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Studies on the Biosynthesis of Cartilage Proteoglycan in a Model System of Cultured Chondrocytes From the Swarm Rat Chondrosarcoma

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Biosynthesis of cartilage proteoglycan was examined in a model system of cultured chondrocytes from a transplantable rat chondrosarcoma. Extensive modification with the addition of chondroitin sulfate glycosaminoglycan, N-linked oligosaccharide, and O-linked oliogosaccharide is required to convert a newly synthesized core protein precursor into a proteoglycan. Kinetic analyses revealed the presence of a large pool of core protein precursor ($t_{1/2} \sim 90$ min) awaiting completion into proteoglycan. The large $t_{1/2}$ of this pool allowed kinetic labeling experiments with a variety of radioactive precursors to distinguish between early biosynthetic events associated primarily with the rough endoplasmic reticulum from late events associated primarily with the Golgi apparatus. The results of a series of experiments indicated that the addition of N-linked oligosaccharide chains occurs early in the biosynthetic process in association with the rough endoplasmic reticulum, whereas the initiation and completion of O-linked oligosaccharides occurs much later, at about the same time as chondroitin sulfate synthesis. This also indicated that keratan sulfate chains, when present in the completed molecule, are added in the Golgi apparatus, as they are probably built on oligosaccharide primers closely related to the O-oligosaccharide chains. Furthermore, when ^{3}H-glucose was used as the precursor, the entry of label into xylose, the linkage sugar between the core protein and the chondroitin sulfate chain, was found to occur within 5 min of the entry of label into galactose and galactosamine in the remainder of the chondroitin sulfate chain. This indicated that the initiation and completion of the chondroitin sulfate chain occurs late in the pathway probably entirely in the Golgi apparatus. Thus, proteoglycan synthesis can be described as occurring in two stages in this system, translation and N-glycosylation of a core protein precursor which has a long half-life in the rough endoplasmic reticulum, followed by extensive rapid modification in the Golgi complex in which the majority of glycosaminoglycan and oligosaccharide chains are added to the core protein precursor with subsequent rapid secretion into the extracellular matrix.

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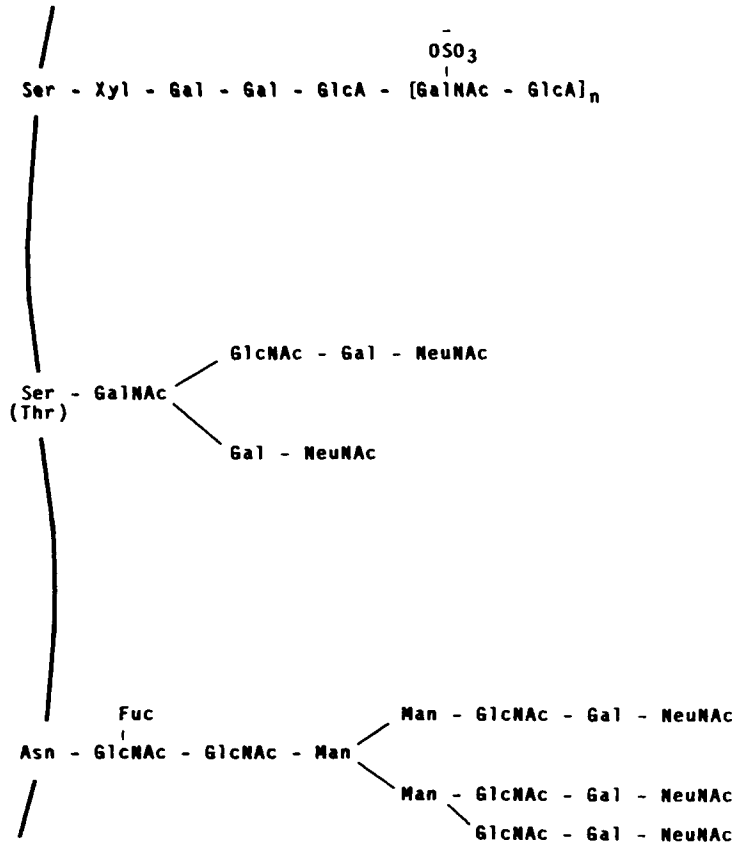

Fig. 1. Schematic representation of the oligosaccharides and glycosaminoglycan chains of proteoglycan monomer from the rat chondrosarcoma. Three types of sugar chains are shown. Both the O-linked oligosaccharide and N-linked oligosaccharide represent only one of several different structures present on the core protein.

The synthesis of cartilage proteoglycan requires extensive post-translational modification of a precursor core protein [1–4]. Several different complex glycoconjugate structures are found on the completed proteoglycan (Fig. 1). They include about 80–100 chondroitin sulfate chains and usually keratan sulfate, 120–150 O-linked oligosaccharides resembling those found in mucins, and 10–15 N-linked oligosaccharides of the complex type found in many glycoproteins. As with other secreted glycoconjugates, the synthetic processes occur in at least two subcellular compartments, the rough endoplasmic reticulum and the Golgi apparatus complex. The completed proteoglycan is about 2.5×10^6 in molecular weight of which more than 90% is added posttranslationally to a 3.5×10^5 molecular weight core protein precursor.

The present study examined this biosynthetic process in a model system using short-term primary cultures of chondrocytes derived from a transplantable rat chondrosarcoma [5–7]. The tumor resembles hyaline cartilage (8,9) and synthesizes

proteoglycans which are similar to cartilage proteoglycan, perhaps of the embryonic type [8,10,11]. High density cultures established from trypsin-collagenase liberated cells [5,12] continue to synthesize a proteoglycan very similar to that found in the tumor [5] at a high rate, approximately 2–4 μg proteoglycan/h/10^6 cells [13].

The schematic diagram in Figure 2 outlines how proteoglycan synthesis proceeds from the rough endoplasmic reticulum (RER), where the core protein precursor is synthesized through the Golgi apparatus compartment where the chondroitin sulfate chains are added [14–20]. The addition of inhibitors of protein synthesis, such as cycloheximide, to the cultures inhibits proteoglycan synthesis by blocking input of core protein precursor into the biosynthetic pathway [21–24], (Fig. 2). Sulfation is one of the last steps in the biosynthetic process occurring concomitantly with glycosaminoglycan chain polymerization [25–27]. When (^{35}S)sulfate was used to assess the rate of appearance of completed proteoglycan at times after a cycloheximide block, it was observed that proteoglycan synthesis continued at a decreasing rate for a relatively long time after protein synthesis was stopped [23,24]. The decrease with time showed nearly first order kinetics with a t½ of ~ 90 min. This suggested that a sizeable pool of core protein precursor existed in the cells, assuming that the rate of radiosulfate incorporation was proportional to the amount of available glycosylation sites (ie, core protein precursor without chondroitin sulfate or with incomplete chains). This was verified further by prelabeling the core protein precursor pool with (^{3}H)serine for 15 min before introducing the cycloheximide block and subsequently following the appearance of labeled core protein in completed proteoglycan (Fig. 2, open circles). The kinetic parameters indicated a first order rate of appearance of label in proteoglycan with a t½ of about 90 min.

