Biophysical Regulation of Matrix Synthesis, Assembly, and Degradation in Dynamically Compressed Calf Cartilage

by

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Abstract

Mechanical forces are thought to modulate the metabolic activity and promote the structural adaptation of a variety of connective tissues including cartilage. However, "abnormal" forces may predispose cartilage to degeneration, as in osteoarthritis, and modeling errors, as in ball and socket ankle joint. During dynamic loading of cartilage, a number of physical phenomena occur naturally and are inexorably coupled; compression of cartilage results in deformation of cells and extracellular matrix, hydrostatic pressurization, fluid flow, streaming potentials, and physicochemical changes associated with increased matrix charge density. The objectives of this thesis were (1) to characterize the effects of static and dynamic compression on the synthesis, assembly, and degradation of extracellular matrix constituents, and (2) to relate metabolic responses to possible biophysical regulatory mechanisms.

Chambers were designed to allow uniaxial radially unconfined compression and mechanical testing of cartilage disks while maintaining an organ culture environment. Dynamic stiffness measurements of 3-mm diameter disks identified a characteristic frequency (0.001 Hz) that separated low- and high-frequency regimes in which different flow and deformation phenomena predominated. At $f \geq 0.01$ Hz, oscillatory strains of only $\sim1\text{–}5\%$ caused hydrostatic pressurization within disks and stimulated biosynthesis ($^{3}$H-proline and $^{35}$S-sulfate incorporation) by $\sim20\text{–}40\%$. At $f \leq 0.001$ Hz, oscillatory compressions of $<5\%$ caused fluid exudation from cartilage disks with little pressurization, but did not affect synthesis. In contrast, static compression depressed synthesis in a dose-dependent manner.

Proteoglycans endow cartilage with a large swelling pressure due to their high fixed charge density. Most cartilage proteoglycans are aggregated on a central
hyaluronate backbone to form a macromolecular complex; the size of the aggregate effectively immobilizes proteoglycans within the collagenous meshwork. Newly synthesized proteoglycans undergo an extracellular conversion process whereby their binding affinity for hyaluronate increases. Static compression slowed the conversion process, as did incubation in acidic media (without compression), both in a dose-dependent manner. Oscillatory compression (f=0.001–0.1 Hz) did not affect the conversion. The similarly delayed kinetics of affinity conversion with compression and acidic media suggest that this delay may be due in both cases to a physicochemical mechanism of decreased intratissue pH. Such a regulatory mechanism may have a physiologic role in the development or remodeling of the cartilage matrix, even in unloaded tissue, favoring deposition of newly synthesized proteoglycans in the low charge (proteoglycan-poor) regions.

The turnover of proteoglycans is increased in experimental osteoarthritis, and may be influenced by physical or biological mechanisms. Slow cyclic compression of cartilage disks, radiolabeled with 35S-sulfate and 3H-proline, led to increases in the release of radiolabeled macromolecules as well as an enrichment of the culture media with collagenous (hydroxyproline-containing) 3H-residues and non-aggregating 35S-proteoglycans. The patterns of release were consistent with several physical phenomena (convection, diffusion, and disruption of the collagen meshwork) as well as biological effects (decreased deposition of proteoglycan).

These studies provide a framework for identifying both physical and biological mechanisms by which static and dynamic compression can modulate the accumulation, maintenance, or degradation of a mechanically functional extracellular matrix in cartilage. The culture and compression methodology potentially allows in vitro evaluation of clinical strategies of physical therapies (e.g., continuous passive motion) to stimulate cartilage remodeling.

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"Physical exercise is not merely necessary to [develop and maintain a healthy] body, but to balance and correct intellectual pursuits as well. The mere athlete is brutal and philistine, the mere intellectual unstable and spiritless. The right education must tune the strings of the body and mind to perfect spiritual harmony."

—Plato
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Chapter I

Introduction

1.1 Cartilage Structure, Function, and Metabolism

In synovial joints, the surfaces of articulating bones are covered by a physically specialized hyaline cartilage connective tissue. This articular cartilage provides a low-friction wear-resistant bearing surface which distributes and transmits stresses generated by body weight and muscle contraction to the underlying bone (Fig. 1.1). In the human femoropatellar and hip joints, cartilage is subjected to contact pressures as high as 3–18 MPa [1,69,73] which result in compression amplitudes of >13% [3]. Femoropatellar contact pressures reach 25 MPa during traumatic impact loads which are just lower than the threshold for bone fracture [65].

The load-bearing properties of cartilage are largely attributable to the proteoglycan and collagen components of the hydrated extracellular matrix. Proteoglycans endow cartilage with a high charge density (~0.1–0.2 mol/l fluid [52,100]) due to acidic carboxyl and sulfate moieties on the glycosaminoglycan chains; the resulting electrostatic repulsion of neighboring charge groups gives cartilage a high osmotic swelling pressure [52]. The balance between this swelling pressure and the tensile strength of collagen as well as the applied stress determines the state of tissue hydration [98]. In tension, the ultimate strength of the surface zone of normal bovine articular cartilage is ~20 MPa [76]. In osteoarthritis, cartilage becomes hyperhydrated [122], perhaps due to fibrillation and loosening of the collagen meshwork [98].

When cartilage is loaded, a variety of electrical, mechanical, and chemical phenomena occur naturally and are inexorably coupled (Fig. 1.2) [52]. Hydrostatic pressure gradients build up and drive fluid to flow out of and to redistribute within cartilage [42,90,97,119]; concomitantly, the tissue becomes consolidated and the
Figure 1.1: Role of articular cartilage in loaded knee joint. Articular cartilage serves as the wear-resistant low-friction material that distributes and transmits load to the underlying bone (adapted from [120]).
Figure 1.2: Mechanical, electrical, and chemical phenomena in cartilage during loading. Physical phenomena include chondrocyte deformation, hydrostatic pressure gradients, fluid flow, streaming currents and potentials, matrix consolidation, and physicochemical alterations (altered ion concentrations and osmotic pressure).

Section 1.1
chondrocytes are deformed [16,144]. Since the extracellular matrix has a fixed net negative charge, there is an excess of mobile positive counterions in solution. The fluid flow entrains these mobile ions and causes streaming currents and potentials [42,90]. In addition, as the extracellular matrix becomes compacted, physicochemical changes occur [48,52,100,173,187]: the fixed charge density increases, which effectively attracts and concentrates positive counterions, and repels and dilutes negative coions; as a result, the total ionic content increases, giving rise to an increased osmotic pressure. Also, the effective pore size decreases, resulting in hindered diffusion of large molecules [193].

In view of these phenomena, mechanical loading of cartilage may influence biological processes through a number of mechanical, electrical or chemical mechanisms. The balanced biosynthesis, assembly, and loss of matrix components (Fig. 1.3) is necessary for the development, remodeling, maintenance, and repair of a mechanically functional cartilage extracellular matrix.

In normal articular cartilage, proteoglycans are actively turned over [101]. One method of estimating proteoglycan turnover time involves measuring the time course of decay in radiolabeled glycosaminoglycans from cartilage during an *in vivo* pulse-chase. Such a study indicated that the glycosaminoglycans of guinea pig costal cartilage exist in two major metabolic pools, a rapidly turning over pool with a half life of 3 days, and a slowly turning over pool with a half life of 60–70 days [93]. Multiple pools have also been identified *in vitro* [23,171]. Each of these pools may correspond to one or several of the multiple proteoglycan populations (e.g., large aggregating proteoglycans, large non-aggregating proteoglycans, and small proteoglycans) that exist in cartilage [64,66].

Another method of estimating proteoglycan turnover time involves quantitating the sulfate incorporation rate *in vivo* (or *in vitro* immediately after explant), measuring the density of sulfate in cartilage, and assuming a first order single-pool rate process [101]. Such studies have yielded estimates of proteoglycan half life

Section 1.1
Figure 1.3: Synthesis, assembly, and loss of cartilage matrix. Chondrocytes synthesize matrix components—predominantly proteoglycan, link protein, hyaluronate, and collagen. These synthetic products assemble into a stable extracellular matrix, and are then released from the tissue at various rates; alternatively, synthetic products may never become incorporated into the matrix, but instead be immediately lost from the tissue. These metabolic processes may be influenced by mechanical, electrical, or chemical phenomena that are induced by physiological levels of loading.
ranging from three years in adult humans, to one year in adult dogs and rabbits, to between two weeks and three months in young rabbits [101].

Proteoglycan metabolism has been studied extensively in cartilage explants in the absence of physical stimuli. When cultured with 10–20% fetal bovine serum, free-swelling cartilage explants from calves (1–6 month old) and adult cows can be maintained in vitro for weeks at steady-state levels of proteoglycan metabolism [23,62,115,117]. In such explant cultures, chondrocyte synthesis of proteoglycan is stimulated by certain endocrine or paracrine serum factors, including insulin-like growth factor-I [105], transforming growth factor β [116], epidermal growth factor [130], and fibroblast growth factor [130]. The loss and catabolism of proteoglycans is largely dependent on active cellular metabolism [23], and can be accelerated by a number of cytokines such as lipopolysaccharides [114,117,182], interleukin 1 [72,114,185], retinoic acid [22], and tumor necrosis factor [163] and reduced by others such as insulin-like growth factor-I [186], transforming growth factor β [116], and γ-interferon [19]. The metabolic effects of interleukin 1 and lipopolysaccharides appear to be fairly specific for large proteoglycans as the synthesis and catabolism of proteins are affected only slightly [114].

The extracellular process by which proteoglycans become assembled into the matrix has also been examined in the absence of physical stimuli. Most cartilage proteoglycans (60–85%) are aggregated on a central hyaluronate backbone in an interaction stabilized by link protein [58,61,122]. Such aggregates are macromolecular complexes which effectively immobilize proteoglycans within the collagenous meshwork, preventing monomers from diffusing out of the tissue [71,122]. Newly synthesized proteoglycans, once secreted from chondrocytes, undergo an extracellular conversion process whereby their binding affinity for hyaluronate increases [125]. This process may be critical for the proper deposition of proteoglycans and the development of a mechanically functional highly-charged matrix.

In contrast to the active metabolism of proteoglycans, collagen is turned over
extremely slowly in mature cartilage since the collagen meshwork is fully assembled before adulthood [122]. In the cartilage of mature dogs, collagen synthesis represents only a few percent of the total protein synthesis [37]. The low turnover rate of collagen in mature tissues may reflect the stabilization and cross-linking of collagen [38]. In developing cartilage, active collagen synthesis does occur; a preliminary report suggested that collagen formation accounts for ~50% of the total protein synthesis in immature (4–5 week old) calf cartilage [110].

1.2 Biophysical Regulation of Cartilage Metabolism during Loading in Vivo

The metabolic activity and structural adaptation of cartilage in vivo appear to be sensitive to the functional demands of the biomechanical environment. For example, several experimental animal studies have demonstrated cartilaginous changes in joints subjected to increased or altered loads. When one foreleg of the sheep was immobilized so that the contralateral foreleg was subjected to increased stress, the glycosaminoglycan (hexuronate) content of the cartilage in the non load-bearing ankle joint decreased, and that in the high load-bearing joint increased [27]. When the anterior cruciate ligament of the dog knee was transected so that the joint was destabilized and the cartilage was subjected to “abnormal” loading, osteoarthritis ensued [103,104] even though collagen synthesis was stimulated [36] and proteoglycan turnover was increased [25,168].

However, in such in vivo studies, it is difficult to identify the physical regulators and biological processes responsible for the alterations in cartilage composition and structure. The complex joint geometries and loading patterns make quantification of physical phenomena difficult. Further, while correlates between biochemical alterations and physical factors can be drawn, the direct effects of loading on cartilage metabolism must still be distinguished from confounding systemic or local

Section 1.2
factors, such as the increased vascularity of subchondral bone [39].

1.3 Biophysical Regulation of Cartilage Metabolism: in Vitro Studies

1.3.1 General Approach

Since cartilage is subjected to mechanical stresses in vivo, one in vitro approach is to first characterize the biological effects of applied static and dynamic mechanical stresses, and then attempt to decipher the mechanisms responsible for observed effects. Test specimens can be of a symmetrical geometry that allows relatively simple quantitative modeling of physical and chemical phenomena. Theoretical models have been developed for the poroelastic mechanical [4,70] and electromechanical [32,43] behavior of cylindrical disks of cartilage subjected to static and dynamic uniaxial compression in both the radially confined [32,43,52,119] and radially unconfined configurations [4,48,78]. In such biophysical studies of cartilage disks subjected to compression, correlations can be made between metabolic responses and physical or chemical phenomena [10,48,173,187].

In designing in vitro protocols to assess the role of putative physical mechanisms in regulating cartilage metabolism, it is useful to consider that loading in vivo results in physical and chemical phenomena that consist of a time-average static component as well as a time-varying dynamic component, either of which may have biological effects. Distinguishing between static and dynamic effects requires assessing the static effects of the hypothesized stimulus, and then assessing the additional effect of a superimposed dynamic component. This can be difficult since the hypothesized stimulus may not be easily controlled, if for example it (1) cannot readily be measured dynamically and non-destructively (e.g., intratissue pH) or (2) exhibits spatial variations (e.g. fluid flow near a free-draining surface versus near an impermeable boundary).

Section 1.3
While physical models exist for cartilage in either the radially confined or unconfined configuration, each has practical advantages and disadvantages. While the confined configuration leads to simpler theoretical analysis due to minimal frictional effects at the interface between the cartilage and platen or chamber (which exist in the unconfined configuration [3,17,78,111]), it has the major disadvantage of allowing nutrient transport only through the porous platen. In preliminary experiments, radiolabel incorporation in radially-confined cartilage disks under a lightly loaded porous polyethylene platen (as used in [42,90]) was much (~50%) lower than in disks directly exposed to medium (data not shown). Thus, the platen appeared to be creating a nutrient diffusion barrier. In addition, the platen has a finite modulus (~10 MPa) which must be accounted for, since this is of the same order as the dynamic modulus of cartilage in confined compression [42]. On the other hand, in unconfined compression, the free circumferential surface for nutrient diffusion is dependent on the compressed cartilage thickness. Overall however, the unconfined configuration appears to be preferable for studying the effects of compression in explant culture.

A major issue is choosing the physical parameter to control during testing. Compression in vitro can be accomplished simply by controlling either mechanical displacement or load. If the hypothesized regulator is the extent of tissue consolidation as has been suggested (i.e., water content [173] and its associated physicochemical parameters such as pH [48]), displacement would be the appropriate test parameter to control. If the hypothesized regulator is osmotic pressure which also has been implicated [173,187], load may be the more appropriate parameter. One advantage of using displacement control is that the time to equilibrium or steady-state during stress relaxation is shorter than the creep time during load (mechanical or osmotic) control.

Another consideration in experimental design is that multiple specimens must be tested simultaneously to nullify the normal topographical variation in tis-

Section 1.3
sue composition and metabolism [53,99,173,179,180]. Indeed, if all samples had the
same mechanical properties, there would be little difference between using either
displacement or load control. To dynamically test multiple specimens with a single
actuator, load control requires all specimens to be in series, whereas displacement
control requires all samples to be in parallel; the later is rather straightforward to
achieve (see Chapter II and [48,50,75]).

Once the metabolic response of cartilage to static and dynamic compres-
sion is characterized, the role of specific biophysical factors can be examined. By
judiciously choosing either a mechanical, electrical, or chemical stimulus with spe-
cific boundary constraints, a number of putative physical regulators can be assessed
independent of others. For example, fluid flow can be generated by several mech-
anisms. Applying a mechanical stress via a porous platen to a cartilage disk in
the radially confined configuration creates axial gradients in pressure-driven flow
[90,119]; alternatively, applying an electric current axially through a cartilage disk
with free draining surfaces creates a uniform axial electroosmotic fluid flow [50,52].
The former method induces all of the phenomena described in Fig. 1.2 whereas the
latter method introduces only electric fields and fluid flow.

1.3.2 Previous Studies on the Effects of Compression on the Synthesis,
Assembly, and Release of Matrix Components in Cartilage

The effects of compression or deformation on biosynthesis by chondro-
cytes in cartilage or cell-laden gels have been examined in several in vitro stud-
ies [10,31,48,49,75,132,173]. These studies have quantitated glycosaminoglycan
and protein synthesis during or after static compression [10,48,75,173], a single
compression-release cycle [49,75], or a few specific cyclic compression protocols
[31,133] (see Chapter II for review). Several studies have addressed the possible
role of physicochemical alterations in affecting biosynthesis [10,48,173,187]. How-
ever, the biosynthetic effects of dynamic compression over a wide range of compres-

Section 1.3
sion amplitudes and frequencies remain relatively unknown.

The effects of compression on the extracellular processing of newly synthesized proteoglycans within cartilage have not been previously examined. Within explant cultures, the conversion of proteoglycans to the high-affinity form is dependent neither on live cells [8] nor on continued protein synthesis [106]; while conversion can be accelerated by cartilage incubation without oxygen [82], and can be slowed or inhibited by low temperature [8,106], no other physical or chemical factors have been examined. Within solutions of purified proteoglycans, the conversion has been further characterized and can be catalyzed by mild base (pH ≥ 7.4) [140] or by incubation of monomer in aggregate [172]. These findings suggest that compression may alter proteoglycan processing in cartilage through physicochemical mechanisms.

The effects of compression on the loss and catabolism of cartilage proteoglycans and proteins has only been addressed in two preliminary studies [75,132] (see Chapter IV). In one study, the maximum loads examined were 0.011 MPa [132] and thus much lower than physiological contact pressures [1,69,73]. In the other study [75], the 35S-constituents released into the medium during a compression and after compress-release were quantitated but not characterized, for example, in terms of their size distribution. Thus, it was not possible to interpret the release in terms of biological or physical mechanisms.

1.4 Objectives and Overview

The overall objectives of this thesis were (1) to characterize the effects of static and dynamic compression of cartilage on the synthesis, assembly, and degradation of extracellular matrix constituents, and (2) to relate these metabolic effects to possible biophysical regulatory mechanisms. The physiologic, pathophysiologic, and potential therapeutic implications of the responses were also considered.

In Chapter II, the effects of mechanical compression on proteoglycan and pro-
tein biosynthesis are described. The effects of static compression, 2-h compression and release, cyclic 2-h compression and release, and 23-h oscillatory compression at frequencies (f) from 0.0001–1.0 Hz were assessed in turn. Dynamic stiffness measurements of 3 mm diameter disks identified a characteristic frequency [0.001 Hz (cycles/sec)] that separated low and high frequency regimes in which different flow and deformation phenomena predominated; for example, at 0.0001–0.001 Hz, significant fluid was exuded from cartilage disks whereas at 0.01–1 Hz, hydrostatic pressure increased within the interior of disks. The biosynthetic responses to low frequency (f \leq 0.001 \text{ Hz}) compression protocols were qualitatively predictable from and consistent with (A) the dosimetry of inhibition by static compression and (B) the kinetics of response to a single 2-h compression and release. The 20–40\% biosynthetic stimulation due to low amplitude (1–5\%) high frequency (f = 0.01–1.0 Hz) compression suggested that several factors (e.g., cell deformation, pressurization, fluid flow, and streaming potentials) that are unrelated to the physicochemical intratissue environment may be biosynthetic stimuli to the chondrocyte.

In Chapter III, the effects of compression on the hyaluronate binding properties of newly synthesized proteoglycans are described. Cartilage disks were pulse labeled with $^{35}$S-sulfate, washed, and then chased while being subjected to compression. The extracellular maturation of newly synthesized proteoglycans was assessed in terms of the acquisition of a high binding affinity by $^{35}$S-proteoglycan for hyaluronate. Oscillatory compression at the amplitude and frequencies which elicited a biosynthetic stimulation had no effect on the maturation of $^{35}$S-proteoglycans. In contrast, static compression was found to markedly slow the conversion of $^{35}$S-proteoglycans. The role of intratissue pH in modulating this conversion was examined; equivalent intratissue acidification by either compression or media titration led to equivalent delays in affinity conversion. Such a physicochemical control mechanism may have a physiologic role, promoting the deposition of newly synthesized proteoglycan in regions of low proteoglycan concentration,
even in the absence of loading.

In Chapter IV, the effects of static and slow cyclic compression on the release of radiolabeled proteoglycans and proteins as well as total tissue proteoglycans are described. The time between radiolabeling and applying compression was chosen to be greater than one day to allow newly secreted $^{35}$S-proteoglycans to be processed into the form with high affinity for hyaluronate (Chapter III) and $^3$H-proline protein precursors to be chased into macromolecules (Appendix A). Static and slow cyclic compression led to increases in the rate of release of macromolecules. The patterns of macromolecular release (kinetics of release, quality of macromolecules released and remaining in the tissue), were consistent with several physical phenomena (convection, diffusion, and disruption of the collagen meshwork) as well as biological effects (decreased accumulation of proteoglycan).

In Chapter V, the effects of static and dynamic compression on cartilage explant metabolism are summarized, along with possible biological and physical mechanisms (Appendices B, C, and E; see below) and in vivo clinical and experimental correlates. In particular, since the results of Chapter II indicated oscillatory compression could stimulate biosynthesis, possible correlates of biosynthetic response with dynamic physical factors were examined. The biosynthetic stimulation appeared coincident with the increase in dynamic stiffness and thus may be related to hydrostatic pressurization, fluid flow, or streaming potentials. Suggestions for future studies to examine these and other possibilities are outlined.

The appendices contain control studies on the calf cartilage explant culture system (Appendices A and D), and some preliminary studies addressing biophysical mechanisms affecting metabolism (Appendices B, C, and E). In Appendix A, control studies on the use of isotopic precursors to assess metabolism by calf cartilage explants are summarized. The kinetics of formation of isotopically labeled macromolecules and their release are determined in these studies, which thereby served as a guide for choosing radiolabel and chase times in all other experiments.

