglycan synthesis and the size of the ATP pool during the initial period of culture. Since the UDP-hexose pool was constant during culture, the decrease in the UDP-glucuronate pool must reflect a decrease in the activity of UDP-glucose dehydrogenase. This enzyme catalyses the irreversible conversion of UDP-glucose into UDP-glucuronate in three steps and requires stoichiometric amounts of NAD^+ [5]. The enzyme is strongly inhibited by NADH, but this effect is largely dependent on the concentration of NAD^+ [27].

The UDP-sugar pool sizes in Swarm chondrocytes are generally higher than in other cells where these have been measured [34], but lower than in some, for example the hepatocyte, which contains 2.2–6.1 nmol of UDP-hexose and 1.7–5.3 nmol of UDP-glucuronate per 10^6 cells [11]. Since the biosynthetic activity of cultures of Swarm chondrocytes can be stimulated considerably by serum factors and insulin, whereas the size of the UDP-sugar pools remains unaffected, the increased requirement of sugar nucleotides for macromolecular synthesis must be met by increasing the rate of flux through the pools. We were able to measure the rate of flux through the pools in stimulated cultures, using [1-^{3}H]glucose as a precursor for labelling UDP-sugars. The ^{3}H in the C-1 position is not lost in UDP-sugar synthesis [35], and this is supported by our finding that UDP-hexose, UDP-*N*-acetylhexosamines and UDP-glucuronate equilibrate to the same ^{3}H specific radioactivity as one another and [^{3}H]glucose in the medium. It is contrary to the proposal made by Lohmander et al. [36]. Both the rate of labelling and the rate of depletion of labelled UDP-hexose and UDP-glucuronate are rapid, reflecting a rapid flux of sugar nucleotides through these pools. If the $t_{\frac{1}{2}}$ for UDP-glucuronate is taken as 12 min and the UDP-glucuronate pool size for day 1 in culture is accepted as being about 0.35 nmol/10^6 cells (Figure 3), it can be estimated that the cells utilize about 205 ng of glucuronate/h from the pool. Since all the glucuronate would be incorporated into chondroitin sulphate chains, and assuming that the monosaccharide accounts for 25% of the mass of the proteoglycan [37], this would lead to an estimate for the synthesis of proteoglycan of about 0.8 μg/h per 10^6 chondrocytes. This is rather lower than the rate of synthesis (2–4 μg/h per 10^6 cells) calculated by Kimura et al. [15,37]. However, the estimates cannot be compared too rigorously, since absolute rates of synthesis in any given series of experiments will depend on variations in the stimulatory activity of different batches of calf serum or insulin and possibly on the way in which the chondrosarcoma has diverged in different laboratories over years of passage.

The $t_{\frac{1}{2}}$ for the UDP-*N*-acetylhexosamine pool in the chondrocytes is much longer, being approx. 50 min, but the pool is much larger than the UDP-glucuronate pool. Thus, on day 1 with a UDP-*N*-acetylhexosamine pool size of 1.8 nmol/10^6 cells, the flux through the pool would be about 1.1 nmol/h per 10^6 cells, a rate 25% greater than the flux through the UDP-glucuronate pool. This additional flux would provide *N*-acetylhexosamines for the synthesis of glycoproteins and proteoglycan oligosaccharides [2,7] as well as for chondroitin sulphate polymerization.

Lohmander et al. [36] made estimates of 6.3 and 14.2 min for the $t_{\frac{1}{2}}$ of UDP-glucuronate and UDP-*N*-acetylgalactosamine respectively in cultures of Swarm chondrocytes. These values were extrapolated from the rate of incorporation of [^{3}H]glucose into the glucuronate and galactosamine components of chondroitin sulphate and suggest a more rapid rate of chondroitin sulphate synthesis than that achieved by our cultures, again perhaps due to differences in foetal-calf serum and/or chondrosarcoma. Their data do, however, confirm that the UDP-*N*-acetylhexosamine pool has a much longer $t_{\frac{1}{2}}$ than the UDP-glucuronate pool.

In conclusion, our data are consistent with the view that increased demand for UDP-sugars as a result of increased synthesis of proteoglycans is met by increasing the rate of flux through the sugar nucleotide pools while maintaining them at a constant size. It is unlikely that the UDP-sugar pools would become rate-limiting on glycosaminoglycan synthesis under any physiological conditions.

C.S. acknowledges the receipt of an SERC postgraduate studentship. We are grateful to the Medical Research Council for financial support.