Continuous labeling protocols were also used to determine the kinetics of synthesis of core protein precursor and completed proteoglycans. In such experiments, there will be time delays in the entry of the label into the macromolecular products associated with equilibration of the labeled precursor in the medium with all the intracellular precursor compartments. Only when these compartments have reached a steady state will incorporation kinetics into the macromolecules under study become linear with time. For example, (^{3}H)serine in the culture medium must progress through intracellular pools of serine, seryl-tRNA, and serine associated with nascent polypeptides in polyribosomes before entering the pool of completed protein. A further delay is introduced before the label enters the completed molecule in the case of molecules which require post-translational modification before completion. This is the case for proteoglycan synthesis, as the core protein precursor must be post-translationally modified by the addition of a large number of glycosaminoglycan chains. The relative contribution of each of the two precursor compartments was examined by comparing the rate of appearance of (^{3}H)serine into total protein (to gain an estimate of the delay produced by the precursor pools to protein synthesis) and into completed proteoglycan (to allow a determination to be made on the delay due to the core protein precursor pool). An experiment was performed in which cultures were labeled with (^{3}H)serine for times from 15–420 min [28]. The amount of ^{3}H-radioactivity in total protein for each culture was determined for both the culture medium and a 4 M guanidine HCl extract of the cell layer as described in Figure 3. The entry of (^{3}H)serine into total protein was linear soon after the introduction of the label (solid line) while the entry of label into completed proteoglycan was significantly delayed. These results indicate that the intracellular precursor pools leading to protein

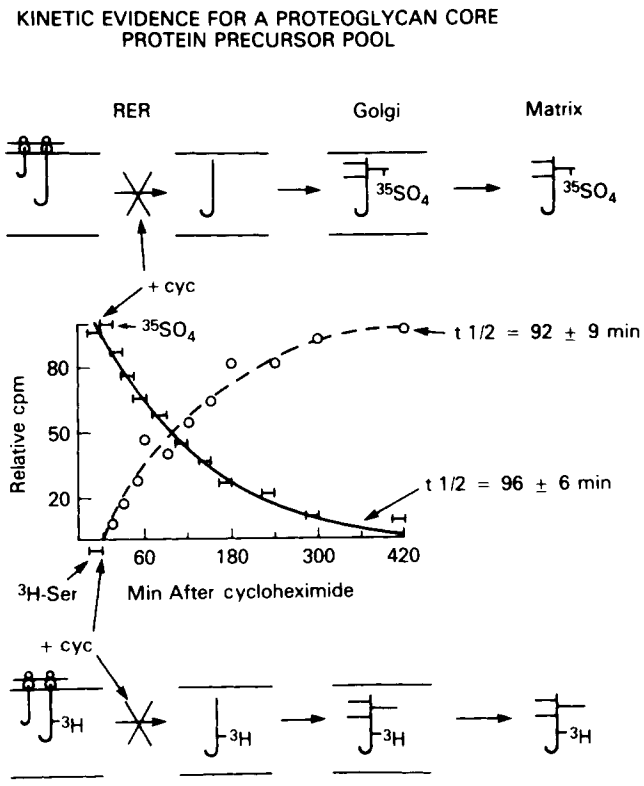

Fig. 2. Inhibition of proteoglycan synthesis by cycloheximide treatment. Chondrosarcoma cell cultures were treated with 100 ug/ml cycloheximide to block translation and hence input of core protein precursor into the biosynthetic pathway flowing from the rough endoplasmic reticulum (RER) through the Golgi apparatus into the extracellular matrix. At this concentration of drug, protein synthesis was inhibited to greater than 95% within 2 min after introduction of cycloheximide as determined by the incorporation of (^{3}H)serine into macromolecules (23). Cultures were labeled with (^{35}S)sulfate for 15 min at various times after the cycloheximide block (horizontal bars), at the end of which the cultures were extracted with 4 M guanidine HCl - 2% Zwittergent 3-12 (23) and the amount of macromolecular radioactivity determined for the combined culture medium and cell extract by chromatography on Sephadex G-25 (solid curve). In a separate experiment, the cultures were labeled with a pulse of (^{3}H)serine 15 min prior to the cycloheximide block (horizontal bar, lower left). At subsequent times following treatment, the amount of radioactivity in completed proteoglycan was monitored after isolating labeled proteoglycan from the bottom fraction of dissociative CsCl density gradients (open circles, dashed curve).

synthesis equilibrated rapidly with the (^{3}H)serine and that the delay in labeling of the proteoglycan indicated that an additional precursor pool not detected for total protein synthesis is involved in proteoglycan synthesis.

Simplified kinetic equations previously used for the kinetic analysis of DNA synthesis [29] and polysaccharide synthesis [30] were used to describe the entry of label into total protein. For continuous labeling studies, the culture medium can be regarded as a large reservoir of labeled precursor, in this case (^{3}H)serine, at constant

specific activity. In steady state, entry of labeled precursor into the final protein products would occur at a constant rate that is proportional to, but in general not equal to the specific activity of the isotope in the culture medium. By treating all of the sequential intermediate precursor pools as a single compartment (PE), the rate of labeling of this compartment can be given by the difference between the rates of input and output, or:

$$dPE/dt = RL - \alpha \times PE$$

where RL = rate of influx of labeled material into the precursor pool from the medium, α = fractional turnover rate of the material in the precursor pool = $\ln 2/t\frac{1}{2}$; PE = concentration of radioactivity in the precursor pool at the time t.

Because at the start of labeling there is no radioactivity in PE (PE(0) = 0), while at equilibrium, dPE/dt = 0, (PE(∞) = RL/α), it can be shown that for any time, t, the amount of radioactivity in the precursor pool is given by:

$$PE(t) = (RL/\alpha) \times (1 - \exp(-\alpha \times t)) \quad (1)$$

The product pool (PD), in this case total protein, will label at a rate equal to the fractional turnover rate of the precursor pool multiplied by the amount of radioactivity in the precursor pool at time t, or:

$$dPD/dt = \alpha \times PE(t)$$

The amount of label accumulated in the product over time is given by integration as:

$$PD(t) = RL \times [t + (1/\alpha) \times (\exp(-\alpha \times t) - 1)] \quad (2)$$

After equilibration of the precursor pool, PE, the equation simplifies into a straight line:

$$PD(\infty) = RL \times t - (RL/\alpha) \quad (3)$$

The data for total protein were fitted by computer to equation 2 and gave a half equilibration time ($t\frac{1}{2}$) of 3 min. This indicated that the precursor pools leading to the synthesis of polypeptide chains equilibrated rapidly and, therefore, the rate of appearance of label in total protein was essentially linear with a slope of 88×10^3 cpm/min. Because the precursor pools leading to completed polypeptide chains equilibrated rapidly, equation 2 also could be applied to the appearance of label into proteoglycan (open circles), as the error introduced by the $t\frac{1}{2}$ for total protein should be slight when compared to the $t\frac{1}{2}$ for proteoglycan synthesis. Computer fitting of the data to equation 2 yielded a $t\frac{1}{2}$ of 97 min with a steady state rate of labeling RL = 10×10^3 cpm/min. A graphical representation of the labeling curve for proteo-