Section 1.4
The characteristic time for incorporation of $^{35}$S-sulfate into $^{35}$S-glycosaminoglycans was rapid ($t_{1/2} = 15$ min). Pulse-chase studies indicated a rapid rate of release of $^{38}$S-macromolecules during the first day, and then a steady release rate of $\sim 1\%$/day. The characteristic time for incorporation of $^3$H-proline into $^3$H-macromolecules was somewhat slower ($t_{1/2} = 65$ min). Thus, $^3$H-proline incorporation during short 1 h radiolabels predominantly reflected cellular uptake, whereas that during longer 8 h radiolabels reflected formation of protein. The kinetics of $^3$H-proline incorporation into collagen was assessed by quantitation of $^3$H-hydroxyproline residues and found to parallel that of $^3$H-macromolecules; $^3$H-collagen accounted for $\sim 80\%$ of the $^3$H-macromolecules. Pulse-chase studies showed a release of $^3$H-constituents which were predominantly non-collagenous molecules; release was rapid during the first day of chase, and subsequently slowed.

In Appendix B, several biological and physical mechanisms by which static and dynamic compression may affect biosynthesis are examined. Control studies verified that $\beta$-xyloside could act as an artificial acceptor for newly synthesized glycosaminoglycan chains which once formed, were able to rapidly diffuse into the medium. The proportion of newly synthesized $^{35}$S-macromolecules which were released into the medium increased from $<1\%$ without $\beta$-xyloside to $>50\%$ with 1 mM $\beta$-xyloside. $\beta$-xyloside had little effect on the overall rates of $^{38}$S-sulfate and $^3$H-proline incorporation into (tissue and medium) macromolecules. $\beta$-xyloside did not alter the compression-induced inhibition of $^{35}$S-sulfate incorporation into $^{35}$S-macromolecules. Thus, the inhibition of glycosaminoglycan synthesis by static compression did not appear to be secondary to an inhibition in synthesis of proteoglycan core protein, to which newly synthesized glycosaminoglycans could attach.

In addition, the effect of compression on $^3$H-proline incorporation into $^3$H-collagen was assessed; neither static nor dynamic compression caused marked changes in the proportion of $^3$H-proline directed into $^3$H-hydroxyproline. The hypothesized relationship of the decreased intratissue pH during compression and decreased biosyn-
thesis was examined [48]. Although $^3$H-proline and, to a lesser extent, $^{35}$S-sulfate incorporation in freely swelling cartilage disks decreased with medium acidification, the magnitude of inhibition did not appear to fully account for the biosynthetic inhibition by compression.

In Appendix C, the effects of applied currents on the release and synthesis of proteoglycans are described. The release of $^{35}$S-proteoglycan from radiolabeled cartilage disks during culture in 37°C medium was stimulated by application of current ($\geq 2$ mA/cm$^2$). Even in the absence of current, there were differences in the size distribution of $^{35}$S-macromolecules released early (0–3 h) after radiolabeling compared to later (24–27 h). The material released early consisted of mostly (~50%) small $^{35}$S-macromolecules (possible a small $^{35}$S-proteoglycan or $^{35}$S-glycosaminoglycan chains), in addition to the large aggregating and non-aggregating $^{35}$S-proteoglycans. However, by 24–27 h, the large $^{35}$S-proteoglycans accounted for ~90% of the released $^{35}$S-macromolecules. Applied current enhanced the release of all three of these components. It was interesting that application of high current (5 mA/cm$^2$) to pulse-labeled cartilage caused a preferential release of aggregatable $^{35}$S-proteoglycans compared with current applied to pulse-chased cartilage; this would be consistent with increased mobility of newly synthesized proteoglycans with a low binding affinity for hyaluronate. The mechanism of the current-induced release appeared to be largely electromechanical rather than cell-mediated since applied current had little effect on cellular activity (glycosaminoglycan synthesis) and also increased proteoglycan release from cartilage in an enzyme-inhibiting solution at 4°C. Importantly, this solution stabilized the newly synthesized $^{35}$S-proteoglycan in the form with low affinity for hyaluronate.

In Appendix D, the effects of serum on biosynthesis and glycosaminoglycan accumulation are described. Medium supplemented with 10% FBS appeared better able to maintain explants at steady state rates of $^3$H-proline and $^{35}$S-sulfate incorporation than 0.1–20% NuSerum. In medium with 10% FBS, disks accumulated

Section 1.4
glycosaminoglycan at a rate of $\sim 3\%$/day.

In Appendix E, the effects of matrix depletion by Chondroitinase ABC on biosynthesis and glycosaminoglycan deposition are described. Chondroitinase treatment (0.1–0.2 U/ml medium) for three days depleted disks of glycosaminoglycan by $\sim 90\%$. In medium with 10% FBS, depleted disks accumulated glycosaminoglycan more rapidly than control disks in the following $\sim 2$ weeks. However, glycosaminoglycan synthesis by depleted disks was initially depressed and only transiently stimulated; thus, the increased glycosaminoglycan deposition appeared primarily a result of decreased rate of proteoglycan loss rather than increased synthesis. Since the Chondroitinase ABC preparation used may have had protease activity (and no protease inhibitors were added to the medium), it was not clear if all effects were due to Chondroitinase excision of matrix proteoglycans, or perhaps due to proteolytic effects on the chondrocytes.
Chapter II

Biosynthetic Response of Cartilage Explants to Dynamic Compression

2.1 ABSTRACT

* The biosynthetic response of calf articular cartilage explants to dynamic compression was examined over a wide range of amplitudes, waveforms, and frequencies. Glycosaminoglycan synthesis was assessed by $^{35}S$-sulfate incorporation, and amino acid uptake and protein synthesis were assessed by $^3H$-proline incorporation. Two culture chambers were designed to allow uniaxial radially unconfined compression and mechanical testing of cartilage disks: one was used inside a standard incubator; the other was used with a mechanical spectrometer and allowed load and displacement to be monitored during compression. Dynamic stiffness measurements of 3 mm diameter disks identified a characteristic frequency [0.001 Hz (cycles/sec)] that separated low- and high-frequency regimes in which different flow and deformation phenomena predominated; e.g. at 0.0001–0.001 Hz, significant fluid was exuded from cartilage disks whereas at 0.01–1 Hz, hydrostatic pressure increased within the interior of disks. At the higher frequencies, oscillatory strains of only ~1–5% stimulated $^3H$-proline and $^{35}S$-sulfate incorporation by ~20–40%. In contrast, at the lower frequencies, (A) compressions of <5% had no effect, consistent with the dosimetry of biosynthetic inhibition by static compression (~25% compression caused a ~20% inhibition of radiolabel incorporation), and (B) higher amplitudes (cycling between 1.25 and 0.88–1.00 mm) stimulated $^3H$-proline and $^{35}S$-sulfate incorporation by ~20–40%, consistent with the kinetics of response to a single 2-h compression and release. None of the compression protocols was associated with detectable alterations in (e.g. compression-induced depletion of) total glycosaminoglycan content. This study provides a framework for identifying both the physical and biological mechanisms by which dynamic compression can modulate chondrocyte biosynthesis. In addition, the culture and compression methodology potentially allows in vitro evaluation of clinical strategies of continuous passive motion therapy to stimulate cartilage remodeling.

*Preliminary results have been described [157,159]. A version of this chapter appears in [162].
2.2 INTRODUCTION

Articular cartilage functions as a weight-bearing wear-resistant material in synovial joints. Mechanical forces are thought to modulate the metabolic activity and influence the structural adaptation of a variety of connective tissues including cartilage [164] and tendon [45]. Clinical observations suggest that abnormal mechanical forces predispose cartilage to degeneration and to modeling errors such as osteoarthritis [145], acetabular abnormalities in congenital dislocation of the hip [131], and ball and socket ankle joint after subtalar ankylosis [68]. However, the physical phenomena and biological processes responsible for these alterations are difficult to identify in vivo. Complexities include quantifying the biomechanics of loading, as well as distinguishing between direct effects of loading on cartilage metabolism and indirect effects due to local or systemic factors such as increased vascularity of subchondral bone [39].

In vitro, cartilage can be maintained in a stable and controlled biochemical and physical environment. Explant culture of cartilage preserves the characteristic chondrocyte biosynthetic phenotype [176] as well as potentially important interactions between the cells and extracellular matrix. When cultured in media supplemented with 10–20% FBS, calf and adult bovine articular cartilage explants synthesize proteoglycans (PG) at steady state levels [62,105]. Thus, most previous in vitro studies of mechanical compression have utilized cartilage explants [10,48,49,75,132,157,162,173].

The physical properties of cartilage can be determined in vitro by testing specimens in well-defined geometries. A number of physical phenomena, which occur naturally during loading and are inexorably coupled, have been identified and quantified; compression of cartilage results in deformation of cells and extracellular matrix [16,144], hydrostatic pressure gradients, fluid flow, streaming potentials and currents [42,90,97,119], and physicochemical changes [48,52,173]. Since any or all of these physical phenomena may have biological consequences, theoretical models

Section 2.2
for poroelastic mechanical [4,70] and electromechanical [32,43] behavior of cartilage provide a framework for establishing protocols for static and dynamic testing of live tissue and interpreting results.

Using such models, the magnitude, direction, and spatial variation of a hypothetical physical stimulus can be predicted. For example, static compression of cartilage explants causes a dose-dependent inhibition of GAG synthesis and amino acid uptake [10,48,49,75,173]. The initiator of this inhibition may be specific physicochemical changes that accompany compression-induced fluid exudation [48,173]; transmembrane pressure [173] and the extracellular ionic milieu (specifically, pH) [48] have been implicated.

*In vivo*, repetitive *loading* causes fluid to be gradually exuded from cartilage during each loading cycle [100,192]. Thus, the resulting cartilage *compression* consists of a time-varying *dynamic* component superimposed on a time-average *static* component. The magnitudes of biomechanical parameters *in vivo* have been estimated; contact pressures in the human femoropatellar and hip joint are 3–18 MPa [1,69,73] and result in compression amplitudes of >13% [3]. Previously, the biosynthetic effects of repetitive loading of cartilage *in vitro* have been examined using only a few specific protocols. Dynamic compressive loads (60 sec on/60 sec off or 4 sec on/11 sec off) applied to adult dog articular cartilage explants could cause an inhibition or stimulation of biosynthesis [132]. However, because the compression that occurred during loading was not measured or estimated, it is not possible to distinguish between the effects of stress versus compression or the effects of time-average static versus dynamic compression. Also, the peak applied stresses (1–11 kPa [132]) were rather low compared to typical *in vivo* stresses [1,69,73]; cartilage explants can withstand 120,000 loading cycles of 3.5 MPa without fissure formation [197]. Cyclic tensile stretching of high-density 14-day chick chondrocyte cultures increased PG synthesis; the single test condition of 5.5% stretching at 0.2 Hz was thought to impart compressive forces to the cells [31]. Another approach
has been dynamic pressurization of the gas phase (13% at 0.3 Hz) overlying the culture medium [86,189]. To interpret these results, it is necessary to first separate the effects of dynamic changes in partial pressure of gas in the medium from hydrostatic pressure, which alone may also elicit a response depending on the amplitude and frequency of pressure change [55,84,92]. Thus, the biosynthetic response of cartilage explants to dynamic loading remains relatively unknown.

In this study of calf articular cartilage explants, our objectives were (A) to distinguish between the biosynthetic responses to static and dynamic components of compression at amplitudes and frequencies of physiologic relevance, and (B) to relate these responses to possible biophysical mechanisms. Glycosaminoglycan (GAG) synthesis was assessed by $^{35}$S-sulfate incorporation, while amino acid uptake and protein synthesis were assessed by $^3$H-proline incorporation. The biosynthetic effects of the static components of loading were examined via (1) 12-h compression, whereas the dynamic components were examined via (2) kinetic studies of a single compression followed by release, (3) slow cycles of compression and release, and (4) oscillations superimposed on a slight static compression. The relative magnitudes of physical phenomena at different compression frequencies were assessed by measuring dynamic stiffness. The results showed that within frequency regimes in which different flow and deformation phenomena predominated, dynamic compression could trigger biosynthetic responses which were dependent on compression amplitude.

2.3 MATERIALS AND METHODS

Dulbecco’s modified Eagle’s medium (DMEM) (low glucose), N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), Dulbecco’s phosphate buffered saline (PBS), L-ascorbic acid, and penicillin/streptomycin were from Gibco, Grand Island, NY. Fetal bovine serum (FBS) was from Hyclone, Logan, UT. L-proline, non-essential amino acids, sodium sulfate, papain (twice crystallized),

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and cycloheximide were from Sigma, St. Louis, MO. Sodium $^{35}$S-sulfate (400-800 mCi/mmol) and L-$[^3]$H]proline (12.9 Ci/mmol) were from New England Nuclear, Boston, MA. ScintiVerse Bio-HP was from Fisher, Boston, MA. 24-well and 48-well culture dishes were from Costar, Cambridge, MA. Tygon tubing (1/16 in inner diameter (ID), 3/16 in outer diameter (OD)) was from Norton Industrial Plastics, Akron, OH. Semi-micro polystyrene cuvets were from VWR, Boston, MA. Acrylic cuvets were from Centaur West, Sparks, NV.

2.3.1 Cartilage Disks: Explant and Culture

The saddle sections (7–14 kg) of 1–2 week old calves were obtained from a local abattoir (J.T. Trelegan, Cambridge, MA, or A. Arena, Hopkinton, MA) directly after slaughter. The intact femoropatellar joint was isolated by transecting the femur and mounting the distal segment in a drilling apparatus. The femoropatellar articular cartilage was then exposed by opening the joint capsule, severing the medial, lateral, and cruciate ligaments, and removing the tibia, patella, and surrounding tissue. Four to six cylindrical cores of cartilage and underlying bone, 9.5 mm in diameter and $\sim$15 mm deep, were drilled from each facet (medial and lateral) of the femoropatellar groove. During this entire process, the cartilage was kept moist and free of blood by frequent rinsing with sterile PBS supplemented with antibiotics (100 U/ml penicillin and 100 $\mu$g/ml streptomycin).

Each core was then inserted into a cylindrical sample holder for a sledge microtome (Model 860, American Optical, Buffalo, NY). After cutting and discarding a $\sim$100 $\mu$m section from the articular surface, two 1 mm thick plane-parallel cartilage slices were microtomed and placed in $\sim$1 ml PBS with antibiotics. After all slices had been cut, the central region of each was punched into four 3.0 mm diameter disks using a stainless steel dermal punch (Miltex Instruments, Lake Success, NY). In each experiment, disks from a single slice were distributed equally between different treatment groups.

Section 2.3
After harvesting, cartilage disks were incubated in media (DMEM with 10 mM HEPES, 10% FBS, 0.1 mM non-essential amino acids, an additional 0.4 mM proline, and 20 μg/ml ascorbate) at 37°C in 5% CO₂:95% air. The total time from slaughter to incubation was less than six h. The media was changed daily, and for the first day, supplemented with antibiotics. Individual wells of 24-well or 48-well culture dishes held up to four disks in 1 ml of media. After 2–6 days of incubation, disks were subjected to the compression and radiolabeling protocols described below.

2.3.2 Chamber for Static and Repetitive Compression

One set of compression chambers allowed cartilage disks to be sandwiched between platens and compressed to the desired thickness in a uniaxial, radially unconfined configuration (Fig. 2.1). These chambers were machined out of polysulphone (Westlake Plastics, Lenni, PA) and used inside a standard incubator. The base of each chamber contained media wells with 6.4 mm diameter fluid-impermeable nubs that protruded up from the base of the wells and served as the bottom compression platens. The lid had similar nubs that protruded down and served as the top compression platens. In a single chamber, 12 cartilage disks were simultaneously compressed by inserting a teflon spacer (0.50–1.25 mm) between the chamber lid and base and applying a weight (6.7 kg, corresponding to a stress of 0.77 MPa) large enough to force together the lid (0.13 kg, 0.02 MPa), spacer, and base. Without a spacer, the top and bottom platens were flush (± 0.025 mm); thus, the thickness of the teflon spacer corresponded to the final thickness of the compressed cartilage. The chamber base had small channels that allowed gas diffusion to the media in each well. The total time to assemble and load each chamber was ~5 min.

Section 2.3
Figure 2.1: Schematic of static compression chamber. Cartilage disks lay in media wells, sandwiched between chamber lid (top platen) and chamber base (bottom platen). A large load is applied to the chamber lid, forcing the top and bottom platens together. A spacer limits the distance between the top and bottom platens and therefore determines the compressed thickness of the cartilage disks.
2.3.3 Chamber for Dynamic Compression and Mechanical Testing

Another chamber allowed dynamic compression and mechanical testing of experimental cartilage disks and static compression of controls (also in uniaxial, radially unconfined compression) within a single culture environment (Fig. 2.2). The polysulphone cylindrical base was mounted onto the actuator of a mechanical spectrometer (Dynastat, IMASS, Hingham, MA). The base contained a single media well with 9.5 mm diameter nubs that protruded up from the base and served as the bottom compression platens for cartilage disks. Each of eight nubs had three indentations (4 mm diameter × 0.1 mm deep) on which a total of 24 cartilage disks rested; these indentations ensured that the cartilage disks remained sandwiched between the platens during the course of the experiment.

Dynamic compression was applied to 12 of the 24 cartilage disks via four polysulphone compression rods (9.5 mm diameter × 50 mm long, ~7 g) that served as top platens. The compression rods protruded up through and slid freely within holes in the 6.4 mm thick stainless steel chamber lid. Equal dynamic displacements were transmitted to the compression rods (and thus the underlying cartilage disks) via four micrometer heads (0.001 in graduation with lock nuts, L.S. Starrett Co., Athol, MA) that were set into a 6.4 mm thick aluminum plate, which in turn was clamped into the upper jaw of the spectrometer. The micrometers enabled application of an initial static compression about which the dynamic compression was superimposed.

The chamber was mounted and aligned parallel to the aluminum plate/micrometers by interposing two equal thickness blocks between the chamber lid and aluminum plate. This ensured that the the applied static offset for all dynamically compressed specimens was repeatable to within ± 25 μm. The dynamic displacement imposed by the spectrometer could be controlled to < 1 μm. Compliance measurements of the dynamic compression apparatus without cartilage samples indicated an elastic deformation of 7.8 μm/kg total load, which was

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Figure 2.2: Schematic of dynamic compression chamber. Chamber creates a culture environment for mechanically testing tissue samples using a conventional mechanical spectrometer. Experimental cartilage disks are dynamically compressed between the chamber base (bottom platen) and compression rods (top platen). The actuator motion (bottom) exerts a force that is transmitted up through the chamber base, cartilage disks, compression rods, and micrometers, and measured at the load cell (top). Control disks, in the same media reservoir, are statically compressed between the chamber base (bottom platen) and polysulphone-covered stainless steel pins (top platen) that are secured to the chamber lid. Media is recirculated through a heat exchanger to maintain the temperature at 37°C; a 5% CO₂:95% air gas mixture is injected into the chamber.

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approximately equally divided between the compression chamber and the spectrometer load cell. This is consistent with the manufacturers' mechanical specifications of 2.5 GPa tensile modulus for polysulphone (Westlake Plastics), and a 4 μm/kg deformation of the spectrometer load cell (IMASS, personal communication). The magnitude of cartilage compression was calculated from the measured load, actuator displacement, and apparatus compliance.

Static compression was applied to the 12 control cartilage disks within this chamber via four polysulphone-covered stainless steel pins (top platens). Each pin was 6.4 mm diameter × 50 mm long; one end was covered with a 1.6 mm thick polysulphone sleeve, while the other end was threaded (40 threads per inch), allowing the pin to be secured to the chamber lid with counter nuts. Before each experiment, the pins were positioned over the bottom platen at the desired height using calibrated spacers in place of the cartilage disks. During an experiment, the chamber lid was clamped down onto the base, thus compressing cartilage disks to the desired thickness.

A sterile culture environment was maintained inside this chamber. A mixture of 5% CO₂:95% air was regulated to ~50 cc/min through a flowmeter (Model R7630, Matheson, Secaucus, NJ), humidified by bubbling through a water bath, and passed through a 0.22 μm filter just before entering the chamber; this buffered the bicarbonate-based media to pH ~7.4. Media was recirculated through a heat exchanger to keep the incubation bath well mixed and at 37°C. Media was pumped at ~15 ml/min (Model 7535-10 peristaltic pump, Cole Palmer, Chicago, IL) through a total of ~2 feet of tygon tubing attached to the chamber via polypropylene pipe adapters (1/16 in ID, Cole Palmer). A polypropylene tee adapter (Cole Palmer) was included in the tubing pathway between the pump and the chamber to allow media changes without disassembling the apparatus; when not in use, the extra port was clamped shut and secured in a sterile wrapping. The heat exchanger consisted of a coiled 15 gauge, 12 in long hypodermic stainless steel needle (Becton Dickinson, Section 2.3
Rutherford, NH) within a stirred water bath, kept at constant temperature via a proportional temperature controller (Model 22, Fisher).

The mechanical spectrometer was interfaced to a computer or a frequency synthesizer (Model 5100, Rockland Systems, West Nyack, NY) to control the compressive displacement; during culture experiments, the displacement and resulting load were recorded on a chart recorder (Model 2200, Gould, Cleveland, OH) and/or digitized and stored on a computer. Fourier decomposition of the load signal into fundamental and harmonic components enabled quantitative assessment of the extent of nonlinearities.

The total time to load cartilage disks into the dynamic chamber under sterile conditions, seal the chamber, mount it in the spectrometer, and begin compression was \(~\)30 min. At the initiation and termination of each experiment, the position of the cartilage disks (under the compression platens) was visually confirmed by transilluminating the chamber.

2.3.4 Compression and Radiolabeling Protocols

In separate experiments, the equilibrium load-displacement relationships were measured for individual cartilage disks using the mechanical spectrometer. Step compressive displacements at 0.1 mm increments were applied to each disk bathed in PBS with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine-HCl, and 5 mM N-ethylmaleimide), and the resulting equilibrium load was measured. Equilibrium was assumed to be reached when the load changed by less than 3 g over 3 min. During the 2–6 day incubation period prior to compression studies on biosynthesis, these and other independent measurements indicated that the calf cartilage disks swelled axially by 25–40% above the cut thickness of 1.0 mm. Therefore, to eliminate ambiguity, static compressions are reported as the final compressed specimen thickness; dynamic axial strain amplitudes are reported as a percentage of the initial (1.0 mm) specimen thickness.