REFERENCES

1 Leloir, L. F. (1951) Arch. Biochem. Biophys. **33**, 186–190
2 Lohmander, S. and Kimura, J. H. (1986) in Articular Cartilage Biochemistry (Kuettner, K., Schleyerbach, R. and Hascall, V. C., eds.), pp. 93–111, Raven Press, New York
3 Maroudas, A. (1975) Philos. Trans. R. Soc. London B **271**, 293–313
4 Venn, G. and Mason, R. M. (1983) Biochem. J. **215**, 217–225
5 Gabriel, O. and Van Lenten, L. (1979) Biochemistry of the Carbohydrates II: Int. Rev. Biochem. **16**, 1–36

6 Kimura, J. H., Hardingham, T. E., Hascall, V. C. and Solursh, M. (1979) J. Biol. Chem. **254**, 2600–2609
7 Mason, R. M., Kimura, J. H. and Hascall, V. C. (1982) J. Biol. Chem. **257**, 2236–2245
8 Singh, J., Schwartz, L. R. and Wiebel, F. (1980) Biochem. J. **189**, 369–372
9 Wice, B., Trugnan, G., Pinto, M., Rousser, M., Chevalier, G., Dussaulx, E., Lacroix, B. and Zweibaum, A. (1985) J. Biol. Chem. **260**, 139–146
10 Krug, E., Zweibaum, A., Schulz-Holstege, C. and Keppler, D. (1984) Biochem. J. **217**, 701–708
11 Aw, T. Y. and Jones, D. P. (1982) Anal. Biochem. **127**, 32–36
12 Bansal, M. K., Ward, H. and Mason, R. M. (1986) Arch. Biochem. Biophys. **246**, 602–610
13 Stevens, R. and Hascall, V. C. (1981) J. Biol. Chem. **256**, 2053–2058
14 Hughes, R. C. (1983) in Glycoproteins (Brammer, W. J. and Edidin, M., eds.), pp. 36–57, Chapman and Hall, London
15 Kimura, J. H., Lohmander, L. S. and Hascall, V. C. (1984) J. Cell Biochem. **26**, 261–278
16 Kuhn, N. J. and White, A. (1977) Biochem. J. **168**, 423–433
17 Perez, M. and Hirschberg, C. B. (1985) J. Biol. Chem. **260**, 4671–4678
18 Perez, M. and Hirschberg, C. B. (1986) Biochim. Biophys. Acta **864**, 213–222
19 Gainey, P. A. and Phelps, C. F. (1972) Biochem. J. **128**, 215–227
20 Handley, C. J. and Phelps, C. F. (1972) Biochem. J. **127**, 911–912
21 Speight, G., Handley, C. J. and Lowther, D. A. (1978) Biochim. Biophys. Acta **540**, 238–245
22 Helting, T. and Rodén, L. (1969) J. Biol. Chem. **244**, 2790–2798
23 Ericksson, G., Sarnstrand, B. and Malmstrom, A. (1984) Arch. Biochem. Biophys. **235**, 692–698
24 Neufeld, E. F. and Hall, C. W. (1965) Biochem. Biophys. Res. Commun. **19**, 456–461
25 Balduini, C., Brovelli, A., Seziale, P. and Castellani, A. (1975) Protides Biol. Fluids **22**, 213–217
26 Castellani, A. A., De Luca, G., Rindi, S., Salvnii, R. and Tira, M. (1986) Ital. J. Biochem. **35**, 296–303
27 Balduini, C., Brovelli, A. and Castellani, A. (1970) Biochem. J. **120**, 719–723
28 Kornfeld, S., Kornfeld, R., Neufeld, E. F. and O'Brien, P. J. (1964) Proc. Natl. Acad. Sci. U.S.A. **52**, 371–379
29 Winterburn, P. J. and Phelps, C. F. (1971a) Biochem. J. **121**, 701–709
30 Winterburn, P. J. and Phelps, C. F. (1971b) Biochem. J. **121**, 711–720
31 Winterburn, P. J. and Phelps, C. F. (1971c) Biochem. J. **121**, 721–730
32 Buchanan, J. M. (1973) Adv. Enzymol. Relat. Areas Mol. Biol. **39**, 91–183
33 d'Arville, C. A. and Mason, R. M. (1983) Biochim. Biophys. Acta **760**, 53–60
34 Yurchenco, R. D., Caccarini, C. and Atkinson, P. H. (1983) Methods Enzymol. **175**, 175–204
35 Hallerstein, M. K., Greenblatt, D. J. and Munro, H. N. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 7044–7048
36 Lohmander, L. S., Hascall, V. C., Yanagishita, M., Kuettner, K. E. and Kimura, J. H. (1986) Arch. Biochem. Biophys. **250**, 211–227
37 Kimura, J. H., Hardingham, T. E. and Hascall, V. C. (1980) J. Biol. Chem. **255**, 7134–7143

Received 12 August 1992/12 October 1992; accepted 20 October 1992