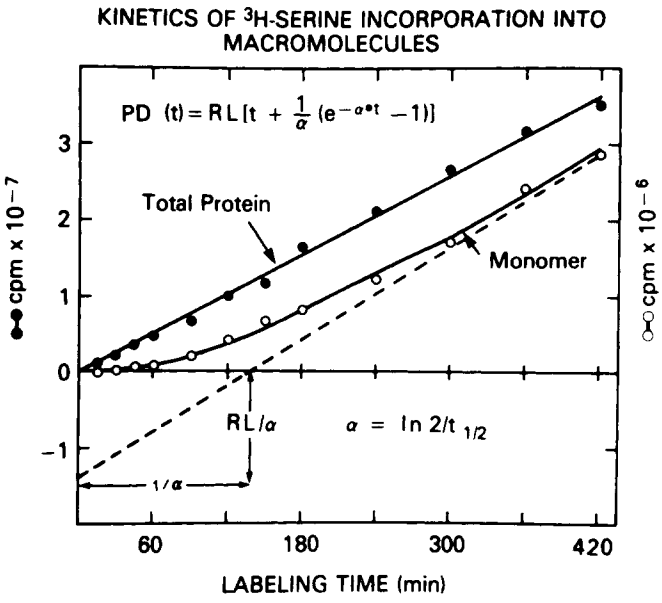

Fig. 3. Incorporation of (^{3}H)serine into macromolecules as a function of time. After labeling, the culture medium was removed and made 4 M in guanidine HCl with protease inhibitors as previously described (28,31). The cell layer was dissociatively extracted with 4 M guanidine HCl also containing protease inhibitors. Both medium and extract contained carrier proteoglycan aggregate. After dialysis of both media and extracts to lower the guanidine HCl concentration, the reconstituted proteoglycan aggregates were isolated by associative CsCl density gradients. Aliquots of the dialyzed media and extracts were taken to determine the amount of incorporation into total protein (filled circles) prior to centrifugation. Aggregates were further fractionated on dissociative isopycnic density gradients and the proteoglycan monomer was recovered and quantitated from bottom (DI) fractions (open circles). The data were fitted to kinetic equation 2 (see text) to determine the kinetic parameters of the labeling by an iterative method of least squares on a PDP-10 computer. After equilibration of the precursor pool, the rate of labeling becomes linear for a system in steady state (dashed line).

glycan after equilibration is given by the dashed line in Figure 3. The linear labeling curve is displaced from the origin by $1/\alpha$ on the ordinate (representing the average lifetime of a molecule in this compartment = t½/ln2) and by - RL/α on the abscissa (representing the size of the precursor pool).

The slopes of the linear labeling curves (RL) were used to compare the relative rates of synthesis of proteoglycan core protein compared to that of total protein. The ratio indicated that proteoglycan synthesis constituted about 11% of the total protein synthesis by the chondrocytes. These findings for (^{3}H)serine labeling confirmed earlier observations, indicating that the amino acid precursor pools equilibrated rapidly and that a significant delay in the appearance of label into completed proteoglycan can be detected by continuous labeling protocols. This delay reflects the time between labeling of core protein precursor and its conversion into completed proteoglycan.

A direct examination of the core protein precursor pool was undertaken in a separate experiment utilizing (^{3}H)leucine as the label. At the end of incubation periods

from 15–420 min, the cell layer was dissociatively extracted with 4 M guanidine HCl and carrier proteoglycan aggregate added (Fig. 4). The culture medium was also made 4 M guanidine HCl with added carrier. Aggregates were reconstituted by dialysis to lower the guanidine HCl concentration to associative conditions [31]. Labeled core protein precursor with functional hyaluronic acid binding region, completed proteoglycan, and functional link protein were recovered in mixed aggregates formed with the carrier proteoglycan from the bottom of CsCl density gradients [32]. Completed proteoglycan then was separated from core protein precursor and link protein by dissociative CsCl density gradient centrifugation. The amount of radioactivity in completed proteoglycan as recovered from the bottom fraction of each gradient for various times of labeling is shown in Figure 4 (open circles) and represents the sum of the labeled proteoglycan monomer in the medium and extract fractions. The kinetic pattern shown here was similar to that detected for the (^{3}H)serine experiments described in Figure 3. Labeling of total protein reached linearity early (data not shown), whereas the labeling of the proteoglycan monomer showed a delay indicating the presence of a core protein precursor pool. The insert in Figure 4 is a fluorogram of a 4% SDS-polyacrylamide gel showing the presence of labeled core protein precursor (upper bands) and link protein (lower bands) which were obtained from the top fractions of the dissociative gradients of reconstituted aggregates from the cell layer extracts used to isolate the labeled proteoglycan. The amount of label in the core protein precursor bands was estimated by counting slices of disk gels run separately for each time of labeling. The results are shown in Figure 4 (solid circles). Fitting the data to a model for a single precursor pool, equation 1, yields the dashed curve; the data points are better approximated by the solid curve. There appears to be a delay in the appearance of label in the core protein precursor pool not taken into consideration by the mathematical analysis. As with the (^{3}H)serine, at least part of this delay represents equilibration of the (^{3}H)leucine pool with its intracellular pool of tRNA. In addition, part of the delay may be due to the time required to synthesize the polypeptide chains of the core protein precursor ($M_r \sim 350{,}000$). These results indicate that the actual labeling kinetics for the core protein precursor pool are complex and cannot be described in terms of the simple model given in equation 1. The data are, however, consistent with the general pattern of labeling expected for a core protein precursor pool.

Further consideration of the fluorogram shown in Figure 3 indicates that additional details can be added to the description of the intracellular pool of precursor macromolecules. The labeled macromolecules shown were all functional in that they were able to bind to hyaluronic acid. They were of low buoyant density as indicated by their recovery from the top fractions of the dissociative density gradients, even though initially they were recovered in the high buoyant density fractions of the associative gradients with the carrier proteoglycan aggregates indicating that they were in ternary complexes with other components of the aggregate. Therefore, the core protein precursor is able to aggregate before glycosaminoglycan chains have been added. Furthermore, the entry of label into functional link protein appears to be nearly as fast as its entry into total protein exhibiting a $t_{1/2} \cong 10$ min (data not shown). Taken together, these data indicate that both the core protein precursor and link protein are able to form aggregates while still within the cell. That they do not assemble into aggregates until after they are secreted [5,33,34] suggests that additional mechanisms are required for controlling the assembly process.