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In control experiments to characterize the kinetics of isotope incorporation into free swelling cartilage, disks were incubated in 24-well dishes for 1, 2, 4, or 8 h in media containing both 20 μCi/ml $^3$H-proline and 10 μCi/ml $^{35}$S-sulfate (1 ml/disk). These isotope concentrations were used in all experiments requiring 1-h radiolabeling pulses; half these concentrations were used during 4–12-h radiolabels. All media (both with and without isotopes) was temperature and CO$_2$ equilibrated by preincubation for 1 h just before contact with cartilage disks. Other control disks were (1) lyophilized to lyse the chondrocytes before 8-h radiolabeling, (2) treated with 100 μg/ml cycloheximide for 1.5 h prior to and during 8-h radiolabeling, or (3) radiolabeled for 8 h, washed 4 times over 2 h with media (1 ml/disk), and chased for 24 h with media (1 ml/disk). Radioactivity in the chase media, as well as in other fractions described below, was determined by mixing portions (0.1–0.5 ml) with 2.5 ml ScintiVerse Bio-HP and measuring $^3$H-cpm and $^{35}$S-cpm in a liquid scintillation counter (RackBeta 1211, LKB, Turku, Finland), with corrections for spillover of $^{35}$S counts into the $^3$H channel and dilution quenching.

The biosynthetic response to compression was examined using five protocols (summarized in Fig. 2.3). Before each culture experiment, the mechanical testing chambers were autoclave-sterilized and prewarmed to 37°C. (A) Static mechanical compressions were applied using the static chambers (Fig. 2.1). In one experiment, experimental disks were radiolabeled (0.8 ml/disk) during 12-h compression to a thickness ranging from 0.50 to 1.25 mm; free swelling control disks were incubated in 24-well culture dishes. In another experiment, the kinetics of response to compression were examined by radiolabeling disks compressed to 0.50 mm and disks just covered with the chamber lid (control) for 1, 2, 4, or 8 h; here, control disks were essentially at their free swelling thickness.

(B) A single compression-release cycle was applied using the static chambers (Fig. 2.1). Experimental disks were compressed to 1.00 or 0.50 mm for 2 h and then released; controls were free swelling in 24-well dishes. At 0, 1.5, 3.5, and 5.5 h after
Figure 2.3: Mechanical testing and radiolabeling protocols. (A) **Single compression.** A load is applied (time = 0) and compression continues until a specified displacement is reached. Cartilage disks are isotopically labeled with both $^3$H-proline and $^{35}$S-sulfate during the entire loading period (up to 12 h). (B) **Single compression-release.** Compression is applied at time = 0 (as in A) and after 2 h, the load is withdrawn and the cartilage disks reswell. (C) **Repetitive (1–9) compression-release.** Same as B except after 6 h of release, compression cycle is repeated. Radiolabeling is for 4 h beginning 1.5 h after 1st, 2nd, or 3rd release. (D) **Cyclic compression-release.** Each compression cycle consists of a 2-h compression and 2-h release. The applied displacement during compression and release are exponentials with 10 and 15 min time constants, respectively. (E) **Oscillatory compression.** A sinusoidal displacement is applied. Protocols A–C are performed in the static chamber (Fig. 2.1), while D and E are in the dynamic chamber (Fig. 2.2).
the release, control and compressed-released disks were radiolabeled for 1 h in a 24-well dish (1 ml/disk). In a separate experiment, the mechanical spectrometer and PBS bath conditions (above) were used to characterize the load-displacement behavior resulting from the compression-release protocol (A–C).

(C) Repetitive (1–3) compression-release cycles were applied using the static chambers (Fig. 2.1). Experimental disks were compressed to 0.50 mm for 2 h and then released; the time before the next compression-release, if any, was 6 h. Control disks were free swelling in 24-well dishes. After applying 1, 2, or 3 compression-releases, compressed and control samples were radiolabeled in a 24-well dish (1 ml/disk) during a 4-h period beginning 1.5 h after the release.

(D) Cyclic compression-release was applied using the dynamic chamber (Fig. 2.2). Experimental disks were cycled between a swollen thickness of 1.25 mm and a maximally-compressed thickness of 0.50–1.13 mm while load was measured. Each cycle consisted of 2 h of compression using an exponential displacement with a time constant of 10 min followed by 2 h of release with a time constant of 15 min. Control disks were held within the chamber at a swollen thickness of 1.25 mm. The chamber contained 25–30 ml of media; during the last 8 h of 23-h cyclic compression, the media was replaced with fresh media including radiolabels.

(E) Oscillatory strain was applied using the dynamic chamber (Fig. 2.2). Experimental disks were subjected to oscillatory compression superimposed on a static compression to 1.00 mm while load was measured; oscillation amplitudes were 1.3–10% at frequencies of 0.0001–1.0 Hz. Control disks were held statically at 1.00 mm. Compression and radiolabel times were as in protocol D.

2.3.5 Biochemical Analysis

After radiolabeling, each disk was serially washed six times over 90 min in 1–1.5 ml PBS supplemented with 0.8 mM sodium sulfate and 1 mM L-proline at 4°C, and then lyophilized. The wet and dry weights of some disks were measured
before and after lyophilization, respectively. Dried disks were then analyzed, as
described below, via either a combination of (I) SDS extraction followed by (II) pa-
pain digestion or (II) papain digestion alone. Radiolabel medium was not routinely
analyzed for biosynthetic products since 12-h control studies showed that >90%
and >95% of $^3$H- and $^{35}$S-macromolecules, respectively, were retained within the
cartilage disks.

(I) In radiolabel control experiments, dried disks were extracted with 2%
SDS, 10 mM dithiothreitol, 50 mM Tris-HCl, pH 8.5 at 100°C for 15 min. The ex-
tracted disks were washed three times over 90 min (as above), lyophilized again, and
further analyzed as described below (II). The extracts were either frozen (−20°C)
for later analysis or immediately cooled to room temperature whereupon a 0.5 ml
portion was separated on a PD10 column of Sephadex G-25 (MW cutoff of 1–5,000;
Pharmacia, Piscataway, NJ). The elution buffer was the same as the extraction
buffer, except the SDS concentration was reduced to 1%. Fractions (0.5 ml) were
collected, or alternatively, the macromolecular ($V_0$) and low molecular weight ($V_i$)
component peaks were pooled after control studies showed that separation of ra-
dioactivity to baseline was achieved. The radioactivity in the $V_0$ fraction and that
remaining in the disk were summed and defined to be macromolecular; no additional
low-MW $^3$H- or $^{35}$S-components were released from SDS-extracted disks during ex-
haustive dialysis against PBS supplemented with 0.8 mM sulfate and 1 mM proline.
The kinetics of formation of macromolecular product, $P(t)$, during radiolabeling
time $t$ was described by fitting $P(t) = R \cdot [t + (e^{-t/\tau_p} - 1)\tau_p]$ to obtain $\tau_p$ using an
iterative method of least squares [174]; $\tau_p$ is the average lifetime of a molecule in a
single lumped intermediate pool (representing all sequential intermediates between
the initial radiolabel added to the media and the macromolecules produced) [83].
To allow comparison with other studies [79,109], this time constant is converted to
$\tau_{1/2} = \tau_p \cdot \ln 2$.

(II) In most compression experiments, dried disks were directly digested with

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0.5–1.0 ml papain (125 μg/ml in 0.1 M sodium phosphate, 5 mM Na₂-EDTA and 5 mM cysteine-HCl, pH 6.0). Portions (100 μl) of the digest were analyzed for radioactivity. Portions (10 μl) were assayed for sulfated GAG by reaction with 1 ml dimethylmethylen blue dye solution [40] in a semi-micro polystyrene cuvet and spectrophotometry (Model λ3B, Perkin Elmer, Norwalk, CT); chondroitin sulfate (0–15 μg of whale/shark cartilage, Sigma) was used as the standard. Portions (100 μl) of some digests were also analyzed for DNA by reaction with 2 ml Hoechst 33258 dye solution in an acrylic cuvet and fluorometry [77]. All assays were performed in duplicate.

Sample quantities are expressed as mean ± SEM except where noted. Differences between sample means were assessed by two-tailed t-test.

2.4 RESULTS AND DISCUSSION

2.4.1 Mechanical Characterization

Fig. 2.4 shows the equilibrium load-compression relationship for calf cartilage disks. A finite load was required to compress most samples to ≤1.2 mm. For compressed thicknesses of 1.1 down to 0.8 mm, load increased linearly with displacement, whereas for greater compression, load increased nonlinearly. Microscopic examination (data not shown) of free-swelling disks showed negligible changes (~1%) in radial dimension from the original 3.00 mm diameter; disks compressed to 0.75–0.50 mm between glass slides increased only ~12% in area, comparable to the ~5% increase reported for human articular cartilage disks [111]. Thus, stress was approximated as the measured load normalized to the unloaded cross-sectional area; the equilibrium unconfined compression modulus corresponding to the linear load-displacement region (Fig. 2.4) was 0.47 MPa. This is similar to that reported for fetal human epiphyseal cartilage (0.70 ± 0.35 MPa) [17].

The load-compression history of disks subjected to the compression-release...
Figure 2.4: Steady-State Compression. Displacements were applied to individual cartilage disks, and the resulting equilibrium loads measured. Equivalent stress is estimated on the right ordinate as the load normalized to the uncompressed cross-sectional area. Samples were originally microtomed to 1.00 mm, but swell by >20% during subsequent culture. Load (mean±SD, n = 12) is reported multiplied by 12 to allow comparison with experiments (e.g. Figs. 2.6–2.8) where 12 cartilage disks are loaded in parallel.
protocol with the static compression chamber (Fig. 2.3A–C) was characterized via a simulation using the mechanical spectrometer (Fig. 2.5). The compression phase consisted of application of constant load, causing creep compression of cartilage to a final fixed thickness (determined by the teflon spacer in the static compression chamber or chosen to be 0.5 mm in the simulation); a 560 g load (0.77 MPa) applied to an individual cartilage disk (equivalent to the 6.7 kg load used for 12 cartilage disks in the static chamber) caused creep compression to 0.5 mm over ~14 min (Fig. 2.5). At this fixed displacement, stress relaxation occurred and was 90% completed after an additional ~15 min. The release phase consisted of a step removal of load (to a small tare load, ~15 g); reswelling was 90% completed over ~17 min.

Dynamic loads were monitored throughout biosynthesis experiments using the dynamic chamber. Fig. 2.6 shows a load measurement during cyclic 2 h on/2 h off (Fig. 2.3D) compression, along with the applied exponential compression and release displacements. The compression phase showed an increase in stress followed by stress relaxation, with peak loads never more than 20% greater than the final load even for the highest compression amplitude; with these peak loads, >90% of the applied displacement was taken up by the cartilage samples (i.e., <10% by the compression apparatus). Therefore, the magnitude of cartilage compression in these experiments (Fig. 2.3D) is reported as the applied displacement.

Fig. 2.7 shows typical loads from biosynthesis experiments during 2.5% oscillatory (Fig. 2.3E) compression at 0.001 and 0.1 Hz. At 0.001 Hz, the resulting load was sinusoidal. The higher strain rates at 0.1 Hz produced a nonlinear load response; e.g., the load measured zero when the micrometers lifted off the compression rods at the peak of the release phase. Fig. 2.8 summarizes the load and stiffness data from all biosynthesis studies where the applied oscillatory compression was 2.5%. Fig. 2.8A shows that the average load was ~1 kg (~0.1 MPa) at all frequencies, while the amplitude of the fundamental (first harmonic) increased

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Figure 2.5: Mechanical characteristics of compression and release in static chamber. (See Figs. 2.1 and 2.3A–C). A 560 g load (0.77 MPa) was applied (time = 0) to a single cartilage disk and the compression (creep) measured. When the cartilage disk became compressed to 0.5 mm (t ~ 14 min), the displacement was held constant while the load (stress relaxation) was measured. After 2 h, the load was decreased to 15 g, while the displacement (reswelling) was monitored.

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Figure 2.6: Loading during cyclic compression. (see Fig. 2.3D). Cyclic compression (2-hr compression/2-hr release) was applied (bottom trace) to 12 cartilage disks in parallel and consisted of exponential compression and release waveforms. A representative load measurement (top trace), taken during a biosynthesis experiment, is shown.

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Figure 2.7: Loading during oscillatory compression. (see Fig. 2.3E). Oscillatory compression was applied to 12 cartilage disks in parallel and consisted of a 2.5% amplitude sinusoidal displacement (bottom traces) superimposed on a static compression to 1.0 mm. Representative loads (top traces) measured during biosynthesis experiments at (A) $f = 0.001$ Hz and (B) $f = 0.1$ Hz are shown. Total harmonic distortion was $<0.2\%$ at 0.001 Hz and $3.1\%$ at 0.1 Hz.
Figure 2.8: Oscillatory compression mechanics during biosynthesis experiments. (see Figs. 2.3E, 2.7, and 2.15, and Table 2.1). Data (mean, n = 1–4 at each frequency) is from experiments where a 2.5% amplitude sinusoidal displacement was superimposed on a static compression to 1 mm and applied to 12 cartilage disks in parallel. (A) Load was monitored and the static (x) and fundamental (first harmonic) components of dynamic (▲) load are plotted as a function of frequency. The equivalent stress, computed as load normalized to unloaded cross-sectional area is indicated on the right ordinate. (B) Dynamic stiffness amplitude (■) and phase angle (○); amplitude is reported as the fundamental load component normalized to the unloaded cross-sectional area of 12 disks and to the computed dynamic strain amplitude (corrected for chamber and load cell compliance).

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four- to eight-fold between 0.0001 and 1.0 Hz. The dynamic load amplitudes were high enough using this protocol, especially at the higher compression frequencies and amplitudes, that a significant portion of the applied displacement was taken up by the chamber and load cell. Therefore in these experiments (Fig. 2.3E), the amplitude of cartilage compression was computed (Table 2.1).

Fig. 2.8B shows the calculated dynamic stiffness magnitude and phase angle: dynamic stiffness was defined as the amplitude of the fundamental frequency component of the Fourier-decomposed load signal divided by the total (unloaded) cross-sectional area of the 12 disks, and by the computed cartilage strain amplitude. This frequency response is qualitatively consistent with certain trends predicted by a linear biphasic model [4] in the sinusoidal steady state: the dynamic stiffness amplitude varied with frequency in a sigmoidal manner, from $\sim$3 MPa at 0.0001 Hz to a plateau of $\sim$37 MPa at 0.1–1.0 Hz; the phase angle between load and displacement showed a peak of 54° at 0.001 Hz, and decreased to 8° at 0.1–1.0 Hz. The frequency of peak phase is theoretically of order $1/\tau_m$, where $\tau_m = a^2/H_\alpha k$ is the characteristic unconfined compression stress relaxation time constant [4] ($a$ is the disk radius, $H_\alpha$ is the equilibrium confined compression modulus, and $k$ is the hydraulic permeability). For $a = 1.5$ mm, $H_\alpha \sim 0.5$ MPa, and $k \sim 2 \times 10^{-15}$ m$^2$/Pa·s, the characteristic frequency $\sim 1/\tau_m \sim 0.001$ Hz. However, we had limited success in quantitatively fitting the measured frequency response to the linear theory [4] (analysis not shown), as have others, who used ramp-plateau displacements [17]. Possible factors not included in the model [4,17] are adhesion at the cartilage/platen interface [4,17,111], nonlinear permeability [70,88], solid phase viscoelastic effects [97], and anisotropy [89,111].
2.4.2 Control Studies of $^3$H-Proline and $^{35}$S-Sulfate Incorporation: Kinetics and Distribution

The dependence of $^3$H-proline and $^{35}$S-sulfate incorporation on viable cells was ascertained. Freeze-drying cartilage disks (to lyse the chondrocytes) before radiolabeling caused a >99% reduction of both $^3$H-proline and $^{35}$S-sulfate incorporation. Inhibition of protein synthesis with cycloheximide for 1.5 h before and during 8-h radiolabeling allowed further characterization of the distribution of incorporated radiolabel. Cycloheximide treatment inhibited incorporation of $^3$H-proline and $^{35}$S-sulfate into macromolecules by 96.1 ± 0.4% and 97.5 ± 0.2%, respectively (each, n = 4). The residual incorporation of radiolabels into macromolecules is consistent with the small amount of cycloheximide-insensitive protein synthesis attributable to mitochondria [79] and a sizable preformed pool of intracellular core protein ($t_{1/2}$ ~1 h [79,109]) available for attachment of sulfated GAG.

Radiolabel uptake by chondrocytes reflects a combination of processes including diffusion of radiolabel to the cell surface, transport across the cell membrane, metabolic conversion to high-energy precursors (i.e., proline to prolyl-tRNA and sulfate to 3'-phosphoadenosine 5'-phosphosulfate), mixing with endogenous precursor pools, and incorporation into protein or GAG. Since previous work indicated compression could induce a rapid biosynthetic response [49,157], we characterized the form of the incorporated radiolabel taken up during 1–8-h isotopic labeling. In this way, biosynthetic responses to compression could be interpreted not only as general effects on total isotope incorporation, but also as likely effects on the specific processes described above.

Fig. 2.9A shows the distribution of $^3$H-proline incorporated into low-MW and macromolecular components during 1–8-h radiolabeling. The appearance of macromolecular $^3$H-cpm corresponded to a $t_{1/2}$ of 65 min. The possibility that a portion of the macromolecular $^3$H-cpm in SDS extracts was in the form of $^3$H-prolyl-tRNA was examined. No macromolecular $^3$H-cpm shifted to the low-MW
Figure 2.9: Distribution of $^3$H-proline and $^{35}$S-sulfate incorporated during 1–8-h isotopic labeling of free-swelling cartilage disks. Total (×) radiolabel, (A) $^3$H-cpm and (B) $^{35}$S-cpm, incorporated into cartilage disks was separated into low-MW (■) and macromolecular (●) components on Sephadex G25. Details of cartilage extraction and fractionation are given in Materials section. All points are mean±SEM (n = 6). Macromolecular cpm were fit to first-order kinetic model [83] to determine time to half maximal (linear) incorporation ($t_{1/2}$).

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fraction after further alkali treatment sufficient to completely deacylate tRNA [79] (data not shown). Thus, the SDS-extraction procedure (15 min at pH 8.5 and 100°C) appeared to degrade prolyl-tRNA, consistent with the macromolecular $^3$H- cpm being exclusively in protein. The low-MW $^3$H-cpm would likely be composed of free $^3$H-proline and $^3$H-biosynthetic intermediates, as well as $^3$H-peptides generated from rapidly degraded $^3$H-proteins. Indeed, incorporation of $^3$H-cpm into low-MW components was relatively insensitive to cycloheximide, compared to the almost complete inhibition of protein synthesis; cycloheximide-treated cartilage was still able to incorporate $^3$H-proline into low-MW $^3$H-components at 66.0 ± 3.2% (n = 4) of low-MW $^3$H-control levels. Thus, incorporation of $^3$H-proline during short 1-h radiolabeling predominantly reflected cellular uptake and early biosynthetic processing of $^3$H-proline.

Fig. 2.9B shows the distribution of $^{35}$S-sulfate incorporated into low-MW and macromolecular components during 1–8-h radiolabeling. The incorporated $^{35}$S-sulfate was >96% in macromolecules, even for the 1 h radiolabel duration. The rate of $^{35}$S-cpm incorporation approached linearity even during the first hour of radiolabel ($t_{1/2} = 15$ min). This is consistent with the rapid incorporation of $^{35}$S-sulfate into secreted PG (>75% during 10 min of chase in cell cultures) [81,109]. The 15 min $t_{1/2}$ is slightly longer than the 5 min delay to linear incorporation into finely sliced cartilage explants [62], and may reflect the diffusion time of $^{35}$S-sulfate into our 1 mm thick cartilage disks [102]. Thus essentially all incorporated $^{35}$S was in the form of macromolecules.

The possibility of macromolecular degradation during the SDS extraction procedure was investigated. Cartilage disks, largely free of low-MW $^3$H-components, were prepared for extraction by 8-h radiolabeling followed by 24 h of chase. The chase allowed low-MW $^3$H-protein precursors to be processed into proteins and $^3$H-peptides (products of rapidly turned over proteins) to diffuse out from the cartilage and into the media. Of the $^3$H-cpm incorporated during 8-h radiolabeling,
21.4 ± 0.4% (n = 4) was released during this chase and only 2.2 ± 0.2% remained in the disks as SDS-extractable low-MW \(^3\)H-molecules, consistent with minimal degradation of macromolecules during SDS treatment. Similarly, of the \(^35\)S-cpm incorporated, 3.2 ± 0.1% was released during chase and <1% remained in disks as SDS-extractable low-MW \(^35\)S-molecules.

2.4.3 Quantitation of Cartilage Constituents and Biosynthetic Rates

In a group of control disks maintained in culture for 2–6 days, dry weight (dw) was 2.03 ± 0.25 mg/disk (mean ± SD, n = 30), wet weight (ww) was 12.19 ± 1.27 mg/disk, dw/ww was 16.7 ± 1.9%, GAG content was 0.679 ± 0.071 mg/disk, GAG/dw was 33.9 ± 4.7%, and GAG/ww was 5.6 ± 0.6%.

Within each compression experiment, the GAG and DNA contents of compressed and control groups were not significantly different (p>0.2). For all animals and experiments, the GAG content ranged from a low of 517 ± 35 to a high of 752 ± 16 μg/disk (mean ± SD, n = 12) and averaged 644 ± 64 μg/disk (mean ± SD) in 28 control sets (1.25 or 1.00 mm control groups in protocols D and E). The DNA content ranged from 4.44 ± 1.04 to 7.19 ± 1.49 μg/disk (mean ± SD, n = 12) and averaged 6.34 ± 0.78 μg/disk (mean ± SD) in 12 control sets. Normalization of radiolabel incorporation to DNA content did not alter the resulting trends (where DNA content was measured).

Assuming identical intracellular and extracellular specific activities, the rate of sulfate incorporation ranged from 0.99 ± 0.25 to 2.88 ± 0.45 nmol sulfate/disk·h (mean ± SD, n = 12) and averaged 1.53 ± 0.54 nmol sulfate/disk·h (mean ± SD) in 28 control sets, while the rate of proline incorporation ranged from 0.43 ± 0.09 to 1.30 ± 0.09 nmol proline/disk·h and averaged 0.69 ± 0.24 nmol proline/disk·h. Assuming the wet weight values above, the average rates of sulfate and proline incorporation were 0.13 and 0.06 nmol/mg ww·h, respectively. Assuming one sulfate molecule per GAG disaccharide, this sulfate incorporation rate corresponds to a
synthesis rate of 18 μg GAG/day or 2.9% of the total GAG/day. This sulfate incorporation rate compares well with rates in cartilage explants from newborn calves (computed to be 0.06–0.12 nmol/mg ww·h) [62].

In three separate control experiments, GAG content and the rates of ³⁵S-sulfate and ³H-proline incorporation were stable or became slightly elevated (0–25%) over seven days of culture (data not shown), consistent with previous findings of steady state PG metabolism by adult bovine cartilage explants in 10% FBS [57] and GAG synthesis by newborn calf cartilage explants in 20% FBS [62]. The ~30% variation in GAG synthesis by disks from a single animal followed a regional variation over the joint surface (data not shown) consistent with the topographical distribution of GAG [179]. Also, the spectrum of proteins synthesized by our cartilage disks over days 0–7 of culture has been found (by SDS extraction and gel electrophoresis) to be qualitatively constant [95].

2.4.4 Biosynthetic Response To Compression

12-h Static Compression. A 12-h static compression caused a dose-dependent decrease in ³H-proline and ³⁵S-sulfate incorporation (Fig. 2.10). There was no significant difference in incorporation into disks freely swelling in 24-well culture dishes and disks compressed to 1.25–1.00 mm (p>0.15). Thus, the compression chambers appeared biologically inert, and occlusion at the cartilage-platen interfaces did not appear to restrict nutrient supply at minimal compressions. At compression to 0.75 mm, ³H-proline and ³⁵S-sulfate incorporation were down, −16.6 ± 3.8% and −21.6 ± 5.5%, respectively (both p<0.001), compared to disks compressed to 1.00 mm (the original cut thickness). This trend is consistent with results from previous studies of mechanical and osmotic compression of articular and epiphyseal cartilage where loading was for 4–12 h [10,48,75,173].

Static 12-h compression to 0.5 mm affected the distribution of ³H-proline incorporated into macromolecular and low-MW components. Macromolecular ³H
Figure 2.10: Radiolabel incorporation during 12-hr static compression. (see Fig. 2.3A). $^3$H-proline (■) and $^{35}$S-sulfate (○) incorporation are expressed relative to incorporation into disks held at 1 mm (the original explant thickness); free-swelling controls were incubated in 24-well culture dishes. All points are mean±SEM (n = 9–12).
accounted for 76.1 ± 2.2% of the total $^3$H-cpm in 1.25 mm controls but only
53.8 ± 5.4% in compressed samples (n = 4, p<0.05). Low-MW $^3$H-cpm in
compressed samples was only 73.6 ± 11.1% of the low-MW $^3$H-cpm in controls (n = 4,
p = 0.17). Thus, static compression appeared to slow the uptake and conversion of
$^3$H-proline into macromolecules. On the other hand, $^{35}$S-sulfate incorporation was
>98% into macromolecules in both compressed and control disks.

**Incorporation Kinetics after Step Compression and Step Release.**
The kinetics of the decrease in incorporation following a step compression to 0.5 mm
is shown in Fig. 2.11. Both $^3$H-proline and $^{35}$S-sulfate incorporation were rapidly
inhibited to low, steady state rates within the first few hours of compression. The
nonlinearity of radiolabel incorporation in both control and compressed disks may
be related to the radiolabel equilibration time (~1 h [102]) in the unconfined com-
pression configuration. When the inhibitory effect (Fig. 2.12A) was fit to a sim-
ple exponential decay, $(1 - C) + Ce^{-t/t_i}$, $^3$H-proline incorporation was inhibited
with $t_i = 16$ min whereas $^{35}$S-sulfate incorporation was affected more slowly with
$t_i = 40$ min. This is in general agreement with a previous study, in which compres-
sion inhibited incorporation of $^3$H-proline before $^{35}$S-sulfate [49].

After releasing a 2-h compression to 0.50 mm, $^3$H-proline incorporation
rapidly rebounded to $+90.7 ± 8.0\%$ above control levels at 1.5–2.5 h after release
(Fig. 2.12A). By 5.5–6.5 h, $^3$H-proline incorporation was only slightly elevated above
control levels ($+15.8 ± 4.0\%$, p<0.025). Similar trends in $^3$H-proline incorporation
were seen after release of a lesser 2-h compression to 1.00 mm (Fig. 2.12B). The
rebound in $^3$H-proline incorporation is consistent with a previous report for epi-
physeal cartilage [49], in which 2- or 12-h loading (0.5 MPa) and release caused a
rebound in $^3$H-proline incorporation that peaked at 0–2 h and decayed to control
levels by 6 h. The magnitude of this effect and its detection during a 1-h labeling
time (see Fig. 2.9A) suggest that compression-release may alter the rate of proline
transport into the cell and hence the incorporation into low-MW $^3$H-species. This is

*Section 2.4*
Figure 2.11: Kinetics of radiolabel incorporation during compression to 0.5 mm. (see Fig. 2.3A). (A) $^3$H-proline and (B) $^{35}$S-sulfate incorporation into control (●), and compressed (■) disks during 1–8-h incubation. All points are mean±SEM (n = 10). Compressed/control ratios are summarized in Fig. 2.12.
Figure 2.12: Kinetics of radiolabel incorporation during compression-release. (see Fig. 2.3A, B). (A) $^3$H-proline (☐) and $^{35}$S-sulfate (○) incorporation into disks compressed to 0.5 mm and free-swelling controls were measured during the entire duration of compression and are expressed as compressed/control±SEM (n = 10). After release of a 2-h compression to 0.5 mm, incorporation of $^3$H-proline (■) and $^{35}$S-sulfate (●) were assessed during 1-h pulse labels at various times after release (n = 12). (B) Incorporation after release was examined as in A, except 2-h compression was to only 1.0 mm.
further supported by the failure of cycloheximide treatment beginning 1.5 h before compression to abolish this stimulation of $^3$H-proline uptake by compression-release (data not shown).

The effect of 2-h compression and release on $^{35}$S-sulfate incorporation depended greatly on the amplitude of compression (Fig. 2.12A, B). Compression to 0.50 mm caused an initial depression in $^{35}$S-sulfate incorporation, followed by a gradual return to non-compressed control levels within 1–3 h and a slight stimulation at 5–6 h (+13.5 ± 3.6%, p<0.025); a lesser compression to 1.00 mm did not affect $^{35}$S-sulfate incorporation over the following 6 h. When $^{35}$S-sulfate incorporation has been measured at specific times after compression and release, a variety of responses have been reported. When calf epiphyseal cartilage disks were loaded for 0.5 or 2 h at 0.5 MPa (~50% compression) and then released, $^{35}$S-sulfate incorporation was unaffected; however, 12 h of loading and then release caused an inhibition up to 6 h later [48]. When adult human articular cartilage was osmotically compressed by ~26% for 4 h and then released, $^{35}$S-sulfate incorporation was at control levels ~1.5–6 h later [10,173]. When calf cartilage slices were compressed (~3 MPa) for 24–48 h, $^{35}$S-sulfate incorporation rebounded 25–32% over control levels during the next two days [75]. Finally, 2 h of extremely low loads (0.001–0.01 MPa) caused a 50–70% depression in $^{35}$S-sulfate incorporation during the 2 h following release [132].

Thus, our results are additional evidence that release of a compression is not simply followed by a return of biological activity to control levels, but can act as a stimulatory signal with prolonged and time-dependent cellular effects. These results further suggested that appropriately timed compression cycles may have a net cumulative effect on synthesis. Therefore, the effect of a variety of longer-term dynamic compression protocols were examined.

Repetitive and Cyclic Compression. The possibility that the marked stimulation of $^3$H-proline and mild stimulation of $^{35}$S-sulfate incorporation after
a single compression and release might be enhanced or reduced after repetitive compression-release cycles was investigated. Fig. 2.13 shows the effect of multiple 2-h compressions to 0.5 mm (at 8-h intervals) on incorporation during 1.5–5.5 h after release. $^3$H-proline incorporation was significantly increased (+37.8 to +51.4%) above control levels after 1–3 compression-release cycles. $^{35}$S-sulfate incorporation appeared to become slightly increased with successive compression-release cycles—from $-6.6 \pm 10.1\%$ below control levels after 1 cycle to $+18.2 \pm 9.1\%$ above controls after 3 cycles ($p<0.1$). The effects of a single compression to 0.5 mm and release on $^3$H-proline and $^{35}$S-sulfate incorporation during the 4-h radiolabeling period here were consistent with the 1-h pulse labels (compare Fig. 2.12A at $t = 3–7$ h and Fig. 2.13).

Fig. 2.14 shows the net effect of 23-h cyclic compression (2 h on/2 h off) on radiolabel uptake during the last 8 h of compression. Cyclic compression between 1.25 and 0.50 mm resulted in a depression ($-59.1 \pm 9.0\%$) in $^{35}$S-sulfate incorporation but no significant change ($+15.9 \pm 10.0\%$) in $^3$H-proline incorporation. Cyclic compression to 0.75 mm resulted in no significant change in $^{35}$S-sulfate incorporation ($-4.4 \pm 7.0\%$), but a significant increase in $^3$H-proline incorporation ($+21.2 \pm 5.7\%$, $p<0.025$). At compression to $\sim 0.88–1.00$ mm, both $^3$H-proline and $^{35}$S-sulfate incorporation were highest, elevated above controls by 31–34% and 17–38%, respectively.

These amplitude-dependent effects on $^3$H-proline incorporation were also consistent with the kinetic response to a single compression and release. During 2-h compression to 0.5 mm and then 2-h release (Fig. 2.12A, $t = 0–4$ h), net $^3$H-proline incorporation was $\sim$control levels whereas net $^{35}$S-sulfate incorporation was diminished; while compression to 1.0 mm had little effect on $^3$H-proline incorporation (Fig. 2.10), release of a compression to 1.0 mm caused a sizable enhancement of $^3$H-proline incorporation in the following 2 h (Fig. 2.12B, $t = 2–4$ h). The slight and delayed increase in $^{35}$S-sulfate incorporation (Fig. 2.12A, $t = 8$ h) is consistent with
Figure 2.13: Effect of repetitive (1–3) compression-release on radiolabel incorporation. (see Fig. 2.3C). Control disks were free swelling (never compressed); experimental disks were compressed to 0.5 mm (2 h) and then released (6 h) for 1–3 cycles. Incorporation of $^3$H-proline (■) and $^{35}$S-sulfate (●) during 1.5–5.5 h after 1$^{st}$, 2$^{nd}$, or 3$^{rd}$ release are expressed as compressed/control±SEM (n = 12). * = p < 0.05.
Figure 2.14: Effect of cyclic compression/release on radiolabel incorporation. (see Fig. 2.3D). Control disks were statically compressed to 1.25 mm; experimental disks were cyclically compressed between 1.25 and 0.5–1.13 mm. Incorporation of \(^3\)H-proline (■) and \(^35\)S-sulfate (○) are shown in the table (inset) and graphed as cyclic/static±SEM (n = 12 disks per experiment). Multiple points at 1 mm represent replicate experiments on different animals. * = p < 0.05; ** = p < 0.01.
GAG synthesis being dependent on and limited by core protein, which may exist as a rather large preformed pool with a relatively long turnover time ($t_{1/2} \sim 1$ h) [79,109].

Oscillatory Compression. The effect of 23-h oscillatory compression on radiolabel uptake during the last 8 h is shown in Table 2.1; these results are graphically summarized in Fig. 2.15 over three frequency regimes that were suggested by the mechanical characterization (Fig. 2.8B). At 0.0001–0.001 Hz, compressive strain amplitudes up to 4% had little effect on $^3$H-proline and $^{35}$S-sulfate incorporation. At 0.01 Hz, compressions of 1.1–4.5% caused a $\sim 40\%$ increase in $^3$H-proline incorporation and a $\sim 30\%$ increase in $^{35}$S-sulfate incorporation while a lesser compression had no effect. Similarly, at 0.1–1 Hz, oscillatory strains of $\geq 0.7\%$ stimulated $^3$H-proline and $^{35}$S-sulfate incorporation by $\sim 20–40\%$. A previous study found that a 0.2 Hz 5.5% stretch of high-density chondrocyte cultures stimulated $^{35}$S-sulfate incorporation 41%, but did not affect glycine incorporation [31].

2.5 GENERAL DISCUSSION

The data in this study show that dynamic compression of calf cartilage disks in the radially-unconfined configuration can (1) stimulate or inhibit biosynthesis depending on the frequency and amplitude of compression, and (2) induce different flow and compression behavior, as inferred by dynamic stiffness measurements, that is characteristic of two distinct frequency regimes ($f \leq \frac{1}{t_{\alpha}} \sim 0.001$ Hz and $f > \frac{1}{t_{\alpha}}$). The biosynthetic responses are summarized here within the framework of these two frequency regimes in order to show the consistency of biosynthetic effects elicited by the different compression protocols and to consider specific physical phenomena as potential regulators of chondrocyte biosynthesis.

The frequency dependence of dynamic stiffness measurements (Fig. 2.8B) and theory [4] suggests the following qualitative physical interpretation of our dynamic compression protocols. At all frequencies, there is deformation of
Table 2.1: Effect of oscillatory compression on $^3$H-proline and $^{35}$S-sulfate incorporation (see Fig. 2.3E).

<table>
<thead>
<tr>
<th>Frequency [Hz]</th>
<th>Strain [%]</th>
<th>Oscillatory: cpm×10$^{-3}$</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$^3$H-proline $^{35}$S-sulfate</td>
<td>$^3$H-proline $^{35}$S-sulfate</td>
<td></td>
</tr>
<tr>
<td>70.1± 4.1</td>
<td>87.1± 7.1</td>
<td>0.0001</td>
<td>2.1</td>
<td>87.2± 4.7*</td>
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<tr>
<td>141.6± 6.1</td>
<td>142.8± 6.5</td>
<td>0.001</td>
<td>1.0</td>
<td>122.1± 4.6*</td>
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<tr>
<td>68.4± 2.9</td>
<td>48.9± 2.5</td>
<td>0.001</td>
<td>1.9</td>
<td>66.9± 4.8</td>
</tr>
<tr>
<td>74.9± 5.2</td>
<td>31.0± 1.4</td>
<td>0.001</td>
<td>1.9</td>
<td>63.8± 3.0</td>
</tr>
<tr>
<td>80.4± 6.7</td>
<td>64.9± 5.0</td>
<td>0.001</td>
<td>2.0</td>
<td>77.7± 4.0</td>
</tr>
<tr>
<td>51.4± 3.3</td>
<td>27.3± 2.0</td>
<td>0.001</td>
<td>2.0</td>
<td>53.4± 2.9</td>
</tr>
<tr>
<td>127.1± 4.6</td>
<td>107.1± 3.9</td>
<td>0.001</td>
<td>4.2</td>
<td>133.1± 7.3</td>
</tr>
<tr>
<td>92.7± 5.6</td>
<td>78.9± 4.5</td>
<td>0.01</td>
<td>0.6</td>
<td>77.3± 5.9</td>
</tr>
<tr>
<td>62.7± 3.5</td>
<td>80.7± 3.8</td>
<td>0.01</td>
<td>1.1</td>
<td>88.2± 4.4**</td>
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<tr>
<td>52.4± 2.0</td>
<td>56.8± 3.4</td>
<td>0.01</td>
<td>2.2</td>
<td>72.8± 4.9**</td>
</tr>
<tr>
<td>51.1± 2.1</td>
<td>54.2± 3.2</td>
<td>0.01</td>
<td>4.5</td>
<td>71.2± 3.0**</td>
</tr>
<tr>
<td>81.3± 4.9</td>
<td>123.5± 8.1</td>
<td>0.1</td>
<td>0.7</td>
<td>107.0± 6.6**</td>
</tr>
<tr>
<td>101.3± 4.7</td>
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<td>4.2</td>
<td>115.7± 5.1</td>
</tr>
<tr>
<td>67.3± 5.4</td>
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<td>0.7</td>
<td>86.7± 4.3**</td>
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<tr>
<td>49.3± 3.6</td>
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<td>0.6</td>
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<tr>
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<td>94.5± 6.1</td>
<td>1.0</td>
<td>1.1</td>
<td>71.1± 3.3**</td>
</tr>
</tbody>
</table>

Control disks were statically compressed to 1 mm. Experimental disks were compressed to 1 mm with an additional superimposed oscillation; the magnitude of oscillation is expressed as % peak strain and accounts for the compliance of the chamber and load cell. All cpm values are mean ± SEM (n=12 or *n=10) per cartilage disk; *p < 0.05, **p < 0.01. Oscillatory/static ratios are graphically summarized in Fig. 2.15.

Section 2.5
Figure 2.15: Effect of oscillatory compression on radiolabel incorporation. (see Fig. 2.3E and Table 2.1). Control disks were statically compressed to 1 mm; experimental disks were compressed to 1 mm with a superimposed oscillatory strain of up to ~5% at frequencies of ≤0.001 Hz (A, B), 0.01 Hz (C, D), and ≥0.1 Hz (E, F). Incorporation of $^{3}$H-proline (■) (A, C, E) and $^{35}$S-sulfate (○) (B, D, F) are expressed as oscillatory/static±SEM (n = 10–12 disks per experiment). Multiple points at the same strain represent separate experiments on different animals. * = p < 0.05; ** = p < 0.01.
chondrocytes and matrix (although the details would vary with frequency). Low frequency \((f \leq 0.001 \text{ Hz})\) compressions included repetitive (2 h on/6 h off), cyclic (2 h on/2 h off), and oscillatory \((f \leq 0.001 \text{ Hz})\) protocols (Fig. 2.3C–E). This regime is consistent with slow fluid exudation from the cartilage disk, accompanied by minimal intratissue pressurization, fluid velocities, streaming potentials, and radial stress. There is ample time for alterations in physicochemical parameters (e.g., decreased pH). In contrast, during higher frequency \((f = 0.01–1 \text{ Hz})\) oscillatory compression (Fig. 2.3E), there is less time for fluid motion relative to the solid extracellular matrix. Hydrostatic pressure increases in the interior of the disk where cartilage mechanical behavior approaches that of an elastic solid near the periphery, however, relatively high radially-directed pressure gradients are induced [4] with correspondingly high fluid velocities, streaming potentials and currents [43].

Perhaps the most exciting result was that higher frequency dynamic compression at 0.01–1 Hz evoked a threshold-dependent stimulation of biosynthesis (Fig. 2.15). Oscillatory compression amplitudes of ~1–5% stimulated incorporation of both \(^3\text{H}\)-proline and \(^35\text{S}\)-sulfate by ~20–40%. Here, the low amplitude displacement control protocol ensured that negligible physicochemical changes could occur, since tissue volume remained almost constant. It is interesting that continuous passive motion protocols in vivo have been conducted with a frequency in this regime \((f = 0.025 \text{ Hz}, \text{ a cycle period of } 40 \text{ s})[124,164]\). In this higher frequency regime, cell deformation, hydrostatic pressure gradients, fluid flow, and streaming currents and potentials occur in a spatially complex profile; while radiolabel incorporation reflects an average effect over the entire cartilage disk. Future correlations of spatial variation in incorporation may help to identify which physical stimuli exert biological effects. Cell shape and cytoskeletal alterations can affect the metabolic activity of chondrocytes [13,14]. Possible mechanoreceptors include cilia [143] and the cytoskeleton [138].

**Lower frequency dynamic compression at \(\leq 0.001 \text{ Hz}\)** triggered biosynthetic
responses that were qualitatively predictable from and consistent with (A) the dosimetry of inhibition by static 12-h compression (Fig. 2.10) and (B) the kinetics of response to a single compression and release (Fig. 2.12). Low amplitudes (cycling between 1.25 and 1.13 mm, Fig. 2.14, or oscillations of <0.05 mm at f<0.001 Hz, Fig. 2.15) had negligible effects, consistent with the insensitivity of cartilage disks to low levels of static compression (1.0–1.2 mm, Fig. 2.10). Moderate amplitudes (cycling between 1.25 and 1.00–0.88 mm, Fig. 2.14) stimulated $^3$H-proline incorporation slightly more than $^{35}$S-sulfate incorporation; this follows from the sizable rebound in $^3$H-proline incorporation after a 2-h compression to 1 mm and release (Fig. 2.12B). High amplitudes (repetitive compression to 0.5 mm, Fig. 2.13 and cycling between 1.25 and 0.75–0.50 mm, Fig. 2.14) led to differential effects on $^3$H-proline and $^{35}$S-sulfate incorporation, consistent with the net effects of 2-h compression to 0.5 mm and 2-h release (Fig. 2.12A); increases in $^{35}$S-sulfate incorporation (Figs. 2.12 and 2.13) were moderate and delayed relative to the increases in $^3$H-proline incorporation. This would be expected if the increase in $^3$H-proline incorporation were largely due to an increase in amino acid uptake (Fig. 2.9) rather than protein synthesis. To determine whether compression-release affects protein synthesis would require extensive analysis of $^3$H-proline pools [129]. Moreover, PG core protein synthesis and general protein synthesis could be regulated by quite different mechanisms.

The physical mechanisms triggering these low frequency biosynthetic responses may be similar to the proposed physical mediators of static compression: decreased pH [48] or increased extracellular osmotic pressure [173]. In these studies [48,173], $^{35}$S-sulfate transport into compressed cartilage did not appear to limit $^{35}$S-sulfate incorporation rates. Our compression protocols (Fig. 2.3) ensured that $^3$H-proline and $^{35}$S-sulfate equilibration times were much shorter than the radiolabel duration. Thus, 1-h radiolabels to assess the kinetics of radiolabel uptake after compression-release were performed post-release in disks free swelling in cul-
ture dishes, whereas 8–12-h radiolabels to assess the longer term effects of static and dynamic compression were performed while disks were compressed between fluid-impermeable platens; the corresponding 90% radiolabel equilibration times are ~6–27 min and ~1 h, respectively [102]. In kinetic studies of a step compression, radiolabeling was initiated ~5 min before compression to allow isotope diffusion into the cartilage disks before the diffusional constraints of the compression configuration were imposed.