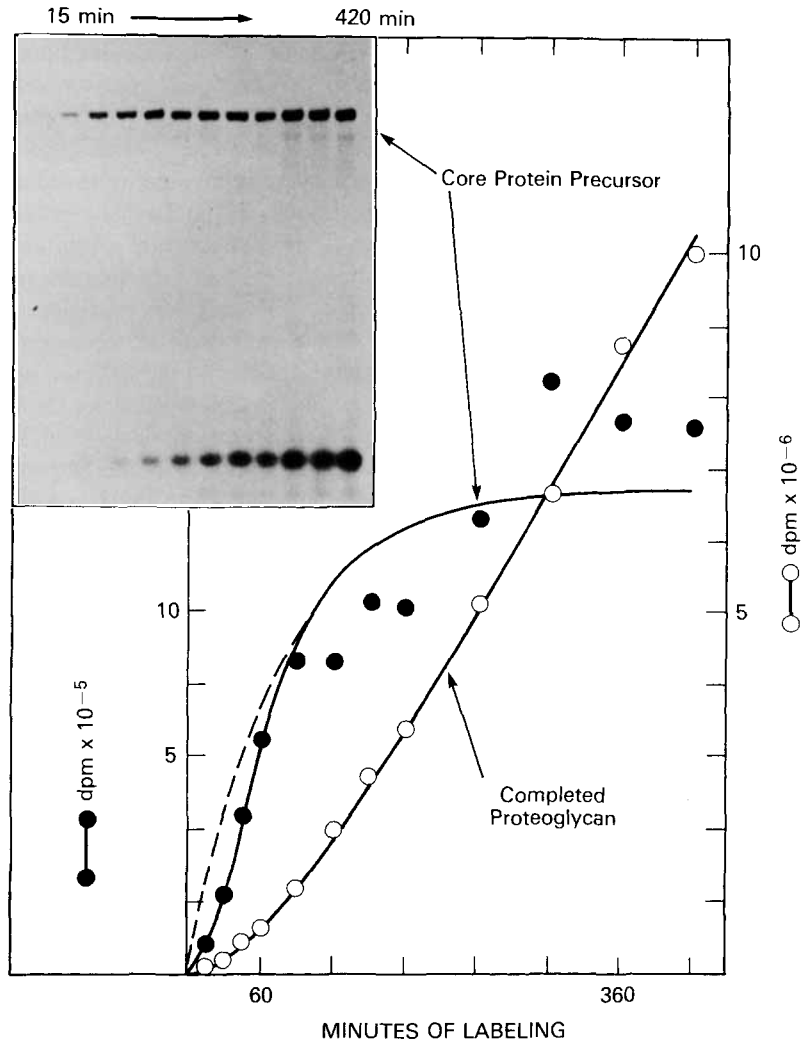

Fig. 4. Incorporation of (^{3}H)leucine into core protein precursor and proteoglycan with time. Cultures were labeled for 15–420 min and proteoglycan core protein precursor and completed proteoglycan were isolated from the culture media and cell extract after reconstitution with carrier proteoglycan aggregate, as described in the legend to Figure 3. The core protein precursor was recovered from the top fraction of the dissociative gradient (AlD4), whereas the proteoglycan was quantitated from the bottom (AlDl) fraction. The radioactivity in core protein precursor was determined after sodium dodecyl sulfate (SDS) gel electrophoresis, slicing and counting the gel and is shown as filled circles. Radioactivity in completed proteoglycan is shown as open circles. The insert is a fluorogram of an SDS polyacrylamide gel of the AlD4 fractions from the cell extracts. The upper bands are the core protein precursor; the lower bands are the link protein.

The fluorogram also shows only one major band for the core protein precursor. No detectable bands of intermediate size were seen between the core protein precursor and the top of the gel where core protein substituted with glycosaminoglycan chains

would be. These results suggest that the core protein precursor represents the major if not the immediate precursor to the completed proteoglycan. In a melanoma cell line [35], a two-stage biosynthetic pattern for proteoglycan synthesis was observed in which an endoglycosidase H sensitive precursor 230,000 in molecular weight was converted into a second precursor 240,000 in molecular weight, now insensitive to endoglycosidase H, before being completed into the proteoglycan with the addition of glycosaminoglycan chains. Our results agree with previous studies indicating that the core protein precursor pool consists predominantly of one major band of apparent molecular weight of 370,000 [32,36], nearly identical to the size of the core protein precursor reported for embryonic chick chondrocytes detected from either cell extracts [37,38] or from cell-free translation of chondrocyte RNA [39–41]. No detectable labeled material was found in the cell extracts, which could constitute an appreciable pool of core protein precursor partially substituted with glycosaminoglycan chains. This is consistent with previous studies [23] which indicated that the distribution of hydrodynamic sizes of newly completed, intracellular proteoglycans labeled by a 2 min pulse of (^{35}S)sulfate was the same as that found after chase times of 15 min, by which time most of the labeled molecules had been secreted from the cells.

The half-life of molecules in the intracellular core protein precursor pool was examined also by pulse-chase labeling protocols with (^{3}H)serine followed by detection of the labeled core protein precursor either by immunoprecipitation of the precursor from detergent-guanidine HCl extracts [32], or by electrophoretic mobility on polyacrylamide gels of subcellular fractions derived from these cells [36]. Both of these studies found a t½ for the core protein precursor pool of 80–100 min. The subcellular fractionation study, in addition, indicated that the core protein precursor was found largely in the rough endoplasmic reticulum, where it remained for about 70–90% of the intracellular half time. Completed proteoglycan was found mainly associated with the Golgi apparatus enriched fraction, consistent with previous reports implicating the Golgi apparatus as the site of glycosaminoglycan synthesis [14–20]. Taken together, the studies summarized above indicate that the biosynthetic half time for completing the proteoglycan is long and can be described as occurring in two major steps: a) synthesis of the core protein precursor and b) subsequent completion of the proteoglycan. These steps, which are widely separated in time, were exploited to determine the timing and to infer the location of particular biosynthetic events.

Besides labeled amino acids to follow synthesis of the core protein precursor and radiolabeled sulfate to follow completion of the chondroitin sulfate chains, a number of other labeled precursors can be used. Some of these are illustrated in Figure 5. The intermediary metabolic pathways leading to the nucleotide sugar precursors for the synthesis of glycosaminoglycan chains has been reviewed [20,42,43]. Events that occur early during the biosynthesis of the proteoglycan, for example, in the rough endoplasmic reticulum, would be expected to introduce a long delay before the label appears in the completed product. Conversely, events which occur late in the biosynthetic process such as sulfation will introduce only a short delay in labeling the completed product.

An example of the former is illustrated in the examination of labeling of N-linked oligosaccharides. The completed proteoglycan contains several complex N-linked mannose-containing oligosaccharides (Fig. 1), which can be detected conveniently by labeling cultures with (2-^{3}H)mannose [44–46]. The position of the initial

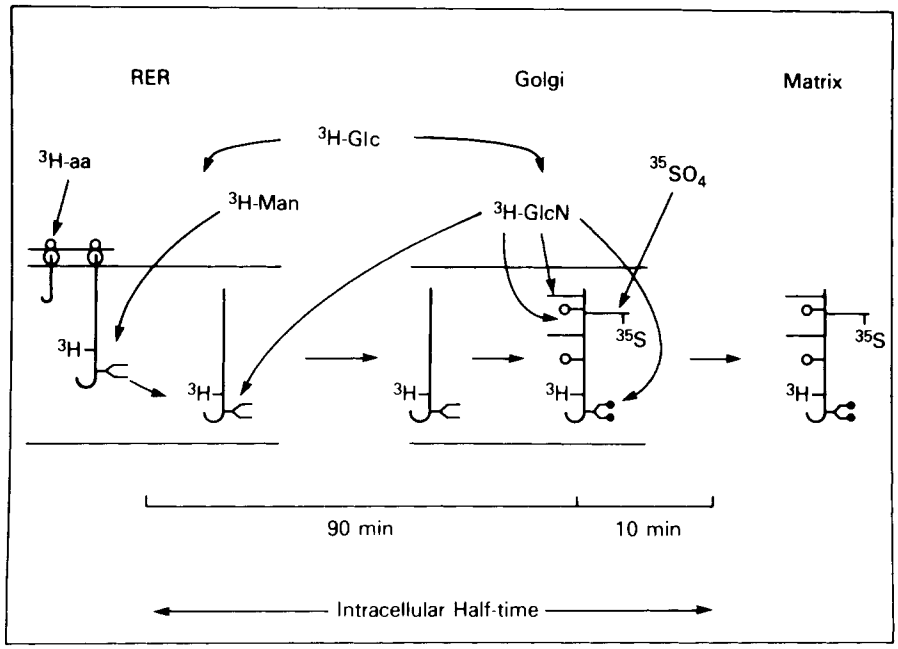

Fig. 5. Schematic representation of the biosynthetic pathway for cartilage proteoglycan. The biosynthetic pathway for the proteoglycan is shown initiated by translation of the mRNA for the core protein in association with the rough endoplasmic reticulum (RER). Following insertion into the RER, the core protein precursor to the proteoglycan is not modified extensively until transfer to the Golgi apparatus, which occurs with a t½ of about 90 min. Addition of the chondroitin sulfate chains in the Golgi apparatus and secretion occur rapidly, with a t½ of about 10 min. A variety of radioactive precursors and their sites of addition to the proteoglycan are indicated by arrows. Mannose (^{3}H-Man) is added to N-linked oligosaccharides in the RER. Glucose (^{3}H-Glc) and glucosamine (^{3}H-GlcN) are added at several points in the biosynthetic pathway (arrows).