Alternatively, compression may modulate cartilage biosynthesis by affecting the transport of macromolecules. Static compression would cause a decrease in tissue hydration (with exudation of fluid) and a diminution of the effective matrix pore size [184]; such a relationship between macromolecular transport and matrix hydration has been quantified in synthetic isotropic polyelectrolyte hydrogels [193]. Affected molecules in the culture media might include, for example, insulin-like growth factor I, which is a potent stimulator of GAG synthesis, as well as its serum carrier protein [105]. In contrast, cyclic compression could increase the convective transport of macromolecules [100] and thereby facilitate access of such stimulatory factors to the chondrocytes.

Other studies have suggested that the concentration of extracellular PG may play a regulatory role in chondrocyte biosynthesis. Depletion of GAG from bovine cartilage explants has been associated with a stimulation of $^{35}$S-sulfate incorporation [169]. However in the present study, neither the inhibitory nor the stimulatory effects of static or dynamic compression was accompanied by detectable changes in total GAG content. In view of the modest rate of GAG synthesis in control disks (2.9%/day), little total accumulation of GAG would be expected during ~24-h compression experiments.

In summary, the in vitro compression of calf cartilage disks via specific waveforms, frequencies, and amplitudes of physiological magnitude can enhance or depress $^{35}$S-sulfate incorporation into GAG and $^3$H-proline uptake into chondrocytes.
and incorporation into proteins. The frequency and amplitude dependence and kinetics of biosynthetic response suggest several possible mechanisms of physical signal transduction and cellular biosynthetic regulation. The cartilage compression-culture methods described here may be used to further probe these biophysical mechanisms as well as the balance between anabolic and catabolic [162] processes.

Finally, it may be of eventual clinical relevance that the in vitro findings here are in general agreement with in vivo studies of joint loading and motion: immobilization or reduced dynamic loading led to a decrease in PG synthesis and a loss of GAG [2,27,33,128,134,154]; increased dynamic loading led to an increase in PG synthesis and content [27,85,135,191]; and abnormal loading associated with anterior cruciate ligament transection led to an increase in collagen [36] and PG metabolism [25,26,168]. Since continuous passive motion appears to stimulate cartilage growth and remodeling in vivo [124,164], studies involving cartilage compression in vitro could potentially serve as a precise means of testing and optimizing therapeutic strategies to combat diseases of cartilage.

2.6 ACKNOWLEDGEMENTS

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Chapter III

Effects of Compression and pH on the Hyaluronate Binding Properties of Newly Synthesized Proteoglycans in Cartilage Explants

3.1 ABSTRACT

* The effects of tissue compression and altered medium pH on the hyaluronate binding properties of newly synthesized proteoglycans in calf cartilage explants were examined. Pulse-chase experiments showed that $^{35}$S-proteoglycans in free-swelling cartilage disks in pH 7.45 medium were converted from low hyaluronate binding affinity to high affinity with a $t_{1/2}$ of about 4.5 h. Static compression during chase (in pH 7.45 medium) slowed the conversion, as did incubation in acidic medium (without compression). Both effects were dose-dependent. For example, the $t_{1/2}$ for conversion was increased to 9 h by either (1) compression from a thickness of 1.25 mm to 0.50 mm or (2) medium acidification from pH 7.45 to 6.99. Oscillatory compression of 2% amplitude at 0.001, 0.01, or 0.1 cycles/sec during chase did not, however, affect the conversion. Changes in the hyaluronate binding affinity of $^{35}$S-proteoglycans in these experiments were accompanied by no marked change in the high percentage (~80%) of molecules which could form aggregates with excess hyaluronate and link protein. Since static tissue compression would result in an increased matrix proteoglycan concentration and thereby a lower intratissue pH [Gray, Pizzanelli, Grodzinsky et al. (1988) J. Orthop. Res. 6, 777-792], it seems likely that matrix pH may influence proteoglycan aggregate assembly by an effect on the hyaluronate binding affinity of proteoglycan monomer. Such a pH mechanism might have a physiologic role, promoting proteoglycan deposition in regions of low proteoglycan concentration.

*Preliminary results have been described [155,160].
3.2 INTRODUCTION

The load-bearing properties of cartilage are largely dependent on the high density of matrix-affixed charge residing on the extracellular matrix proteoglycans [52,100,192]. The ionized carboxyl and sulfate groups of the glycosaminoglycan side chains endow cartilage with a large osmotic swelling pressure which is balanced by the tensile strength of the collagen framework [98]. Most cartilage proteoglycans (60–85%) appear to exist in aggregates, in which monomers are non-covalently bound to hyaluronate in an interaction stabilized by link protein [58,61,122]. Such aggregates are macromolecular complexes that can consist of ~30–100 proteoglycan monomers affixed to a ~1.3 μm long hyaluronate backbone [18,61]. The size of the aggregate effectively immobilizes proteoglycans within the collagenous meshwork, preventing monomers from diffusing out of the tissue [71,122]. In contrast, catabolized monomers which are unable to aggregate with hyaluronate are able to diffuse within the matrix and are preferentially released from thin cartilage slices in vitro [23,59].

The mechanism by which proteoglycan aggregates are assembled biosynthetically has been extensively studied in chondrocyte culture [80,81]. In studies of cartilage explants, it has been shown that monomers, once secreted from chondrocytes, appear to undergo a maturation process in the extracellular matrix whereby their binding affinity for hyaluronate increases [125]. This conversion process occurs in articular cartilage in vivo [170] and in purified proteoglycan solutions [172]. Within explant cultures, this process is dependent neither on live cells [8] nor on continued protein synthesis [106], can be accelerated by incubation without oxygen [82], and can be slowed or inhibited by low temperature [8,106]. Within solutions of purified proteoglycans, the conversion can be catalyzed by mild base (pH ≥ 7.4) [140] or by incubation of monomer in aggregate, but not by incubation with link protein alone [172]. Other studies have shown that the conversion can be blocked by N-ethyl maleimide [8], consistent with a rearrangement or formation of disulphide

Section 3.2
bonds.

The maturation of newly synthesized proteoglycans may be affected by the mechanical forces to which cartilage is naturally subjected. Mechanical forces in vivo are thought to influence the structural adaptation of cartilage, while abnormal forces predispose cartilage to degeneration and osteoarthritis [162]. In vitro, both static and dynamic compression of cartilage explants at physiologic magnitudes can alter proteoglycan metabolism [75,162]. Static compression of cartilage can inhibit proteoglycan biosynthesis [10,48,75,162,173], possibly through a physicochemical alteration of intratissue ion concentrations [48,188]. Compression increases the concentration of negatively charged glycosaminoglycans, leading to a decreased intratissue pH. Such an intratissue acidification can independently account for the observed inhibition of proteoglycan synthesis [48]. On the other hand, physiologic levels of dynamic compression, consisting of oscillatory displacements of ~1-5% at frequencies of 0.01-1 cycles/s, can stimulate synthesis [162]. The physical loading protocols in these studies are relevant to cartilage in vivo, where compression can be considered to be a combination of a time-average static component and a time-varying dynamic compressive component.

In the present study, we used pulse-chase methods to examine the effects of static compression and oscillatory compression on the acquisition of hyaluronate binding affinity by newly synthesized proteoglycans, as well as the role of altered intratissue pH in mediating the effects of static compression.

3.3 EXPERIMENTAL PROCEDURES

3.3.1 Materials

Materials for cartilage explant and culture and proteoglycan isolation and analysis were obtained as described previously [162,170]. In addition, sterile filtered 1.0 M NaOH and 1.0 M HCl were from Sigma, St. Louis, MO. Tissue-Tek embedding
solution (O.C.T. compound 4583) and Tissue-Tek II disposable specimen molds were from Miles Diagnostics, Elkhart, IN. Sepacryl S-1000 was from Pharmacia, Piscataway, NJ.

3.3.2 Cartilage Explant, Culture, and Radiolabeling

Cartilage disks, 1 mm thick × 3 mm diameter, were explanted from the femoropatellar grooves of 1–2 week old calves, and cultured in DMEM (44 mM NaHCO₃) with 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), 0.1 mM non-essential amino acids, an additional 0.4 mM L-proline, 20 μg/ml ascorbate and 10% FBS in a humidified 5% CO₂:95% air incubator at 37°C, as previously described [162]. Medium (pH 7.45) was changed daily, and on the first day, supplemented with 100 μg penicillin/ml and 100 U streptomycin/ml. All medium was prepared by temperature and CO₂ equilibration in the incubator for 1–2 h just before use. After 2–6 days of culture, disks swelled axially to a thickness of 1.25–1.4 mm and attained relatively steady state rates of biosynthesis [162]. Disks were then pulse radiolabeled for 1.5 h in medium including 100 μCi ³⁵S-sulfate/ml (1 ml/4 disks) before use in the various chase protocols, described below.

Static Compression. Pulse-labeled disks were washed three times (1 ml/disk) over a total of 0.5 h with culture medium (pH 7.45), and then placed in individual wells of compression chambers (0.8 ml medium/disk) where they were subjected to uniaxial, radially unconfined, static compression between two impermeable platens essentially as described previously [162]. Cartilage disks were compressed to a thickness of 1.25, 1.00, 0.75, or 0.50 mm. In such a configuration, stress relaxation (and concomitant fluid flow relative to the solid matrix) is 90% complete within ~15 minutes [162]; thus, mechanical equilibrium was attained rapidly during the 3–48 h of chase.

Oscillatory Compression. Pulse-labeled disks were washed as above, and then

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placed in a dynamic compression chamber (25 ml medium/12 disks) held within a mechanical spectrometer (Dynastat, IMASS, Hingham, MA), as described previously [162]. This apparatus allowed cartilage disks to be subjected to a time-varying uniaxial, radially-unconfined compression; the resulting load was continuously monitored and the static and dynamic compressive stress amplitudes were computed [162]. Control studies showed that injection of a 10% CO₂:90% air mixture into the chamber fixed the bicarbonate-buffered medium at pH~7.4. During the 8.5-h compression and chase period, disks were subjected to a static compression to a thickness of 1.00 mm and a superimposed 2% (0.02 mm peak) oscillatory strain. Oscillations were at frequencies of 0 (static control), 0.001, 0.01, or 0.1 cycles/s.

**pH Variation.** Pulse-labeled disks were washed and then compressed to a static thickness of 1.25 mm as above, except pH-adjusted medium was used during wash and chase. Medium with pH of 6.99–7.70 in the 5% CO₂ incubator was prepared from the above medium formulation by adding combinations of 1 M HCl, water, and 1 M NaOH in a total added volume of 2.24 ml/l medium such that 18.8, 11.2, or 0 mmol HCl/l medium or 22.4 mmol NaOH/l medium was added. Since medium pH was not significantly altered during the 24-h chase period (pH change ≤0.02 pH units), the pH of medium was routinely measured after chase using medium pooled from 4 disks with a Ross combination pH electrode (Orion Research, Boston, MA).

In all pulse-chase protocols, tissue incubations were either on a rotary shaker (Model 3520, Labline Instruments, Melrose Park, IL) at 60 rpm (during static compression) or with medium recirculation (during dynamic compression [162]) to minimize stagnant films. In each protocol, disks from adjacent anatomical locations within a single joint were evenly distributed among the different treatment groups, and each treatment group consisted of 4–6 disks. For a given chase duration in the static compression protocols, separate chambers were used to impose the different treatment conditions in order to allow samples to be tested simultaneously. In

*Section 3.3*
oscillatory compression protocols, a single dynamic chamber was used in sequential
12-h experiments for the treatment conditions.

After chase, disks were rinsed over 0.5 h with two changes (1 ml/disk)
of a phosphate buffered saline solution (137 mM NaCl, 2.7 mM KCl, 8.1 mM
Na₂HPO₄·7H₂O, 1.5 mM KH₂PO₄) at 4°C including protease inhibitors (0.1 M
6-aminohexanoic acid, 10 mM Na₂·EDTA, 10 mM benzamidine-HCl, 10 mM N-
ethylmaleimide, 1 mM phenylmethanesulfonyl chloride, 1 µM pepstatin, and 1 µM
leupeptin) [67] at pH 7.4 and stored at −20°C.

3.3.3 Extraction and Isolation of Proteoglycans from Cartilage Disks

Groups of 4–6 disks (~48–72 mg wet weight of cartilage) from each
pulse-chase condition were then frozen in Tissue-Tek embedding solution within
10×10×5 mm specimen molds and cryostat sectioned to 20 µm. Cartilage slices
were then extracted for 36–42 h at 4°C by addition of 4 M guanidinium-HCl, 50 mM
Na acetate, 10 mM HEPES, and protease inhibitors, pH 7.0. The tissue residue was
treated with 0.5 ml of 0.5 M NaOH for 1 day at 22°C to solubilize essentially all
remaining glycosaminoglycans, and then neutralized with an equal volume of 0.5 M
HCl. Control studies where slices were rinsed free of embedding solutions indicated
that the small volume (~0.2 ml) of embedding solution had no effect on subsequent
proteoglycan analysis procedures.

Cartilage extracts were filtered over glass wool, adjusted to a density of
1.45 g/ml with solid cesium chloride, centrifuged at 38,000 rev/min in a Beckman
50Ti rotor (rₑ=8.02 cm) for 72 h at 12°C, and sliced into four equal gradient
fractions † (D1, 1.56 g/ml; D2, 1.48 g/ml; D3, 1.43 g/ml; D4, 1.38 g/ml). D1
fractions were dialyzed exhaustively against 0.15 M Na acetate, pH 6.8 at 4°C, and
then stored at −20°C.

†The terms D1, D2, D3, and D4 refer to the fractions of highest to lowest buoyant density
obtained by subjecting cartilage extracts to cesium chloride density gradient centrifugation under
dissociative conditions and are based on the terminology of Heinegård [67].

Section 3.3
3.3.4 Analytical Procedures

Samples were assayed for proteoglycan as sulfated glycosaminoglycan by reaction with dimethylmethylene blue [40]. The concentration of hyaluronate in Healon was taken as given by the supplier (10 mg/ml). Radioactivity was determined by liquid scintillation counting after addition of 2 ml ScintiVerse BioHP; guanidinium-HCl samples were prepared to a volume of 0.5 ml by addition of 2 M guanidinium-HCl, 0.5 M Na acetate, pH 6.8, whereas Triton X-100 samples (~0.5 ml) were prepared by addition of 1 ml of 10% (w/v) sodium dodecyl sulfate. DNA was determined by reaction with Hoechst 33258 [28].

3.3.5 Proteoglycan Aggregation Experiments with Hyaluronate and Link Protein

Portions of D1 samples (containing 250 µg of proteoglycan and ~75,000 cpm of radioactivity in ~0.6 ml) were fractionated on Sephacryl S-1000 columns (1×45 cm) eluted at 9 ml/h (11.5 cm/h) in 0.5 M Na acetate, 0.1% (v/v) Triton X-100, 0.02% Na₂ azide, pH 6.8; fractions (0.6 ml) were collected and assayed for both proteoglycan and radioactivity. To determine the affinity of tissue proteoglycans and newly synthesized ³⁵S-proteoglycans for hyaluronate (see [172]), samples were mixed with 0.8% or 1.6% (w/w) Healon, allowed to aggregate for 2 h at 22°C, stored frozen at ~20°C, and subsequently analyzed by chromatography as above. To determine maximum aggregability, samples were mixed with 5% (w/w) Healon and 5% (w/w) link protein, adjusted to 4 M guanidinium-HCl, allowed to dissociate for 2 h at 4°C, dialyzed for 24 h against 0.15 M Na acetate, pH 6.8 at 4°C, stored frozen, and later analyzed by chromatography. The percentage of proteoglycans in aggregate was computed by considering all proteoglycans eluting earlier than the local minimum at elution volume, Vₑ, ~16 ml as aggregate, and all eluting between this minimum point and that near Vₑ ~28 ml as monomer. Recovery of proteoglycan and ³⁵S on chromatography was typically 90-100%. In some samples,
4 μg DNA and 0.04 μCi $^3$H$_2$O were added as markers for the void ($V_0$) and total column ($V_t$) volumes, respectively.

3.3.6 Analysis of Medium

Chase and wash solutions were analyzed for $^{35}$S-macromolecules by adjusting samples to a buffer of 2 M guanidinium-HCl, 0.5 M Na acetate, 1 mM Na$_2$ sulfate, pH 6.8 and fractionating 0.5 ml portions on a PD-10 column of Sephadex G-25 in the same buffer.

3.4 RESULTS

3.4.1 Radiolabeling and Isolation of Proteoglycans

Newly synthesized proteoglycans were pulse radiolabeled with $^{35}$S-sulfate and isolated after 0–48-h chase. Virtually all of the $^{35}$S-proteoglycans remained in the tissue during pulse and subsequent chase protocols; during 1.5-h radiolabeling and 24-h chase, <1% and <2%, respectively, of the $^{35}$S-macromolecules were released into the medium. In addition, a high proportion of proteoglycan extraction from the cartilage explants was achieved by cryostat sectioning disks to 20 μm before 4 M guanidinium extraction; under all compression and pH conditions, this procedure yielded a >93% extraction of both proteoglycans and $^{35}$S, consistent with yields from adult human articular cartilage [11].

From these extracts, proteoglycans were purified by cesium chloride equilibrium density gradient centrifugation. Under all compression and pH conditions, the distribution of proteoglycan and $^{35}$S in the gradient fractions was similar, with >90% of the proteoglycan and >93% of the $^{35}$S in the D1 fraction. Consistent with the slow rate of $^{35}$S loss during chase, the specific activity of D1 proteoglycans ($^{35}$S cpm/μg proteoglycan) was not significantly altered after 0–48 h of chase (typical standard deviation~15% within an experiment). Thus, the D1 proteoglycans...
represented the majority of the proteoglycan population within the tissue.

When D1 samples (without addition of hyaluronate) were analyzed on Sephacryl S-1000 (Fig. 3.1), the majority of both proteoglycans and $^{35}$S-proteoglycans eluted as a single peak ($K_{av} \sim 0.38$). Since these proteoglycan monomers were more included on Sephacryl S-1000 than on Sepharose CL-2B (peak $K_{av} \sim 0.28$, data not shown), fractionation on Sephacryl S-1000 allowed a more clear resolution of proteoglycan aggregates from monomers. Since only a small fraction of D1 samples (1–8% of tissue or $^{35}$S-proteoglycans) eluted as $V_0$ aggregates, the D1 proteoglycans appeared to be monomers predominantly free from hyaluronate and from self-aggregation, which can occur in the absence of detergent [172].

The small peak ($K_{av} \sim 0.85$) of dimethylmethylene blue reactive material, which eluted just ahead of the total column volume, has not been identified but may be free chondroitin or dermatan sulfate chains. A similarly eluting component from bovine cartilage D1 preparations has also been observed by uronic acid assay after fractionation on Sepharose CL-2B [82]. The possibility that this might be DNA fragments binding to the dimethylmethylene blue dye was excluded when specific analysis for DNA by Hoechst 33258 indicated only a peak at the $V_0$.

### 3.4.2 Hyaluronate Binding Properties of Proteoglycan Monomers

The affinity of D1 proteoglycans for hyaluronate was assessed by aggregation with limiting amounts of hyaluronate before chromatography on Sephacryl S-1000 in 0.5 M Na acetate, 0.1% Triton X-100, 0.02% Na$_2$ azide pH 6.8. When pulse and 3-h chase proteoglycan samples were mixed with 1.6% (w/w) hyaluronate (Figs. 3.2A, 2B, 3A, and 3B), tissue proteoglycans eluted with a high proportion as aggregate compared to newly synthesized $^{35}$S-proteoglycans showing that newly secreted monomers were mostly in the low affinity form. When cartilage was chased for increasing times in medium at normal pH while held at a swollen thickness of 1.25 mm, $^{35}$S-proteoglycans attained a markedly increased affinity for hyaluronate.
Figure 3.1: Size distribution on Sephacryl S-1000 of a D1 proteoglycan sample from cartilage disks labeled with 35S-sulfate. Free swelling disks were radiolabeled and then held at a swollen thickness of 1.25 mm during 3-h chase in pH 7.45 medium. Proteoglycans were extracted, D1 purified and fractionated on a Sephacryl S-1000 column internally calibrated with calf thymus DNA and 3H2O using an elution buffer of 0.5 M Na acetate, 0.1% Triton X-100, 0.02% Na2 azide, pH 6.8 (see Experimental section for details); fractions were assayed for proteoglycan (−) and 35S (+). The percentage of total proteoglycan and radiolabel are identified above and below, respectively, the indicated regions.

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Figure 3.2: Effect of static compression on the hyaluronate binding properties of proteoglycan monomers from cartilage disks at various times after radiolabeling with $^35$S-sulfate. Portions of proteoglycan monomer samples were reacted with 1.6% (w/w) hyaluronate and fractionated on Sephacryl S-1000; fractions were assayed for proteoglycan (−) and $^35$S (●). The elution profiles were from cartilage radiolabeled for 1.5 h and then held at a swollen thickness of 1.25 mm (A, C, E) or compressed to 0.5 mm (B, D, F) in pH 7.45 medium during a chase period of 3 h (A, B), 9 h (C, D), or 24 h (E, F). The ratio $R$ is the percentage of radiolabeled monomers eluting as aggregate divided by the percentage of total tissue monomers eluting as aggregate.

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Figure 3.3: Effect of medium pH on the hyaluronate binding properties of proteoglycan monomers from cartilage disks at various times after radiolabeling with \(^{35}\)S-sulfate. Portions of proteoglycan monomer samples were reacted with 1.6% (w/w) hyaluronate and fractionated on Sephacryl S-1000; fractions were assayed for proteoglycan (\(\cdot\)) and \(^{35}\)S (\(\circ\)). The elution profiles were from cartilage radiolabeled for 1.5 h and then bathed in medium at pH 6.99 (A, C, E) or pH 7.70 (B, D, F) and held at a swollen thickness of 1.25 mm during a chase period of 3 h(A, B), 9 h(C, D), or 24 h(E, F). The ratio \(R\) is the percentage of radiolabeled monomers eluting as aggregate divided by the percentage of total tissue monomers eluting as aggregate.

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(Fig. 3.2A, C, E) such that by 24 h, their affinity was similar to (or even slightly higher than) that of tissue proteoglycans. In all samples, the ability of tissue proteoglycans to interact with hyaluronate was essentially the same with 49-59% eluting as aggregate. Thus, the relative affinity of $^{35}$S-proteoglycans for hyaluronate, compared to that of tissue proteoglycans could be computed as a ratio, $R$, which was the percentage of radiolabeled monomers eluting as aggregate to the percentage of total tissue monomers eluting as aggregate [82].

**Static Compression**

Static compression during chase markedly slowed the acquisition of hyaluronate binding affinity by $^{35}$S-proteoglycans. With compression to 0.50 mm, the maximum compression tested (Fig. 3.2B, D, F), the chase time to reach half of the maximal affinity change was ~9 h, compared to about 4.5 h in control samples at 1.25 mm (Fig. 3.2A, C, E). This delay in conversion was also shown to be dependent on the degree of compression in a dose-dependent fashion such that a measurable delay was observed even at a minimal compression to 1.00 mm (Fig. 3.4A).

Samples compressed to 0.50 mm were fully converted to the high affinity form by 48 h of chase (data not shown), showing that compression delayed but did not prevent conversion. In addition, virtually identical trends were obtained whether chromatography was done with 0.8% or 1.6% (w/w) hyaluronate and whether samples were run on Sephacryl S-1000 or Sepharose CL-2B (data not shown).

**Medium pH**

To determine the effect of intratissue pH on $^{35}$S-proteoglycan affinity conversion, the effect of altered medium pH (holding cartilage thickness constant at 1.25 mm in the slightly swollen state) during chase was examined. Medium pH of 6.99, 7.24, 7.45, or 7.70 (SD ~ 0.02, n=3) altered the kinetics of affinity acquisition by $^{35}$S-proteoglycans for hyaluronate (compare Fig. 3.2A, C, E with Fig. 3.3A–F),

*Section 3.4*
Figure 3.4: Dose dependence of the effect of (A) static compression and (B) medium pH on the acquisition of hyaluronate binding properties by $^{35}\text{S}$-proteoglycan monomers in $^{35}\text{S}$-sulfate labeled cartilage disks. Cartilage was radiolabeled for 1.5 h and then, during 0–24 h chase, either (A) compressed to a thickness of 1.25–0.50 mm and bathed in medium at pH 7.45 or (B) bathed in medium at pH 6.99–7.70 and held at a swollen thickness of 1.25 mm. Portions of proteoglycan monomer samples were reacted with 1.6% (w/w) hyaluronate and fractionated on Sephacryl S-1000; the ratio ($\mathcal{R}$), the percentage of radiolabeled monomers eluting as aggregate divided by the percentage of total tissue monomers eluting as aggregate, was determined as in Figs. 3.2 and 3.3.

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as summarized in Fig. 3.4B. Medium at acidic pH (6.99 and 7.24) delayed the affinity acquisition relative to medium at normal pH (7.45) at all chase times (3, 9, and 24 h). In contrast, medium at basic pH (7.70) accelerated the affinity acquisition (Figs. 3.3B, D, F and 3.4B) such that as early as 9 h of chase, the affinity of $^{35}$S-proteoglycans for hyaluronate was similar to that of tissue proteoglycans for hyaluronate. The effect on affinity acquisition of medium acidification to pH 7.24 and 6.99 of cartilage disks at 1.25 mm was therefore very similar to the effect of static compression to 0.75 and 0.50 mm, respectively (Fig. 3.4A and B). In the two different experiments of Fig. 3.4A and B, $^{35}$S-proteoglycans in control disks at 1.25 mm and pH 7.45 showed virtually identical kinetics of affinity conversion.

Oscillatory Compression

Oscillatory compressions of 2% strain were superimposed on a static offset compression to 1.00 mm during 9-h chase. None of these compression protocols markedly altered the $^{35}$S-proteoglycan affinity conversion compared to the 1.00 mm static control condition where no oscillation was superimposed (Fig. 3.5). The degree of affinity conversion in the 1.00 mm static controls within this dynamic compression apparatus ($R \sim 0.76$) compared well to the conversion in samples treated similarly within the static compression apparatus ($R = 0.72$, Fig. 3.4).

3.4.3 Aggregability of Proteoglycan Monomers with Excess Hyaluronate and Link Protein

The maximum capacity of D1 proteoglycans to form aggregates was assessed by mixing portions with excess (5% w/w) hyaluronate and (5% w/w) link protein before chromatography. The high aggregability of both tissue and $^{35}$S-proteoglycans (range, 77–86% in 8 samples) was not markedly altered after different chase conditions (compression and pH) and chase durations (3–24 h). The chromatographic

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Figure 3.5: Effect of oscillatory compression during 9-h chase of \( ^{35} \text{S}-\text{sulfate} \) labeled cartilage disks on the hyaluronate binding properties of \( ^{35} \text{S}-\text{proteoglycan} \) monomers. Cartilage disks were radiolabeled for 1.5 h and then, during a 9-h chase period, subjected to a 2% (0.02 mm peak) oscillatory compression at the indicated frequency, superimposed on a static compression to 1.0 mm. Proteoglycans were extracted and portions of monomer samples were reacted with 1.6% (w/w) hyaluronate and fractionated on Sephacryl S-1000. Fractions were assayed for proteoglycan and radioactivity and the ratio \( R \), the percentage of radiolabeled monomers eluting as aggregate divided by the percentage of total tissue monomers eluting as aggregate, was computed. The mean and range of \( R \) from duplicate monomer preparations is graphed.

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Figure 3.6: Aggregability of proteoglycan monomers from $^{35}$S-sulfate labeled cartilage with excess hyaluronate and link protein. Portions of monomer samples were mixed with 5% (w/w) hyaluronate and 5% (w/w) link protein under dissociative conditions, dialyzed to associative conditions, and separated on Sephacryl S-1000 into proteoglycan (−) and $^{35}$S-proteoglycan (●) that eluted as aggregates (PG-AGG) or monomers (PG-MON). The ratio $R$ is the percentage of radiolabeled monomers eluting as aggregate divided by the percentage of total tissue monomers eluting as aggregate. The elution profiles were from cartilage chased for 24 h, while being held at a swollen thickness of 1.25 mm or compressed to 0.5 mm, and bathed in medium at pH 6.99, 7.45, or 7.70.

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profiles, showing the aggregability of proteoglycans after 24-h chase under the most extreme compression (cartilage thickness of 0.50 mm) and altered pH conditions (pH 6.99 and 7.70) as well as control conditions, are shown in Fig. 3.6.

3.5 DISCUSSION

The results described here clearly demonstrate that the hyaluronate binding properties of newly synthesized proteoglycans within the extracellular matrix of immature cartilage explants can be altered by mechanical and chemical stimuli. Static compression and acidic medium pH were both shown to slow the acquisition of hyaluronate binding affinity normally seen with newly secreted monomers in explants. On the other hand, basic pH accelerated the conversion. Since the proteoglycans extracted and purified in the D1 fraction represented a high proportion of the radiolabeled and total tissue proteoglycan population, differences in \(^{35}\)S-proteoglycan properties after different chase conditions were attributable to effects on the majority of newly synthesized proteoglycans, and not to a selective extraction or a loss into the medium of \(^{35}\)S-proteoglycans. Further, alterations in \(^{35}\)S-proteoglycan affinity for hyaluronate were not due to degradation of hyaluronate binding sites, since neither compression nor altered pH affected the maximum capacity of tissue or \(^{35}\)S-proteoglycans to form aggregates with excess hyaluronate and link protein.

Since cartilage has a highly negatively charged matrix due to the ionized acidic carboxyl and sulfate moieties on proteoglycans, the intratissue concentration of cations (e.g., H\(^+\)) is increased (and that of anions is decreased) compared to concentrations in medium [52,100]. Compression of cartilage would increase the density of tissue charge and thereby further decrease intratissue pH. The charge density of our calf articular cartilage disks is \(~0.2–0.3\) M as assessed by chemical titration [139], GAG analysis [162], or NMR measurements of interstitial ion concentrations (Leann Lesperance, personal communication). Thus, a similar acidic intratissue pH

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of pH~6.7 in our cartilage disks would be achieved by either (1) compressing disks to 0.50 mm in normal pH~7.45 medium, or (2) holding disks at 1.25 mm in pH~7.0 medium [48].

The similar delay in affinity conversion under these comparable intratissue pH conditions and the dose-dependence of delay at intermediate levels of compression or medium acidification (Fig. 3.4A, B) are consistent with the hypothesis that static compression delays the extracellular processing of newly synthesized proteoglycans by decreasing the intratissue pH. This would also be consistent with the observation [140] that the proteoglycan conversion process in solutions is markedly slower at pH 7.4 than at higher pH (up to pH~9.2). Further, the pH dependence of proteoglycan affinity conversion both in cartilage and in solution suggests that altered intratissue pH within explants affects the hyaluronate binding domain of the proteoglycan directly. Such a direct effect, which would be independent of chondrocyte activity, would be in accord with the null effects of low-amplitude oscillatory compression on proteoglycan affinity conversion.

It is interesting that identical oscillatory compression protocols of 2% strain at frequencies of 0.001–0.1 cycles/sec were previously observed to stimulate chondrocyte biosynthesis [162]. The dynamic stiffness amplitudes at 0.001, 0.01, and 0.1 cycles/s were 4, 15, and 18 MPa, respectively (data not shown), and are comparable to values from previous dynamic stiffness measurements of calf cartilage disks [162]. Even during a single oscillation cycle, the peak amplitude of dynamic compression (0.02 mm) would result in a negligibly small pH change, compared to the magnitude of the static offset compression (1.25 – 1.00 mm = 0.25 mm). Further, the dynamic component of compression has 0 average value over a complete cycle, thus giving an essentially 0 time-average pH change. The differential effects of dynamic compression on proteoglycan synthesis and affinity change are also consistent with the hypothesis that physical stimuli other than changes in intratissue pH (such as hydrostatic pressure, fluid flow, cell deformation, or streaming potentials) can alter

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cell-dependent processes in cartilage [162].

The physicochemical modulation of affinity conversion described here could have an important physiologic role in the formation and remodeling of a mechanically functional extracellular matrix. Proteoglycan monomers with a low affinity for hyaluronate may be able to diffuse through proteoglycan-rich regions (of relatively low intratissue pH), and then become stabilized into aggregates with hyaluronate in proteoglycan-poor regions (of relatively high intratissue pH), where they are functionally required. Also, during static compression, low affinity proteoglycans may have a longer time to diffuse through the compacted and dense matrix meshwork before being converted to the high affinity form. It remains to be investigated whether the low affinity proteoglycans are actually more mobile within the extracellular matrix than the high affinity proteoglycans.

3.6 ACKNOWLEDGEMENTS

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Chapter IV
Effects of Compression on the Release of Newly Synthesized Proteoglycans and Proteins from Cartilage Explants

4.1 ABSTRACT

- The effects of mechanical compression and altered medium pH on the release and catabolism of proteoglycans and proteins radiolabeled with $^{35}$S-sulfate and $^3$H-proline from calf cartilage explants were examined. A single 2- or 12-h compression from a cartilage thickness of 1.25 to 0.50 mm, or slow cyclic compression (2-h on/2-h off) from 1.25 to 1.00, 0.75, or 0.50 mm for 24 h led to transient alterations and/or sustained increases in release of radiolabeled macromolecules. The effects of imposing or removing compressional loads were consistent with physical phenomena, including fluid flow, hindered diffusion, and matrix disruption, as well as metabolic alterations. Compression-induced fluid flow appeared to enhance the release of $^{35}$S- and $^3$H-macromolecules from tissue into medium (e.g., during cyclic compression). In contrast, compression-induced matrix consolidation appeared to hinder the diffusional transport and release of $^{35}$S- and $^3$H-macromolecules (e.g., during prolonged static compression). The acidification of intratissue pH associated with compression appeared to have little effect on release rates, although alkalinization of free-swelling disks did increase release rates. In disks subjected to high amplitude cyclic compression, there was a sustained increase in the rate of release of $^3$H- and $^{35}$S-macromolecules as well as collagenous ($^3$H-hydroxyproline containing) residues; these effects may be due to disruption of the collagen meshwork. In these compressed disks, a 21% decrease in glycosaminoglycan accumulation was accounted for by both an increase in release of glycosaminoglycans and a decrease in synthesis. The size distribution of the remaining tissue and $^{35}$S-proteoglycans was not affected, although a slightly higher proportion of these proteoglycans could form aggregates with excess hyaluronic acid and link protein than control proteoglycans. The $^{35}$S-proteoglycans released during such cyclic compression were of smaller average size than those from controls, but contained a similarly low proportion (~15%) with a functional hyaluronic acid binding region. This study provides a framework for studying the effects of different biophysical phenomena on the loss and catabolism of cartilage macromolecules.

*Preliminary results have been described [158,160].
4.2 INTRODUCTION

The maintenance or accumulation of a mechanically functional extracellular matrix in cartilage is dependent on the balance between synthesis and loss of matrix components. Tissue loading may modulate extracellular matrix catabolism and release either through direct physical or chemical processes or biological (cell-mediated) mechanisms. In experimental osteoarthritis, the turnover of proteoglycans is increased [25,168]. However, in vivo, it is difficult to quantify physical parameters, and therefore correlate biological alterations with, for example, possible physical initiators or biochemical mechanisms.

In vitro, both immature and mature cartilage explants can maintain steady state rates of proteoglycan metabolism [23,62,117]; the proteoglycans released into the medium as well as those remaining in the tissue have been extensively characterized [23,185]. The balance can be shifted towards proteoglycan loss by a number of agents such as lipopolysaccharides [114,117,182], interleukin 1 [72,114,185], retinoic acid [22], and tumor necrosis factor [163], or towards proteoglycan accumulation by others, such as insulin-like growth factor-I [105,186], transforming growth factor β [116], epidermal growth factor [130], fibroblast growth factor [130], and γ-interferon [19]. The catabolic effects are largely dependent on active cellular metabolism and reduced, for example, by cycloheximide inhibition of protein synthesis [23]. The metabolic effects may be mediated through intracellular pathways involving cyclic AMP [12], but appear to be independent of prostaglandin E₂ release [5,117,196].

There have been only a few preliminary in vitro studies on the effects of controlled physical stimuli on release and catabolism of cartilage matrix components [75,132]. In one study [75], compressional loads, corresponding to stresses of 0–3 MPa, for 1–4 days were found to cause a decrease in the release of ³⁵S from ³⁵S-sulfate labeled cartilage slices; upon unloading, the release of ³⁵S increased to levels above non-loaded controls. However, the ³⁵S-constituents released into the medium were not characterized, for example, in terms of their size distribution;
thus, it was not possible to interpret the release in terms of biological or physical mechanisms. In another study, compressional stresses of up to 0.011 MPa for 2 h were found to have no effect on release of $^{35}$S-macromolecules. However, these latter loads are quite low compared with typical in vivo stresses; contact pressures in the human femoropatellar and hip joint are 3–18 MPa [1,69,73] and result in compression amplitudes of $>$13% [3].

*In vitro*, the effects of both static and dynamic compressions of physiologic magnitudes on cartilage explant biosynthesis have been studied. Static compression of cartilage can inhibit proteoglycan biosynthesis [10,75,162,173], possibly by acidifying the intratissue environment [48]. Dynamic compression of calf cartilage disks can stimulate biosynthesis [162]. Thus, static and dynamic compression can have differential effects on cartilage metabolism.

The objectives of the present study were to examine the effects of graded levels of mechanical stimuli on the release of $^{35}$S-labeled proteoglycans and $^{3}$H-labeled proteins from cartilage explants during culture. We characterized the effects of (1) static compression and release and (2) slow cyclic compression as well as the possible role of a physicochemical acidification of intratissue pH in mediating the compression effects. Further, we interpreted the release patterns in terms of biophysical mediators including fluid flow and diffusion.

### 4.3 EXPERIMENTAL PROCEDURES

#### 4.3.1 Materials

Materials for explant culture and proteoglycan and protein isolation and analysis were obtained as described previously [162,170]. In addition, 0.22 μm Millex-GS filters were from Millipore, Bedford, MA. Tissue-Tek embedding solution (O.C.T. compound 4583) and Tissue-Tek II disposable specimen molds were from Miles Diagnostics, Elkhart, IN. Centricon-10 microconcentrators were from Amicon,
4.3.2 Explant, Culture, and Pulse-Chase Labeling of Calf Cartilage Disks

Cartilage disks, 1 mm thick × 3 mm diameter, were explanted from the femoropatellar grooves of 1–2 week old calves, and cultured in DMEM supplemented with 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes), 0.1 mM non-essential amino acids, an additional 0.4 mM L-proline, 20 μg/ml ascorbate and 10% FBS in a humidified 5% CO₂/95% air incubator at 37°C, as previously described [162]. All medium was prepared by temperature and CO₂ equilibration in the incubator for 1–2 h just before use. On the day of explant, disks were incubated for 20–25 h in medium (1 ml/4 disks) including (A) 160 μCi/ml L-[5-³H]proline and 100 μCi/ml [³⁵S]sulfate or (B) 200 μCi/ml [³⁸S]sulfate, along with 100 μg penicillin/ml and 100 U streptomycin/ml. Disks were then washed with 4–6 changes of medium (1 ml/disk) over 2 h before further incubation. Throughout the culture period, spent medium (0.5 or 1.0 ml/disk) was collected and stored at −20°C with protease inhibitors (0.1 M 6-aminohexanoic acid, 10 mM Na₂-EDTA, 10 mM benzamidine-HCl, 10 mM N-ethylmaleimide, 1 mM phenylmethanesulfonyl chloride, 1 μM pepstatin, and 1 μM leupeptin) [67] and replaced with fresh medium every 24–26 h, or more often during and just following certain mechanical compression protocols.

After 1.5–2 days (except where noted), disks were transferred to fresh medium and subjected to 2–24 h of mechanical or chemical stimuli as described below. At the termination of these protocols, disks were then returned to the free-swelling state and normal medium (pH~7.45) and further incubated for an additional 3–5 days. Finally, disks were rinsed over 0.5 h with two changes of a 4°C phosphate buffered saline solution (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄·7H₂O, 1.5 mM KH₂PO₄) including protease inhibitors, at pH 7.4 and

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stored at −20°C until later extraction. Alternatively, disks were rinsed without protease inhibitors and digested with papain [162].

4.3.3 Mechanical and Chemical Stimulation

Cartilage explants were subjected to one of three physical or chemical stimuli. The tissue used for each protocol was from a single, but different animal.

(1) Disks were placed in individual wells of compression culture chambers (0.8 or 1.1 ml medium/disk) where they were subjected to a single step compression between two impermeable platens, in a uniaxial, radially unconfined geometry [162]. Cartilage disks were compressed to a thickness of 0.50 mm for 2 or 12 h; the static compression chamber was clamped together to enable orbital shaking (Model 3520, Labline Instruments, Melrose Park, IL) of the apparatus at 60 rpm to minimize stagnant films. Since cartilage disks swelled axially to a thickness of 1.25–1.4 mm during initial culture [162], controls were held at a swollen thickness of 1.25 mm during the test period. In this compression configuration, stress relaxation (and concomitant fluid flow relative to the solid matrix) is 90% complete within ~15 minutes [162]; thus, mechanical equilibrium was attained rapidly during the test period, and the compression was essentially static.

(2) Disks were placed in the same chambers as in (1) and subjected to 24-h cyclic compression: 2-h compression to 1.00, 0.75, or 0.50 mm (2 h on), followed by a release to 1.25 mm (2 h off); controls were held at 1.25 mm. Alternatively, in an experiment to characterize the radiolabeled macromolecules both released into the medium and remaining in the cartilage, disks were placed in a specially designed dynamic compression chamber (25 ml medium/12 disks) held within a mechanical spectrometer (Dynastat, IMASS, Hingham, MA), and and subjected to computer-controlled cyclic compression for 24 h as described previously [162]. Here, groups of disks were subjected to different compression amplitudes in sequential runs, 2–6 days after radiolabeling.
(3) Disks were transferred to pH-adjusted medium (0.5 ml/disk) for 24-h incubation. Medium with pH between 6.99-7.77 in the 5% CO₂ incubator was prepared by addition of combinations of 1 M HCl, water, and 1 M NaOH in a total added volume of 2.24 ml/l medium such that 18.8, 11.2, or 0 mmol HCl/l medium or 22.4 mmol NaOH/l medium was added [155]. Control studies showed that pH was not significantly altered (≤0.02 pH units) by 24-h incubation with cartilage disks.

4.3.4 Measurement of Release of ³⁵S-Proteoglycans and ³H-Proteins

Portions of spent medium from individual disks were analyzed for total ³⁵S and ³H radioactivity. The remaining spent media from groups of 4-12 similarly treated disks were then pooled and analyzed by fractionating a portion (0.5-1.0 ml) on a PD-10 column of Sephadex G-25 (MW cutoff of 1-5,000) into ³⁵S- and ³H-macromolecular (V₀) and low molecular weight (V₁) component peaks. The separations were performed under dissociative conditions with columns equilibrated and eluted with 2 M guanidinium-HCl, 0.5 M Na acetate, pH 6.8, although similar separations were achieved with 1% SDS, 10 mM dithiothreitol, 50 mM Tris-HCl, pH 8.5.

Previous control studies [158] indicated that by one day of chase after radiolabeling with ³H-proline and ³⁵S-sulfate, virtually all of the ³H and ³⁵S remaining in the cartilage disks was incorporated into macromolecular proteins and proteoglycans, respectively; after chase, only ~2% of the ³H remaining in the disk and < 1% of the ³⁵S were SDS-extractable as low-molecular weight components (<1-5,000) on Sephadex G-25 [158]. Thus, equivalent trends were observed whether the total ³⁵S-proteoglycan and ³H-protein in each sample were calculated as the radioactivity remaining in the cartilage disks at the termination of the experiment plus the summed total radioactivity released into the media after the first day of chase or plus the summed macromolecular radioactivity released into the medium each day.
after the first day of chase. The latter method was chosen to allow comparison to previous studies, (e.g. [23,63,117]). From this total (typically \( \sim 10 \times 10^5 \) \(^3\text{H}\)-cpm and \( \sim 5 \times 10^5 \) \(^35\text{S}\)-cpm per disk), the percentage release (both the daily rate and cumulative total) of \(^35\text{S}\)-proteoglycans and \(^3\text{H}\)-proteins was computed. No corrections were made for radiolabeled components in the medium eluting in the V, which may have arisen from extensive degradation of \(^35\text{S}\)-proteoglycan or \(^3\text{H}\)-protein. The data is plotted as % released from the tissue, rather than % remaining in the tissue [22,23,63,117], since the overall release over the course of the experiments was low (and, for \(^35\text{S}\), essentially linear).