label in mannose ensures labeling of primarily mannose containing oligosaccharides, as its conversion to other saccharides such as glucose or galactose involves an epimerization about the C2 carbon with a concomitant loss of the ^{3}H label. The biosynthetic pathway for the N-linked oligosaccharides is known for glycoproteins where it proceeds through a dolicohol diphosphate associated oligosaccharide which is transferred as a unit [47–49] to an acceptor protein concomitant with translation in association with the rough endoplasmic reticulum [50–52]. The entry of this label into completed marcomolecules was examined in a series of cultures incubated with the radioisotope for various times. The appearance of radioactivity in total glycoprotein and in completed proteoglycan is shown in Figure 6. In this case the equilibration kinetics for the dolichol diphosphate oligosaccharide precursor pool appears to be slow, t½ $\cong$ 60 min, introducing a significant delay in labeling of the total mannose oligosaccharides in glycoproteins (Fig. 6, filled circles). Nevertheless, even with this delay, the large t½ of the core protein precursor pool introduces a significantly longer delay for the appearance of label in completed proteoglycan, t½ $\cong$ 3–4 hr (Fig. 6, open circles).

An examination of the kinetics of addition of O-linked oligosaccharide chains to the core protein precursor can be made using radioactive precursors, such as (^{3}H)glucosamine or (^{3}H)glucose, which label more than one saccharide in the final structure. For example, (^{3}H)glucosamine will label glucosamine, galactosamine, and sialic acid in the structure shown in Figure 1. Therefore, the labeled oligosaccharides must be purified and then the amount of label in each sugar measured to determine the different equilibration kinetics. Previous work with (^{3}H)glucosamine as a precursor monitored the appearance of label into completed proteoglycan. Chondroitin sulfate and O-oligosaccharide chains were prepared from labeled proteoglycan after different labeling times and the amount of label in each determined. The results demonstrated that the t½ for the entry of label into both chondroitin sulfate and O-linked oligosaccharides occurred within 5 min of each other, exhibiting t½'s of about 10 min [30]. Because the glycosaminoglycan chains are known to be added in the Golgi apparatus, these results indicated that the O-linked oligosaccharides also are added in the Golgi apparatus. This was consistent with similar findings obtained with glycoproteins containing O-linked oligosaccharides [53–56]. Furthermore, entry of label into the two hexosamines in the O-linked hexasaccharide also was examined (Figure 1). The N-acetyl galactosamine serving as the linkage sugar was isolated as galactosaminitol, whereas the N-acetyl glucosamine, located in an interior position, was isolated as glucosamine following alkaline-borohydride release and acid hydrolysis. The ratio of radioactivity in the two sugars was constant at all labeling times indicating that both the initiation and completion of O-oligosaccharide chains occurred concurrently. These results also suggest that skeletal keratan sulfate, which appears to be linked to the core protein via structures closely related to the O-linked oligosaccharides [1–4], is also synthesized in the Golgi apparatus at about the same time as synthesis of chondroitin sulfate.

Additional information can be obtained when (^{3}H)glucose is used, because this precursor will label all of the sugar components of the proteoglycan via conversion by intermediary metabolic pathways [42,43,57–61]. Of particular interest are the galactose and xylose residues in chondroitin sulfate and the galactose in the oligosaccharides, Figure 1. Cultures were labeled for various times and then chondroitin sulfate and O-oligosaccharide chains were prepared from purified proteoglycan monomer as indicated in Figure 7. The kinetics of labeling of chondroitin sulfate and O-linked oligosaccharide are shown in Figure 8. Labeling of both reaches linearity rapidly, exhibiting t½ values of approximately 10 min and 15 min, respectively. This agrees well with previous results using (^{3}H)glucosamine as a precursor and confirms the suggestion that both substituents are added to the core protein precursor at about the same time [30]. The ratio of label in chondroitin sulfate to that in oligosaccharide exhibited an exponential decrease with a half time of about 1–1.5 hr (dashed line, Fig. 8). A similar observation was made earlier with (^{3}H)glucosamine as the labeling precursor [30] and could be explained by the slow equilibration of the labeled precursor with the intermediate pools involved in metabolism of sialic acid which terminates the oligosaccharide chains (Fig. 1). Therefore, the sialic acid residues label more slowly than the other sugar residues and give a proportional increase to radioactivity in the oligosaccharides relative to chondroitin sulfate at later times. Thus, the results using (^{3}H)glucose are in excellent agreement with previous results using (^{3}H)glucosamine as the precursor.

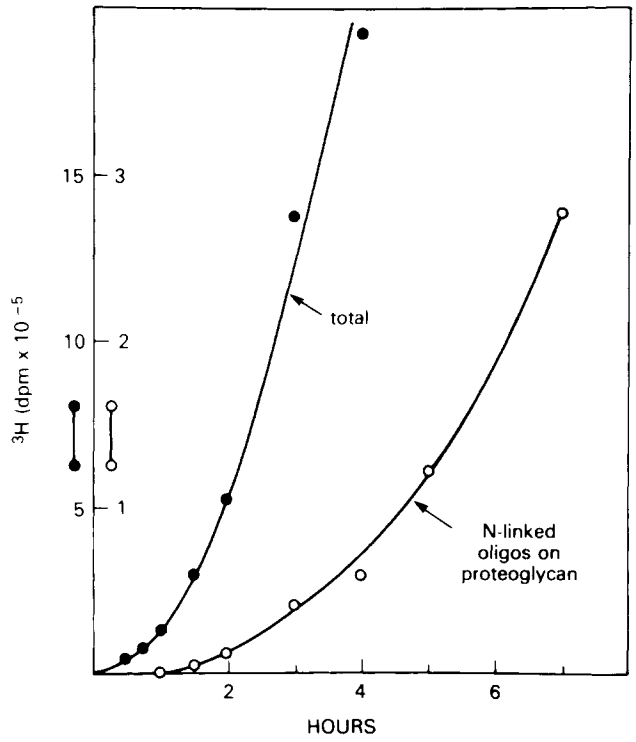

Fig. 6. Incorporation of (2-^{3}H)mannose into macromolecules. Cultures were labeled for various times up to 7 hr. Total incorporation of (^{3}H)mannose into macromolecules was measured by exclusion of aliquots of the culture medium and extract of the cell layer on small Sephadex G-50 columns. Proteoglycans were isolated by direct dissociative CsCl density gradient centrifugation of culture medium and extracts. Radioactivity in proteoglycan is given by open circles, that in total, by filled circles.

The labeled chondroitin sulfate chains were examined further to determine the kinetics of labeling of the linkage region between the glycosaminoglycan chains and the core protein shown in Figure 1. An illustration of the procedure used is shown in Figure 9 for a culture labeled 30 hr with (^{3}H)glucose before extraction and purification of the proteoglycans. The chondroitin sulfate chains, recovered from the ethanol precipitate as indicated in Figure 7, were treated with chondroitinase AC to remove the repeating disaccharide units from the glycosaminoglycan chains and to liberate the linkage region. The digests for each labeling time were chromatographed on BioGel P-4 and gave elution profiles similar to the example shown in Figure 9. Peaks A and B, each containing approximately equal amounts of radioactivity, correspond to two forms of the linkage region. The former was shown by Oegema et al [63] to contain xylitol-2-phosphate. This was confirmed when peak A and B were treated separately with alkaline phosphatase and then rechromatographed on BioGel P-4. The elution profile for each is shown in the lower panels of Figure 9. After treatment, peak A material chromatographed in the same place as Peak B material, which was not altered by the enzymatic treatment.

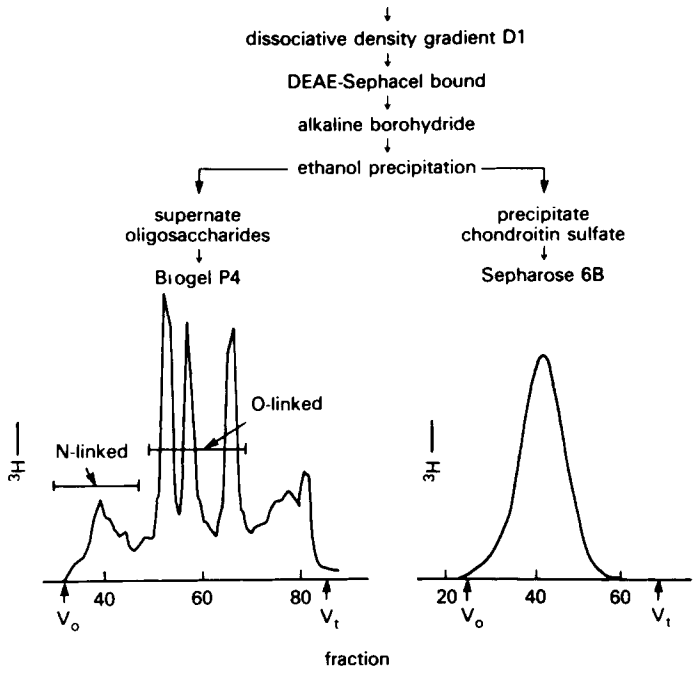

Fig. 7. Protocol for the determination of incorporation of (^{3}H)glucose into saccharide substituents of the proteoglycan. Cultures were labeled for various times from 30 min to 8 hr with (1-^{3}H)glucose at 150 uCi/ml in complete culture medium containing 20% fetal bovine serum. At the end of each labeling period, the cell layer was extracted with 4 M guanidine HCl-2% CHAPS containing protease inhibitors [62]. Carrier proteoglycan was present in both the medium and extract fractions. Labeled proteoglycan monomer was isolated from the combined medium and extract fractions by dissociative CsCl density gradient centrifugation in 4 M guanidine-2% CHAPS and further purified by DEAE-Sephacel chromatography as indicated. The chondroitin sulfate and O-linked oligosaccharide chains were liberated from the proteoglycan by sodium hydroxide treatment in the presence of sodium borohydride. The chondroitin sulfate was separated from the oligosaccharides by ethanol precipitation. Oligosaccharides were recovered from the supernatant after removing the ethanol by evaporation under reduced pressure. They were further fractionated by chromatography on BioGel P-4 to separate N-linked from O-linked oligosaccharides (left panel). The chondroitin sulfate was chromatographed on Sepharose 6B (right panel) to determine chain size and to assess purity.

The material recovered from both peaks subsequent to phosphatase treatment and rechromatography on BioGel P-4 was analyzed further after trifluoracetic acid hydrolysis and HPLC chromatography for radioactivity in neutral sugars. The ratio of labeled galactose to labeled xylitol was nearly identical for both peaks A and B, 1.7 to 1.9, respectively, consistent with two galactoses to one xylose in the linkage structure. If the alkaline phosphatase treatment was omitted, the ratio of galactose to xylitol in Peak A was found to be 5.7 indicating that the recovery of xylitol is low when the xylose is substituted with phosphate, a result also observed by Oegema, et al [63]. A direct examination of the relative amount of phosphorylated xylose residues was done by examining the proportion of label in xylitol in Peak A versus Peak B

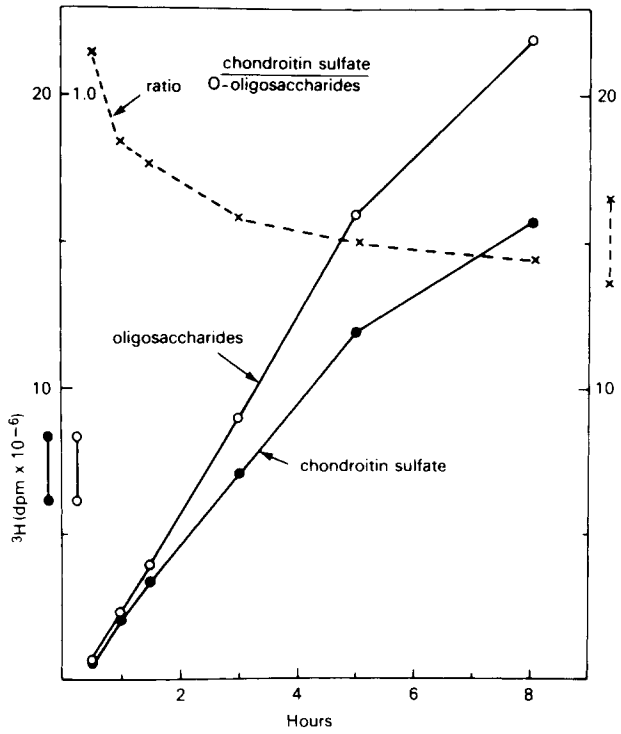

Fig. 8. Rate of entry of (1-^{3}H)glucose into chondroitin sulfate and O-oligosaccharides. Cultures were labeled and fractionated as described in Figure 7. The total radioactivity in chondroitin sulfate is shown as filled circles, that in O-oligosaccharide as open circles. The ratio of label in chondroitin sulfate to O-oligosaccharide is shown as the dashed line.

after treatment with alkaline phosphatase, hydrolysis and HPLC analysis. The proportion was nearly constant from 30 min to 8 hr ($\bar{x}$ = 4.3), indicating that about 80% of the newly synthesized chondroitin sulfate chains were phosphorylated, suggesting that phosphorylation of xylose is a biosynthetically related event.*

The radioactivity in the unsaturated disaccharide residues also was examined. The proportion of radioactivity in chondroitin sulfate disaccharides to that found in the linkage region (Peaks A and B) was constant for labeling times ranging from 30 min to 8 hr, 18.4 to 19.4, respectively. This suggests that the sugars for the linkage region were added to the core protein at the same time as the chondroitin sulfate chains. This was examined directly by measuring the entry of label into xylose and galactose isolated from the linkage region of chondroitin sulfate chains treated with alkaline phosphatase. The data are shown in Figure 10. The t½ to linearity for galactose (~ 10 min) was within 5 min of that of xylose, recovered as xylitol (~ 15 min). These results are very similar to the labeling kinetics reported for the entry of

*The relative content of phosphorylated xylose residues was lower for the 30 hr labeling, 60% compared to 80% immediately after synthesis. The decrease in the proportion of modified chains with increasing times after synthesis probably indicates phosphatase activity in the extracellular compartment.