4.3.5 Extraction and Isolation of Proteoglycans remaining in Cartilage Disks

Groups of 4–6 disks (~48–72 mg wet weight of cartilage) from a single experimental protocol were frozen in Tissue-Tek embedding solution within 10×10×5 mm Tissue-Tek II molds. The block was cryostat sectioned to 20 \( \mu \)m and the cartilage slices were extracted for 36–42 h at 4°C by gently rocking in 50–100 volumes of 4 M guanidinium–HCl, 50 mM Na acetate, 10 mM Hepes, and protease inhibitors, pH 7.0. The tissue residue was treated with 0.5 ml of 0.5 M NaOH for 1 day at 22°C to solubilize essentially all remaining glycosaminoglycans, and then neutralized with an equal volume of 0.5 M HCl.

Portions of extracts were filtered over glass wool, adjusted to a density of 1.45 g/ml with solid cesium chloride, centrifuged at 38,000 rev/min in a Beckman 50Ti rotor \( (r_{ev} = 8.02 \text{ cm}) \) for 72 h at 12°C, and sliced into four approximately equal gradient fractions \(^1\) (D1, 1.56 g/ml; D2, 1.48 g/ml; D3, 1.43 g/ml; D4, 1.38 g/ml). D1 fractions were dialyzed exhaustively against 0.15 M Na acetate, pH 6.8 at 4°C, and then stored at −20°C.

\(^1\)The terms D1, D2, D3, and D4 refer to the fractions of highest to lowest buoyant density obtained by subjecting cartilage extracts to cesium chloride density gradient centrifugation under dissociative conditions and are based on the terminology of Heinegård [67].

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4.3.6 Sepharose CL-2B Chromatography

Portions of medium (0.5 ml) were mixed with 250 μg carrier proteoglycan (50 μl of a 5 mg/ml 4 M guanidinium-HCl extract of calf cartilage), and fractionated on columns (0.65×95 cm) under dissociative conditions [26] eluted at a flow rate of 2 ml/h; fractions (0.6 ml) were collected and assayed for both proteoglycan and radioactivity. Portions of guanidinium extracts of radiolabeled cartilage containing 250 μg endogenous tissue proteoglycan in ~0.4 ml were similarly fractionated. In some samples, 4 μg DNA and 0.04 μCi ^3H_2O were added as markers for the void (V_0) and total column (V_t) volumes, respectively.

4.3.7 Aggregability of Proteoglycans with Excess Hyaluronic Acid and Link Protein

To determine aggregability of tissue and ^35S-proteoglycans extracted from cartilage, portions of D1 samples (containing 250 μg of proteoglycans and about 75,000 cpm of radioactivity in ~0.6 ml) were mixed with 5% (w/w) hyaluronate and 5% (w/w) link protein, adjusted to 4 M guanidinium-HCl, allowed to dissociate for 2 h at 4°C, dialyzed for 24 h against 0.15 M Na acetate, pH 6.8 at 4°C, frozen at −20°C, and later analyzed by chromatography. To determine aggregability of ^35S-proteoglycans in medium samples, portions (2.5 ml) were mixed with 300 μg of bovine nasal cartilage proteoglycan monomer (BNC-A1D1D1) and protease inhibitors, concentrated to ~0.7 ml and buffer exchanged to 4 M guanidinium-HCl, 50 mM Na acetate, 10 mM Hepes, and protease inhibitors, pH 7.0 with a Centricon-10 microconcentrator; half of the retentate was then mixed with hyaluronate and link protein and prepared for chromatography, as above. Samples were applied to Sephacryl S-1000 columns (1×45 cm) eluted at 9 ml/h (11.5 cm/hr) in 0.5 M Na acetate, 0.1% (v/v) Triton X-100, 0.02% Na azide, pH 6.8; fractions (0.6 ml) were collected and assayed for both proteoglycan and radioactivity. Recovery of proteoglycan and ^35S on chromatography was 90–100%.

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4.3.8 Quantitation of ³H-hydroxyproline

Portions of papain digests or medium (~1 ml with 250 μg added carrier hydroxyproline) (equivalent to ~6 mg wet weight of cartilage) were analyzed for ³H-hydroxyproline residues essentially as described previously [178]. Samples were lyophilized, hydrolyzed with 0.2-0.4 ml 6 N HCl at 105°C in teflon-lined pyrex tubes for 16 h, diluted to 0.9 M HCl, and filtered through a 0.22 μm membrane. A 0.5 ml portion of the filtrate was then run on a 0.7×10 cm column of Dowex 50W (X8; 200-400 mesh), equilibrated and eluted with 0.9 N HCl. Fractions (1 ml every 3 minutes) were collected and analyzed for radioactivity. ³H-hydroxyproline and ³H-proline eluted at 7-11 ml and 16-24 ml, respectively. Recovery of ³H was ~90%.

4.3.9 Analytical Procedures

Samples were assayed for proteoglycan as sulfated glycosaminoglycan by reaction with dimethylmethylene blue [40], using appropriate blanks of buffer or medium. The amount of dye-reactive material in the FBS was small compared to the amount of proteoglycan released into medium. The concentration of hyaluronate in Healon was taken as given by the supplier (10 mg/ml). Radioactivity was determined by liquid scintillation counting in an LKB Rackbeta 1211 scintillation counter [162]; before adding 2 ml ScintiVerse BioHP to samples, those with guanidinium-HCl were prepared by adjusting to 0.5 ml with 2 M guanidinium-HCl, 0.5 M Na acetate, pH 6.8, whereas 0.5 ml samples with Triton X-100 were prepared by mixing with 1 ml of 10% (w/v) sodium dodecyl sulfate. DNA was determined by reaction with Hoechst 33258 [28,77].

4.3.10 Statistical Analysis

In most experiments, the media from 6-12 individual disks was combined to form one or two pooled samples before more detailed analysis (e.g., G-25 chromatography to quantitate radiolabel in macromolecular form). In those cases, an

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estimate of the standard error of the mean (SEM) of each pooled sample was made from the SEM of the total (i.e., macromolecular and low molecular weight) radioactivity in each individual medium sample before pooling; typically, the SEM for media from 12 disks was <5%. Similar estimates of variability were obtained from the glycosaminoglycan content remaining in cartilage disks at the end of the experiment. Thus, relative differences of >10% in the relative rates of release (e.g., \(^{35}\text{S}\)-macromolecules released per day) were judged significant. No judgements were made on the significance of absolute differences in the cumulative percentage of material released. In comparing the glycosaminoglycan and DNA content of cartilage disks, differences between sample means were assessed by two-tailed t-test.

4.4 RESULTS

4.4.1 Effect of 2- or 12-h Static Compression on Release of \(^{35}\text{S}\)-Proteoglycans

The cumulative release of \(^{35}\text{S}\)-macromolecules from control disks, maintained at a swollen thickness of 1.25 mm during the 2- or 12-h test period, was ~5.7% after 4.5 days (Fig. 4.1A, D)—an average of 1.3%/day. During this 4.5-day period, the cumulative release of \(^{35}\text{S}\)-macromolecules from samples subjected to 2- and 12-h static compression to 0.50 mm was increased over that from controls to 6.2% and 7.2%, respectively.

During the 2-h compression to 1.25 mm or 0.50 mm, the rates of release of \(^{35}\text{S}\)-macromolecules (Fig. 4.1B) were increased compared to the rates during the previous 24 h (+55% and +72%, respectively). These were accompanied by increases in the proportion of \(^{35}\text{S}\) macromolecules in the medium (Fig. 4.1C). However, during prolonged 12-h compression to 1.25 mm or 0.50 mm, the rates of release of \(^{35}\text{S}\)-macromolecules (Fig. 4.1E) were decreased compared to the rates during the previous 24 h (−39% and −60%, respectively); for disks compressed to 0.50 mm,
Figure 4.1: Effect of 2- or 12-h compression to 0.5 mm on release of $^{35}$S from cartilage explants. Cartilage disks were dual-labeled with $^{35}$S-sulfate and $^3$H-proline, washed, and incubated for 1.5–2 days, and then subjected to static compression to 0.5 mm (●, ■) or held at a swollen thickness of 1.25 mm (○, □) for 2 h (A–C) or 12 h (D–F). The arrows (△, A, D) indicate the time during which compression was applied. Disks were then released from compression and cultured free swelling. Media from individual disks (12 per treatment group) was collected daily and pooled. The cumulative release (A, D) and rate of release (B, E) of macromolecular $^{35}$S, and the proportion of $^{35}$S in the media that was macromolecular (C, F) were determined as described in Materials and Methods.

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this was accompanied by a marked decrease in the proportion of $^{35}$S-macromolecules released into the medium (Fig. 4.1F).

Following unloading of disks compressed to 0.50 mm for 2 or 12 h, the cumulative release of $^{35}$S-macromolecules was increased (Fig. 4.1A, D); during the 12 h after unloading, the rates of release from 2- and 12-h compressed disks were increased over that from 1.25 mm controls by +34% and +117%, respectively (Fig. 4.1B, E). At the same time, the proportion of $^{35}$S in the medium that was macromolecular was elevated in 12-h compressed samples compared to controls (Fig. 4.1F). The release rates returned to near 1.25 mm control levels over the next 3 days.

### 4.4.2 Glycosaminoglycan Content of Cartilage Disks after 2- or 12-h Static Compression

After this period of elevated $^{35}$S-proteoglycan release, the glycosaminoglycan content in the cartilage at the termination of the experiment, 3.5 days after compression, was significantly lower (−7.4%) in 12-h compressed samples than 12-h controls (619±21 vs 669±12 μg/disk, mean ± SEM, n = 12, p = 0.05), but not significantly different in 2-h compressed samples and controls (666±18 vs 680±17 μg/disk).

### 4.4.3 Effect of Altered Medium pH on Release of $^{35}$S-Proteoglycans and Tissue Proteoglycans

The cumulative release of $^{35}$S-macromolecules from control disks, incubated at pH 7.45 during the 24-h test period, was 4.7% after 5.3 days—an average rate of 0.89±0.04 %/day (mean±range for duplicate groups of 6 disks, Fig. 4.2A, B). While incubation at altered pH did not greatly alter the cumulative release of $^{35}$S-macromolecules (Fig. 4.2A), the rate of release of $^{35}$S-macromolecules during incubation at pH 7.77 was elevated by ~40% over that at pH 7.45, with an increased proportion of released $^{35}$S in the form of macromolecules (Fig. 4.2C). Acidifica-
Figure 4.2: Effect of altered pH on release of $^{35}$S from cartilage explants. Cartilage disks were dual-labeled with $^{35}$S-sulfate and $^3$H-proline, washed, and incubated for 2 days in media at pH 7.45, and then incubated in media at pH 6.99 (●), 7.20 (▲), 7.45 (□), and 7.77 (★) for 24 h during the period (△pH) indicated in (A). Disks were then returned to medium at pH 7.45. Media from individual disks (2 groups of 6 disks per treatment group) was collected daily and pooled. The cumulative release (A) and rate of release (B) of macromolecular $^{35}$S, the proportion of $^{35}$S in the media that was macromolecular (C), and the rate of glycosaminoglycan release per disk (D) were determined as described in Materials and Methods. Bar indicates average range.
tion of the medium to pH 6.99 or 7.20 had little effect on the rate of release of $^{35}$S-macromolecules. The rate of release of glycosaminoglycan into the medium (Fig. 4.2D) generally paralleled that of $^{35}$S-macromolecules, although there was a slight decrease in glycosaminoglycan release at acidic pH.

4.4.4 Glycosaminoglycan Content of Cartilage Disks after Altered Medium pH

At the termination of the experiment, 3 days after alteration of medium pH, there was a trend of decreased glycosaminoglycan content with increasing pH treatment, averaging 825, 799, 800, and 761 μg/disk (average range, 38 μg) at pH 6.99, 7.20, 7.45, and 7.77, respectively; however the differences between treatment groups were not statistically significant.

4.4.5 Effect of 24-h Cyclic Compression on Release of $^{35}$S-Proteoglycans and Tissue Proteoglycans

The cumulative release of $^{35}$S-macromolecules from control disks, maintained at a swollen thickness of 1.25 mm during the 24-h test period, was ~6.6% after 7 days (Fig. 4.3A)—an average of 0.9%/day. During this 7-day period, the cumulative release of $^{35}$S-macromolecules from samples subjected to cyclic compression to 0.75 mm and 0.50 mm was increased over that from controls to 7.2% and 10.5%, respectively.

During the 24 h of cyclic compression to 1.00, 0.75, and 0.50 mm, the rates of release of $^{35}$S-macromolecules (Fig. 4.3B) were increased when compared to 1.25 mm controls (+23%, +32%, +124%, respectively) or when compared to rates during the previous 24 h. These were accompanied by increases in the proportion of $^{35}$S-macromolecules in the medium (Fig. 4.3C), and consistent with parallel trends in the rate of glycosaminoglycan released into the medium (Fig. 4.3D).

In the 12-h period just after cyclic compression, the rate of release of $^{35}$S-
Figure 4.3: Effect of 24-h cyclic compression on release of $^{35}$S from cartilage explants. Cartilage disks were dual-labeled with $^{35}$S-sulfate and $^3$H-proline, washed, and incubated for 2 days, and then subjected to cyclic 2 h on/2 h off compression from 1.25 mm to 0.50 mm (■), 0.75 mm (▲), or 1.00 mm (●), or static compression to a swollen thickness of 1.25 mm (○). The arrow (†, A) indicates the 24-h period during which cyclic compression was applied. Disks were then cultured free swelling. Media from individual disks (12 per treatment group) was collected daily and pooled. The cumulative release (A) and rate of release (B) of macromolecular $^{35}$S, the proportion of $^{35}$S in the media that was macromolecular (C), and the rate of glycosaminoglycan release per disk (D) were determined as described in Materials and Methods.
macromolecules and glycosaminoglycans from samples cycled to 0.50 mm (Fig. 4.3B, D) remained high; over the next 4 days, the release rates remained elevated. In contrast, in this 12-h period, the rates of release of $^{35}$S-macromolecules and glycosaminoglycans from samples cycled to 0.75 or 1.00 mm, (Fig. 4.3B, D) returned to their previous rates more quickly, and were transiently lower than the rate of release from 1.25 mm controls. In the experiments performed in the dynamic compression chamber 2–6 days after radiolabeling, a similar trend of a sustained increase in release of $^{35}$S-macromolecules following 24-h cyclic compression to 0.50 mm (here, applied on day 4 after labeling) was observed (data not shown).

4.4.6 Glycosaminoglycan Content of Cartilage Disks after 24-h Cyclic Compression

At the termination of the experiment, 5 days after compression, the glycosaminoglycan content of 1.25 mm control cartilage was 804±32 μg/disk (mean±SEM, n = 12) and the total cumulative glycosaminoglycan released was 47 μg/disk. Samples cycled to 1.00, 0.75, and 0.50 mm, contained 791±30, 741±20, and 636±16 μg/disk, respectively, and contained fewer glycosaminoglycans than controls by 1.6%, 7.8% (p<0.1) and 20.9% (p<0.001), respectively; these samples released a total of 46, 46, and 60 μg/disk, respectively. Since the 0.50 mm compressed disks released only 13 μg/disk more than the 1.25 mm controls into the media over the 6 day period after compression, the 168 μg difference in glycosaminoglycan content of the tissue at the termination of the experiment must be due to decreased rates of glycosaminoglycan synthesis [63,117] in the more compressed disks. This difference between 1.25 mm controls and 0.50 mm compressed samples amounted to 26 μg/day; by similar reasoning, the difference between 1.25 mm controls and 0.75 mm compressed samples was 11 μg/day.

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4.4.7 DNA Content of Cartilage Disks after 24-h Cyclic Compression

At the termination of the experiment, the DNA content of 1.25 mm control disks was 7.22±0.38 µg/disk (mean±SEM, n = 12) and that of 1.00 mm, 0.75 mm, and 0.50 mm compressed disks was 6.43±0.46, 6.69±0.29, and 5.61±0.52, respectively. The 22% decrease in DNA of 0.50 mm compressed samples compared to 1.25 mm controls was significant (p<0.05).

4.4.8 Characterization of $^{35}$S-Proteoglycans Released During and Following Cyclic Compression

The material released into the medium was eluted on Sepharose CL-2B under dissociative conditions to characterize the size distribution of $^{35}$S components. The $^{35}$S-macromolecules released during 24-h cyclic compression to 0.50 mm (Fig. 4.4A) were polydisperse proteoglycans of smaller average size (peak $K_{av}$ ~0.42) than those released from 1.25 mm controls (peak $K_{av}$ ~0.33); these, in turn, were smaller and more polydisperse than carrier calf proteoglycans (peak $K_{av}$ ~0.23). The $^{35}$S-macromolecules released during lesser amplitude cyclic compression showed distributions more similar to those of 1.25 mm controls than those of 0.50 mm compressed. The amount of $^{35}$S material eluting at the total column volume was only increased slightly at compression to 0.50 mm; thus, there appeared to be no marked increase in degradation of proteoglycan to free sulfate via cellular uptake and lysosomal enzymes [63].

In the 12 h following the compression period, the $^{35}$S-proteoglycans released by samples which had been compressed to 0.50 mm (Fig. 4.4B) were of slightly smaller average size than those released by 1.25 mm controls; $^{35}$S-proteoglycans from samples compressed to 1.00 or 0.75 mm were similar to those from 1.25 mm controls.

The maximum capacity of $^{35}$S-proteoglycans released into the medium to form aggregates was assessed using pooled medium from the 24-h period during

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Figure 4.4: Sepharose CL-2B profiles of $^{35}$S-components released into the medium during and just following 24-h cyclic compression. Portions of medium (from the experiment in Fig. 4.3) taken during (A) or in the 12 h following (B) cyclic compression to 0.50 mm (■), 0.75 mm (◆), or 1.00 mm (▲), or static compression to 1.25 mm (○), were eluted under dissociative conditions with added carrier calf proteoglycan and calf thymus DNA (as a $V_o$ marker). Fractions were assayed for (-) proteoglycan and $^{35}$S (■, ◆, ▲, ○).

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Figure 4.5: Aggregability with excess hyaluronate and link protein of $^{35}$S-macromolecules released into media during and just following 24-h cyclic compression. Portions of medium (from an experiment such as that of Fig. 4.4) collected during and in the 24 h just following static compression to 1.25 mm (A) or cyclic compression to 0.75 mm (B) or 0.50 mm (C) were mixed with BNC-A1D1D1 proteoglycan monomer as carrier, mixed with 5% (w/w) hyaluronate and 5% (w/w) link protein under dissociative conditions, dialyzed to associative conditions, and run on Sephacryl S-1000. Fractions were assayed for (-) $^{35}$S and proteoglycan (○, ♦, □). To simplify graphical presentation, data for both proteoglycan and radioactivity have been normalized to the maximum values obtained. The ratio $R$ is the percentage of radiolabeled monomers eluting as aggregate divided by the percentage of total tissue monomers eluting as aggregate.

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cyclic compression and the 24-h period just following compression. Media from
disks cycled to 0.75 mm and 0.50 mm as well as 1.25 mm static controls (Fig. 4.5A–
C) showed a similarly low proportion (13–16%) of $^{35}$S-macromolecules that were able
to aggregate. This appeared to be due to non-functional hyaluronic acid binding
region in the $^{35}$S-macromolecules rather than interfering molecules in the media
since 57–66% of the carrier proteoglycan formed aggregates. The aggregability of
proteoglycans in the preparation from 0.50 mm cycled samples was slightly lower
than those from 0.75 mm cycled or 1.25 mm control samples (55 vs 66%); this could
be accounted for by the higher amount of tissue proteoglycan (presumably, mostly
non-aggregating) released into the medium under that condition. Similar degrees
of aggregation occurred whether samples were run on associative Sepharose CL-2B
or Sephadryl S-1000 (data not shown).

4.4.9 Characterization of $^{35}$S-Proteoglycans and Tissue Proteoglycans

Remaining after Cyclic Compression

The majority (range, 93–94%) of both tissue and $^{35}$S-proteoglycans residing
in the cartilage after cyclic compression were extracted from cryostat sectioned carti-
lage disks by 4 M guanidinium. Portions of the extract were eluted on Sepharose CL-
2B under dissociative conditions (Fig. 4.6A–C). Under all cyclic compression and
static control conditions, the population of large $^{35}$S-proteoglycans (peak $K_{av}$ ~0.28)
eluted just ahead of the large tissue proteoglycans (peak $K_{av}$ ~0.30). There was
also evidence of smaller tissue and $^{35}$S-proteoglycan (peak $K_{av}$ ~0.7) populations.

The aggregability of the large proteoglycans was examined after purifica-
tion of a portion of the cartilage extracts by cesium chloride equilibrium density
gradient centrifugation. The high-buoyant density (D1) fractions ($\rho$ >1.56 g/ml)
contained 91–92% of the glycosaminoglycan and 96% of the $^{35}$S, and had specific
activities of 1100–1200 $^{35}$S cpm/µg glycosaminoglycan. Portions of the D1 proteo-
glycan were mixed with excess (5% w/w) hyaluronic acid and (5% w/w) link protein,

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Figure 4.6: Characterization of $^{35}$S-proteoglycans remaining in matrix after 24-h cyclic compression. Cartilage disks (from the experiment in Fig. 4.5) were statically compressed to 1.25 mm (A, D) or cyclically compressed to 0.75 mm (B, E) or 0.50 mm (C, F), further incubated 24 h without compression, and then extracted under dissociative conditions. Portions of the crude extract were eluted on Sepharose CL-2B (A, B, C) under dissociative conditions. High buoyant density proteoglycans were purified by cesium chloride density gradient centrifugation, mixed with 5% (w/w) hyaluronate and 5% (w/w) link protein, and run on Sephacryl S-1000 under associative conditions (D, E, F). Fractions were assayed for ($^-$) $^{35}$S and proteoglycan ($\bullet$, $\phi$, □). The ratio $R$ is the percentage of radiolabeled monomers eluting as aggregate divided by the percentage of total tissue monomers eluting as aggregate. To simplify graphical presentation, data for both proteoglycan and radioactivity have been normalized to the maximum values obtained. Section 4.4
and chromatographed on Sephacryl S-1000 under associative conditions. Under all compression conditions (Fig. 4.6D-F), a high proportion (65-74%) of the tissue and $^{35}$S-proteoglycans were able to aggregate. However, there did appear to be a slight increase (3-5%) in the aggregability of both tissue and $^{35}$S-proteoglycans remaining in samples cycled to 0.75 or 0.50 mm compared to 1.25 mm control samples.