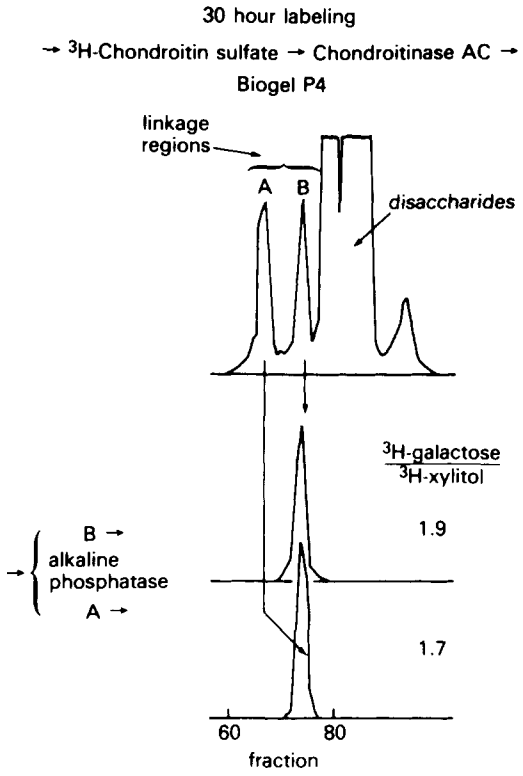

Fig. 9. Characterization of label in the linkage region of chondroitin sulfate. A culture was labeled continuously for 30 hr with (1-^{3}H)glucose, followed by isolation of labeled chondroitin sulfate chains from proteoglycan monomers prepared as described in Figure 7. The chains were further processed by digestion with chondroitinase AC to depolymerize the glycosaminoglycans chains leaving the linkage region (see Fig. 1). The digestion mixture was chromatographed on BioGel P4 to separate the linkage region oligosaccharides from the digestion products. Two peaks (A and B) were observed. Peaks A and B were digested with alkaline phosphatase and then rechromatographed on the same column (lower two panels). Each peak was recovered and hydrolyzed with trifluoroacetic acid and then analyzed for radioactivity in (^{3}H)galactose and (^{3}H)xylitol by HPLC. The ratio of labeled galactose to xylitol in each peak is shown on the right.

glucosamine into chondroitin sulfate and O-linked oligosaccharide [30]. It therefore appears likely that the entire chondroitin sulfate chain is initiated, polymerized, and completed over a short period of time in the Golgi apparatus.

However, since the data indicate that the xylose equilibration precedes that of galactose by about 5 min, it is possible that xylosylation of the core protein precursor occurs at a very late stage in transit through the endoplasmic reticulum before the core protein precursor reaches the Golgi. However, it is also possible, and perhaps likely, that the slight delay observed between xylose and galactose labeling could be accounted for entirely by differences in the equilibration of the labeled glucose with two different nucleotide sugar pools. In this case, the addition of xylose and galactose to the core protein precursor would be nearly concurrent. In any event, the data

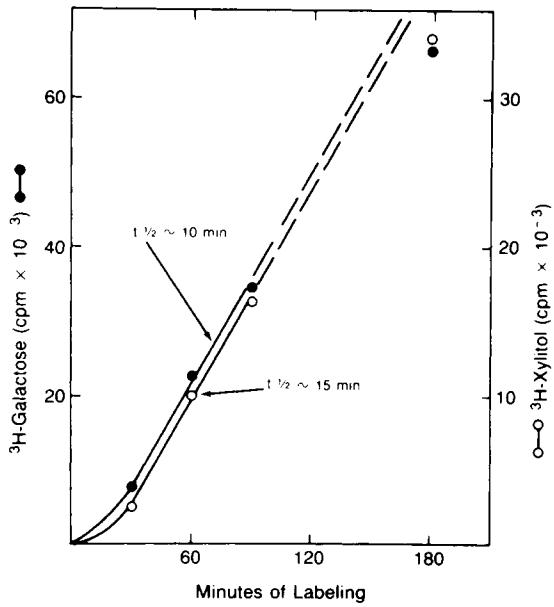

Fig. 10. Entry of labeled glucose into xylitol and galactose of the linkage region of chondroitin sulfate. Radioactivity in the linkage region of chondroitin sulfate chains for labeling periods from 30 min to 8 hr, as described in Figure 7, were prepared as described in Figure 9, and analyzed for label in galactose and xylitol. Only the time points up to 180 min are shown.

clearly indicate that the addition of xylose is a late event of the biosynthetic process and could only precede the addition of galactose by about 5 min, far less than the 90 min half life of the core protein precursor. Thus, the possibility of a biosynthetic mechanism where the core protein precursor is substituted with xylose and perhaps other elements of the linkage region while the core precursor is still a nascent polypeptide chain attached to polysomes, suggested previously [61], can be excluded by these results.

In summary, a model system of cultured chondrocytes from the Swarm rat chondrosarcoma was used to examine the timing and subcellular location of post-translational modifications to the core protein leading to the elaboration of a proteoglycan molecule. The half time for the transit of newly synthesized core protein precursor to move from the rough endoplasmic reticulum through the Golgi apparatus and into the extracellular matrix was determined to be about 90–120 min. Transit through the rough endoplasmic reticulum to the Golgi occupied 70–90% of the biosynthetic time with the elaboration of glycosaminoglycan chains and secretion of the completed molecule accounting for only 10–20%. The delay between the time of translation of the core protein precursor and its completion into proteoglycan was found to be long relative to the time required to equilibrate most metabolic precursor pools. Thus, labeled precursors such as amino acids or monosaccharides could be used as tracers to determine kinetic parameters of post-translational modifications to the core protein precursor without the necessity to correct for equilibration times of metabolic pools. The kinetics of incorporation of labeled monosaccharides into the completed proteoglycan indicated that most of the modifications to the core protein

precursor occurred over a period of a few minutes late in the biosynthetic pathway. The results confirmed earlier reports that chondroitin sulfate chains were synthesized in the Golgi apparatus and that secretion occurred soon after with a t½ of about 5 min. In addition, it was found that the initiation and completion of O-linked oligosaccharides also occurred at nearly the same time as did chondroitin sulfate synthesis. These results fix the time of synthesis of keratan sulfate chains as occurring at nearly the same time as the chondroitin sulfate chains, as the asialyl O-linked oligosaccharides serve as the linkage regions between keratan sulfate and the core protein [1–4,64]. Finally, preliminary results presented above suggest that the addition to the core protein precursor of the linkage region of chondroitin sulfate occurs at about the same time as the elongation of chondroitin sulfate chains, with the possible exception of xylose, which may be added at most 5 min before the galactose residues. This indicates that the chondroitin sulfate chains are both initiated, elongated, and completed within a short period of time late in the biosynthetic process.

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REFERENCES

1. Hascall VC, Hascall GK: In Hay ED (ed): "Cell Biology of Extracellular Matrix," New York: Plenum Press, 1981, pp. 39–63.
2. Hascall VC: In Ginsburg V (ed): "Biology of Carbohydrates," Vol 1. New York: John Wiley and Sons, 1981, pp. 1–49.
3. Roden L: In Lennarz WJ (ed): "The Biochemistry of Glycoproteins and Proteoglycans," New York: Plenum Press, 1980, pp. 267–371.
4. Dorfman A: In Hay ED (ed): "Cell Biology of Extracellular Matrix," New York: Plenum Press, 1981, pp. 115–138.
5. Kimura JH, Hardingham TE, Hascall VC, Solursh M: J Biol Chem 254:2600, 1979.
6. Hascall VC, Kimura JH: Ala J Med Sci 18:29, 1981.
7. Hascall GK, Kimura JH: Anat Rec 200:287, 1981.
8. Oegema TR, Hascall VC, Dziewiatkowski DD: J Biol Chem 250:6151, 1975.
9. Hascall GK: Anat Rec 198:135, 1980.
10. Choi HU, Meyer K, Swarm R: Proc Natl Acad Sci USA 68:877, 1971.
11. Oegema TR, Brown M, Dziewiatkowski DD: J Biol Chem 252:6470, 1977.
12. Handley CJ, Bateman JF, Oakes BW, Lowther DA: Biochim Biophys Acta 386:441, 1975.
13. Kimura JH, Hardingham TE, Hascall VC: J Biol Chem 255:7134, 1979.
14. Godman GC, Lane N: J Cell Biol 21:353, 1964.
15. Horwitz AI, Dorfman A: J Cell Biol 38:358, 1968.
16. Kimata K, Okayama M, Suzuki S, Suzuki I, Hoshino M: Biochim Biophys Acta 273:606, 1971.
17. Freilich LS, Lewis RG, Reppucci AC, Silbert JE: J Cell Biol 72:655, 1977.
18. Martinez A, Arguelles F, Cervera J, Gomar F: Virchows Arch Cell Pathol 23:53, 1977.
19. Silbert JE, Freilich LS: Biochem J 190:307, 1980.
20. Silbert JE, J Invest Dermatol 79:31s, 1982.
21. Telser A, Robinson HC, Dorfman A: Proc Natl Acad Sci USA 54:912, 1965.
22. Cole NN, Lowther DA: FEBS Lett 2:351, 1969.
23. Kimura JH, Caputo CB, Hascall VC: J Biol Chem 256:4368, 1981.
24. Mitchell D, Hardingham T: Biochem J 196:521, 1981.

25. Richmond ME, DeLuca S, Silbert JE: Biochem 12:3898, 1973.
26. Richmond ME, DeLuca S, Silbert JE: Biochem 12:3904, 1973.
27. DeLuca S, Richmond ME, Silbert JE: Biochem 12:3911, 1973.
28. Kimura JH, Thonar EJM, Kuettner KE: In Chester MA, Heinegard D, Lundblad A, Svenson S (eds.): "Glycoconjugates," Lund: Rahams, 1983, pp. 402–403.
29. Rubinow SI, Yen A: Nature New Biol 239:73, 1972.
30. Thonar EJMA, Lohmander LS, Kimura JH, Fellini SA, Yanagishita M, Hascall VC: J Biol Chem 258:11564, 1983.
31. Hascall VC, Kimura JH: In Cunningham LW and Frederickson DW (eds.): "Methods in Enzymology Vol 82A." New York: Academic Press, 1982, pp. 769–800.
32. Kimura JH, Thonar EJM, Hascall VC, Reiner A, Poole AR: J Biol Chem 256:7890, 1981.
33. Bjornsson S, Heinegard D: Biochem J 199:17, 1981.
34. Sommarin Y, Heinegard D: Biochem J 214:777, 1983.
35. Bumol TF, Reisfeld RA: Proc Natl Acad Sci USA, 79:1245, 1982.
36. Fellini SA, Hascall VC, Kimura JH: J Biol Chem 259:4634, 1984.
37. Pacifici M, Soltesz R, Thal G, Shanley DJ, Boettiger D, Holtzer H: J Cell Biol 97:1724, 1983.
38. Nishimoto SK, Kajiwara T, Ledger PW, Tanzer ML: J Biol Chem 257:11712, 1982.
39. Vertel BM: Biochem J 217:259, 1984.
40. Upholt W, Vertel BM, Dorfman A: Proc Natl Acad Sci USA 76:4847, 1979.
41. Upholt WB, Kravis D, Sandell L, Sheffield VC, Dorfman A: In Kelly RO, Goetinek PF, MacCabe JA (eds): "Limb Development and Regeneration, Part B." New York: Alan R. Liss, 1983, pp. 175–182.
42. Schachter H, Roden L: In Fishman WH (ed): "Metabolic Conjugation and Metabolic Hydrolysis." New York: Academic Press, 1973, pp. 2–149.
43. Handley CJ, Phelps CF: Biochem J 126:417, 1972.
44. Lohmander LS, DeLuca S, Nilsson B, Hascall VC, Caputo CB, Kimura JH, Heinegard D: J Biol Chem 255:6084, 1980.
45. Nilsson B, DeLuca S, Lohmander LS, Hascall VC: J Biol Chem 257:10920, 1982.
46. Lohmander LS, Fellini SA, Kimura JH, Stevens RL, Hascall VC: J Biol Chem 258:12280, 1983.
47. Behrens NH, Carmmatti H, Staneloni RJ, Leloir LF, Cantarella AI: Proc Natl Acad Sci USA 70:3390, 1973.
48. Hsu AF, Baynes JW, Heath EC: Proc Natl Acad Sci USA 71:2391, 1974.
49. Waechter CJ, Lennarz WJ: In Snell EE, Boyer PD, Meister A, Richardson CC (eds): 'Annual Review of Biochemistry," Palo Alto: Annual Reviews Inc, 1976, pp. 95–111.
50. Kiely ML, McKnight GS, Schimke RT: J Biol Chem 251:5496, 1976.
51. Bergmen LW, Kuehl WM: Biochem 16:4490, 1977.
52. Struck DK, Lennarz WJ: In Lennarz W (ed): "Biochemistry of Glycoproteins and Proteoglycans," New York: Plenum Press, 1980, pp. 35–83.
53. Kim YS, Perdomo J, Nordberg J: J Biol Chem 248:5466, 1971.
54. Ko GKW, Raghupathy E: Biochim Biophys Acta 264:129, 1972.
55. Hanover JA, Lennarz WJ, Young JD: J Biol Chem 255:6713, 1980.
56. Johnson DC, Spear PG: Cell 32:987, 1983.
57. D'Abramo F, Lipmann F: Biochim Biophys Acta 25:211, 1957.
58. Pearlman RL, Telser A, Dorfman A: J Biol Chem 239:3623, 1964.
59. Feingold DS, Neufeld EF, Hassid, WZ: J Biol Chem 235:910, 1960.
60. Neufeld EF, Hall CW: Biochem Biophys Res 19:456, 1965.
61. Stoolmiller AC, Dorfman A: In Florkin M, Stotz EH (eds): "Comprehensive Biochemistry," New York: Elsevier, pp 241–275, 1969.
62. Chang Y, Yanagishita M, Hascall VC, Wight TN: J Biol Chem 248:5679, 1983.
63. Oegema TR, Kraft EL, Jourdian GW, VanValen TR: J Biol Chem 259:1720, 1984.
64. Hascall VC: In Kelley RO, Goetinck PF, MacCabe JA (eds): "Limb Development and Regeneration, Part B," New York: Alan R. Liss, 1983, pp 3–15.