4.4.10 Effect of 2- or 12-h Static Compression on Release of $^3$H-Proteins

In parallel with the analysis of release of $^{35}$S-components from $^{35}$S-sulfate and $^3$H-proline labeled cartilage disks, the release of $^3$H was examined. The cumulative release of $^3$H-macromolecules from control disks, maintained at a swollen thickness of 1.25 mm during the 2- or 12-h test period, was $\sim$9% after 4.5 days (Fig. 4.7A, D). During this 4.5-day period, the release of $^3$H-macromolecules from samples compressed to 0.50 mm for either 2 or 12 h was increased over controls to $\sim$16% (Fig. 4.7A and D, respectively).

During 2-h compression to 0.50 mm, the rate of release of $^3$H-macromolecules (Fig. 4.7B) was increased (+131%) compared to the rate from control disks compressed to 1.25 mm. This was accompanied by a slight increase in the proportion of $^3$H-macromolecules in the media (Fig. 4.7C). In contrast, during prolonged 12-h compression to 1.25 mm or 0.50 mm, the rates of release of $^3$H-macromolecules (Fig. 4.7E) were decreased compared to the rates in the previous 24 h. For disks compressed to 0.50 mm, this was accompanied by a slight decrease in the proportion of $^3$H-macromolecules in the media (Fig. 4.7F).

Following unloading of disks compressed to 0.50 mm for 2 or 12 h, the release of $^3$H-macromolecules was greatly increased (Fig. 4.7A, D); in 2- and 12-h compressed disks, the rates of release during the next 12 h were elevated by +317% and +258% over the rates in 1.25 mm control disks (Fig. 4.7B, E). At the same time, the proportion of $^3$H in the medium that was macromolecular was elevated to $\sim$80% (Fig. 4.7C, F) compared to $\sim$50% in the medium of control disks. While the release

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Figure 4.7: Effect of 2- or 12-h compression to 0.5 mm on release of $^3$H from cartilage explants. Cartilage disks (in the experiment of Fig. 4.1) were dual-labeled with $^{35}$S-sulfate and $^3$H-proline, washed, and incubated for 1.5–2 days, and then subjected to static compression to 0.5 mm ($\bullet$, ■) or held at a swollen thickness of 1.25 mm (○, □) for 2 h (A–C) or 12 h (D–F). The arrows (ψ, A, D) indicate the time during which compression was applied. Disks were then released from compression and cultured free swelling. Media from individual disks (12 per treatment group) was collected daily and pooled. The cumulative release (A, D) and rate of release (B, E) of macromolecular $^3$H, and the proportion of $^3$H in the media that was macromolecular (C, F) were determined as described in Materials and Methods.

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rates returned to near 1.25 mm control levels over the next 3 days, the proportion of $^3$H in the medium that was macromolecular remained elevated at $\sim$75%.

4.4.11 Effect of Altered Medium pH on Release of $^3$H-Proteins

The cumulative release of $^3$H-macromolecules from disks, incubated at pH 6.99–7.70 for 24 h, was $\sim$3% after 5 days (Fig. 4.8A). The effects of pH on the release rate of $^3$H-macromolecules were apparent only during the 24-h incubation at altered pH (Fig. 4.8B); in medium at pH 6.99 and 7.24, the release rates were decreased by $-17.3\%$ and $-22.5\%$, respectively, compared to incubation at pH 7.45, and increased by $+7.7\%$ at pH 7.77. During and after treatment at pH 7.77, there was a slight decrease in the proportion of $^3$H in the media that was in macromolecular form, compared to normal pH$\sim$7.45 and acidified media (Fig. 4.8C).

4.4.12 Effect of 24-h Cyclic Compression on Release of $^3$H Components

Cyclic compression led to an increase in the release of $^3$H-macromolecules which was dependent on the amplitude of compression (Fig. 4.9A). While 3.4% of the $^3$H-macromolecules were released from 1.25 mm control disks in 7 days, 4.4%, 5.3%, and 8.4% were released from disks cycled to 1.00 mm, 0.75 mm, and 0.50 mm, respectively; the rates of release (Fig. 4.9B) were increased most during 24-h cyclic compression ($+100\%$, $+148\%$, and $+422\%$, respectively, over 1.25 mm control rates). The release rates remained substantially elevated in the next 24 h and remained at elevated levels ($+19\%$, $+37\%$, and $+82\%$, respectively, over 1.25 mm control rates) at the termination of the experiment, 5 days after compression. During the 24-h compression and in the subsequent 24 h, the media was enriched with macromolecular $^3$H constituents (Fig. 4.9C) as well as $^3$H-hydroxyproline residues (Fig. 4.9D). These effects of cyclic compression continued over the 5 day period following compression until the termination of the experiment (Fig. 4.9C, D).

Of the $^3$H remaining in the disks, 41–42% was in the form of $^3$H-

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Figure 4.8: Effect of altered pH on release of $^3$H from cartilage explants. Cartilage disks (in the experiment of Fig. 4.2) were dual-labeled with $^{35}$S-sulfate and $^3$H-proline, washed, and incubated for 2 days in media at pH 7.45, and then incubated in media at pH 6.99 (●), 7.20 (▲), 7.45 (□), and 7.77 (★) for 24 h during the period ($\Delta$ pH) indicated in (A). Disks were then returned to medium at pH 7.45. Media from individual disks (2 groups of 6 per treatment group) was collected daily and pooled. The cumulative release (A) and rate of release (B) of macromolecular $^3$H, and the proportion of $^3$H in the media that was macromolecular (C) were determined as described in Materials and Methods. Bar indicates average range.

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Figure 4.9: Effect of 24-h cyclic compression on release of $^3$H from cartilage explants. Cartilage disks (in the experiment of Fig. 4.3) were dual-labeled with $^{35}$S-sulfate and $^3$H-proline, washed, and incubated for 2 days, and then subjected to cyclic 2 h on/2 h off compression from 1.25 mm to 0.50 mm (■), 0.75 mm (♦), or 1.00 mm (▲), or static compression to a swollen thickness of 1.25 mm (○). The arrow (†, A) indicates the 24-h period during which cyclic compression was applied. Disks were then released from compression and cultured free swelling. Media from individual disks (12 per treatment group) was collected daily and pooled. The cumulative release (A) and rate of release (B) of macromolecular $^3$H, the proportion of $^3$H in the media that was macromolecular (C), and the ratio of $^3$H-hydroxyproline to $^3$H-proline in acid hydrolyzed media (D) were determined as described in Materials and Methods.

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hydroxyproline. Assuming 45% of the proline to be hydroxylated in type II collagen [36] and all of the $^3$H-hydroxyproline in the cartilage disks to be in type II collagen, $\sim$90–92% of the $^3$H radiolabel remaining in the cartilage disks at the end of this experiment was in type II collagen. By also determining the total media $^3$H in the form of $^3$H-hydroxyproline and $^3$H-proline residues, the $^3$H in collagen accounted for $\sim$80% of the $^3$H radiolabel in the cartilage disks, one day after radiolabeling.

4.4.13 Characterization of $^3$H-Components Released during and following 24-h Cyclic Compression

The material released into the medium during 24-h cyclic compression and in the 24 h following cyclic compression were eluted on Sepharose CL-2B under dissociative conditions. The $^3$H-components (Fig. 4.10A and B) eluted as two components, one with a peak $K_{av} \sim$0.85, and the other at the total column volume.

4.5 DISCUSSION

The results described here demonstrate that single or multiple cycles of compression and unloading of immature calf cartilage disks, which were radiolabeled with $^{35}$S-sulfate and $^3$H-proline to tag newly synthesized proteoglycans and proteins, respectively, during in vitro culture, can lead to alterations in the quality of released $^3$H- and $^{35}$S-macromolecules as well as the rate of release. With some protocols, the release rates continued to be elevated up to 5 days after compression. The patterns of macromolecular release both during and after static and cyclic compression are consistent with several physical phenomena (convection and diffusion) which may be associated with disruption of the collagen meshwork.

The interpretation of the rates of release of radiolabeled macromolecules is influenced by the size and mobility of radiolabeled material released into the medium as well as that remaining in the tissue. In calf cartilage, radiolabeled sulfate is incorporated almost exclusively into proteoglycans, with $>$95% incorporated into the

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Figure 4.10: Sepharose CL-2B profiles of $^3$H-components released into the medium during and just following 24-h cyclic compression. Portions of medium (from the experiment in Figs. 4.3 and 4.9) taken during (A) or in the 12 h following (B) cyclic compression to 0.50 mm (■), 0.75 mm (♦), or 1.00 mm (△), or static compression to 1.25 mm (○), were eluted under dissociative conditions with added carrier calf proteoglycan and calf thymus DNA (as a $V_0$ marker). Fractions were assayed for $^3$H.
large high buoyant density proteoglycans, and <5% incorporated into the small low buoyant density proteoglycans (Fig. 4.6 and [23,117]). The majority of released ³⁵S-macromolecules are large proteoglycans (Fig. 4.4) without a functional hyaluronate binding region (Fig. 4.5), whereas most tissue and ³⁵S-proteoglycans remaining in the cartilage are able to aggregate (Fig. 4.6D–F), consistent with previous studies [23,59]. The parallel trends in the rate of release of ³⁵S-macromolecules and glycosaminoglycans (Figs. 4.2B, D and 4.3B, D) suggest that effects of compression are not specific to the newly synthesized population of proteoglycans, but rather are generalized to all the tissue proteoglycans.

The fate of L-[5-³H]proline is more complex. Much of the ³H within control cartilage disks (24-h at 1.25 mm) was in the form of collagen one day after radiolabeling (~80%) and seven days later (~90%), as deduced by the content of ³H-hydroxyproline. The released ³H-macromolecules from these control disks, as well as from cyclically compressed disks, eluted with a Kₘ ~0.85 on CL-2B and thus were much smaller than the released ³⁵S-proteoglycans.

Compression-induced fluid flow appears to entrain radiolabeled molecules and thereby cause a convective redistribution of molecules within and transport of molecules out from cartilage. During 24-h cyclic compression, significant fluid is reversibly exuded; consistent with a convection mechanism, the release of radiolabeled macromolecules into the medium is dependent on the compression amplitude (Figs. 4.3B and 4.9B). The release of ³H-macromolecules appeared very sensitive to the degree of compression (Fig. 4.9B). The released ³⁵S-macromolecules, which were predominantly composed of the large non-aggregatable mobile ³⁵S-proteoglycans (Figs. 4.4 and 4.5), appear to be derived from the pool of mobile proteoglycans within the tissue, since after cyclic compression to 0.75 mm or 0.50 mm, the aggregability of the tissue and ³⁵S-proteoglycans in the cartilage after such treatment was higher in compressed samples (Fig. 4.6D–F). Although the ³⁵S-proteoglycans lost during cyclic compression to 0.5 mm were of slightly smaller average size than those

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from controls (Fig. 4.4), the overall loss of $^{35}$S-proteoglycans was small (Fig. 4.3A); thus, it was not surprising that the size distribution of $^{35}$S-proteoglycans remaining in the tissue was not markedly altered by cyclic compression (Fig. 4.6A–C). Further, it is possible that the convective transport of $^{35}$S-proteoglycans through the collagen meshwork during cyclic compression to 0.50 mm caused shearing of and thus a size decrease in the released molecules. In addition, preliminary experiments indicate that cyclic compression of cartilage disks, bathed in a saline solution at 4°C with protease inhibitors, still augments the release of $^{35}$S-macromolecules (data not shown).

Compression may have induced stresses or strains large enough to disrupt the collagen meshwork, and thereby allow more rapid diffusion of macromolecules. Cyclic compression caused an amplitude-dependent release of collagenous ($^3$H-hydroxyproline-containing) residues (Fig. 4.9D). Also, the medium of samples subjected to 2- or 12-h static compression or 24-h cyclic compression to 0.50 mm contained $^3$H-components (and to a lesser extent, $^{36}$S-components) which had a higher proportion of radiolabeled macromolecules than the medium of control samples (Figs. 4.3B and 4.9B). Alternatively, the increased release of macromolecules may have resulted from increased enzymatic activity, liberating more mobile fragments of radiolabeled macromolecules.

The patterns of release during and just following 12-h static compression and 24-h cyclic compression suggest that diffusion of some $^3$H- and $^{36}$S-macromolecules out of the cartilage matrix can be impeded by physical barriers at the cartilage surface (e.g., the impermeable compression platens) and by decreased matrix pore size. After either 2- or 12-h compression to 0.5 mm, a marked rebound in the rate of release of $^3$H-macromolecules occurred (Fig. 4.7); in contrast, after prolonged 12-h compression to 0.5 mm, but not after 2-h compression, a marked rebound in the rate of release of $^{36}$S-macromolecules occurred (Fig. 4.1). The former effect would be consistent with disruption of the collagen network and release of entrapped $^3$H-

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macromolecules after unloading. The latter effect could represent diffusion of $^{35}\text{S}$-proteoglycans during the 12-h period of compression to 0.5 mm, redistribution of these macromolecules towards the surfaces with the compression platens, and a rebound release after unloading (removal of the platens); during 0.5 mm compression, diffusion of macromolecules would be hindered by compaction of the matrix, but aided by the shorter distance to the axial surface. As discussed above, the possibility of increased degradation of proteoglycans within the tissue seems unlikely.

When control disks were maintained at a thickness of 1.25 mm for a prolonged 12- or 24-h period, the rates of release of $^{35}\text{S}$- and $^3\text{H}$-macromolecules were slightly decreased compared to rates of the previous day when disks were free-swelling (Figs. 4.1E, 4.3B, 4.7E, and 4.9B); this is consistent with the decreased surface area through which molecules can escape from the cartilage matrix in the uniaxially compressed configuration compared to the free swelling state. Static compression to 0.5 mm further hindered diffusion of $^{35}\text{S}$-proteoglycan macromolecules within and out from the disks (Fig. 4.1E), but affected smaller $^3\text{H}$-proteins to a lesser extent (Fig. 4.7E), consistent with a compression-induced consolidation of matrix.

In addition, in the 12 h following the 24-h test period, the rate of release of $^{35}\text{S}$-labeled macromolecules from 1.25 mm control samples (Fig. 4.3B, D) was increased compared to rates during the previous 1–2 days. This would be expected if during the 24-h period when samples were held between the impermeable compression platens, mobile $^{35}\text{S}$-proteoglycans near the center plane of the disk had diffused axially, toward the surface impeded by the platen; then, after unloading and removal of the platens, these $^{35}\text{S}$-proteoglycans, which were close to a cartilage surface, could rapidly diffuse out into the medium. A similar trend occurred in control samples held at 1.25 mm for 12 h (Fig. 4.1E). The absence of such a rebound in release after moderate cyclic compression to 1.00 mm or 0.75 mm may reflect the competing effects of convection.

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Other recent studies have suggested that static compression induces intratissue acidification, which in turn alters cartilage biosynthesis [48] as well as the extracellular processing of proteoglycans [155]. Thus, it was of interest to determine if the effects of static compression on the rate of release of $^3$H- and $^{35}$S-macromolecules (Figs. 4.1 and 4.7) might be elicited via this physicochemical mechanism. Here, compression did not appear to cause increased release through acidification of the intratissue environment; in contrast, at low pH, there was a slight decrease in release of radiolabeled macromolecules, whereas at high pH, there was a slight increase (Figs. 4.2 and 4.8).

In this study, we did not directly assess the biosynthetic activity of chondrocytes in cartilage disks at times (days) after compression. However, differences in the biosynthetic activity of disks subjected to different treatments may be inferred by comparing differences in the total glycosaminoglycans within a culture (i.e., the sum of the glycosaminoglycans in the tissue at the end of the experiment and the total glycosaminoglycans lost from the tissue into the medium) [63,117]. Such a calculation suggests that over the 5-day period following compression, synthesis of proteoglycans was decreased by 11 and 26 µg/disk per day after cyclic compression to 0.75 mm or 0.50 mm, respectively; this, together with the increased release of proteoglycan (Fig. 4.3), resulted in an altered balance of glycosaminoglycan metabolism. This would be consistent with the decreased cell viability, as judged by DNA content, in disks cycled to 0.50 mm. This is also consistent with the results of previous short-term studies where 24 h of cyclic 2 h on/2 h off compression led to a ~60% decrease in $^{35}$S-sulfate incorporation during the last 8 h of cyclic compression, and $^{35}$S-sulfate incorporation returned to ~control values in the 6–12 h after a single 2-h ~50% compression [49,162].

While these compression protocols produced sizable alterations in the release of macromolecules, quantitative correlation of physical phenomena with biological effects would require detailed physical modeling of compression in the radially un-
confined configuration [4], as well as detailed characterization and localization of
the radiolabeled molecules both released into the medium and remaining in the
tissue. In addition, although there appears to be clear movement and redistribution
of macromolecules within the cartilage matrix, the actual release of material
from cartilage disks in vitro may be affected by surface effects associated with the
excision of the cartilage disks; the release through sliced surfaces could be different
than that through the intact articular surface, which may provide more of a barrier
to release of macromolecules.

In summary, compression caused an increase in release of proteoglycan and
protein macromolecules, consistent with a “loosening” or disruption of the cartilage
meshwork, and altered proteoglycan turnover. Even though these studies were on
cartilage explants from immature animals, such a mechanism is hypothesized to be
involved in osteoarthritis [104]. Thus, it would be of interest to test adult cartilage
explants for similar responses. In addition, certain physical phenomena may have
differential dose-response effects on proteoglycan synthesis and release, as has been
observed for insulin-like growth factor-I [94]. Thus, it would be of interest to ascer-
tain the longer-term effects of more specific biophysical factors (e.g., pressurization
associated with oscillatory compression [162]) on net matrix accumulation, and test
the ability of rapidly growing cartilage to repair or remodel mechanically-induced
matrix changes.

4.6 ACKNOWLEDGEMENTS

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Chapter V

Summary and Future Work

The objectives of this thesis were to further characterize the effects of static and dynamic compression of cartilage on the synthesis, assembly, and release of extracellular matrix constituents, and to relate the compression-induced metabolic effects to possible biophysical regulatory mechanisms. In this chapter, each compression protocol (static compression, compression-release, slow cyclic compression, and oscillatory compression) is considered; specific metabolic effects are summarized and possible biophysical mechanisms of action are described and analyzed. In addition, although extrapolations from the in vitro studies summarized here to the in vivo situation are admittedly speculative, some in vivo analogues are suggested and physiological implications are proposed; validation of these hypotheses would require specific in vivo investigation (e.g., as in [168,170]). Finally, some avenues for future study are outlined.

5.1 Regulation of Matrix Metabolism by Static and Dynamic Compression

5.1.1 Step Compression

Biosynthesis

The application of a static compression leads to a general inhibition of proteoglycan and protein biosynthesis as assessed by $^{35}$S-sulfate and $^3$H-proline incorporation into macromolecules (Chapter II, [10,48,49,75,173]). The inhibition of glycosaminoglycan synthesis appears related neither to a limitation in core protein nor to an impediment of proteoglycan secretion and transport away from the chondrocytes since $^{35}$S-sulfate incorporation was still inhibited in the presence of

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\(\beta\)-xyloside (Fig. B.4). The inhibition of protein synthesis does not appear to be markedly preferential for collagen since there was only a slight decrease in the proportion of \(^3\)H-proline converted into \(^3\)H-hydroxyproline residues (Appendix B). The inhibition occurs with a \(t_{1/2}\) of \(\sim 1-4\) h (Chapter II and [49]). The inhibition is dose-dependent; a 25\% decrease in proline and sulfate incorporation was brought about by a \(\sim 30\%\) compression, equivalent to a static stress of 0.25 MPa (Figs. 2.4, 2.10).

This biosynthetic inhibition during static compression appears to involve several biophysical mechanisms.

(1) The decrease in intratissue pH (or the coupled decrease in \(HCO_3^-\)) that occurs with increased density of fixed charge and Donnan exclusion appears to account for part of the biosynthetic inhibition (Fig. B.7 and [48]). The relation between extracellular (intratissue) pH within cartilage and intracellular pH (pH\(_i\)) in chondrocytes is not clear; in many cell types, the steady state pH\(_i\) is strongly influenced by the extracellular HCO\(_3^-\) and activity of the Cl\(^-\)/HCO\(_3^-\) antiport [96]. Since pH\(_i\) is well-suited to be the intracellular coordinator of diverse metabolic processes [20], the hypothesized biosynthetic regulation by intratissue pH or HCO\(_3^-\) induced alteration of pH\(_i\) is attractive; increased pH\(_i\) usually causes a general stimulation of cellular metabolic activity and can advance the cell cycle from the \(G_0/G_1\) state to the S state [96].

(2) The increase in matrix osmotic pressure that occurs with increased fixed charge density (primarily due to the increase in intratissue sodium concentration) may account for part of the biosynthetic inhibition [173,187]. When the concentration of sodium in the bathing medium was increased above 1.2 times the physiologic concentration, biosynthesis fell [187]; further, in medium at 0.5 times the physiologic sodium concentration, compression (osmotic stress) caused a marked increase in biosynthesis [187]. In addition, it has been observed that there is a slight increase in biosynthesis at very low stress [48]; this is consistent with a biosynthetic peak in medium having a sodium concentration slightly greater than physiologic levels

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[187]. It is interesting that osmotic perturbation of cell volume can alter the activity of the Na⁺/H⁺ antiport and thus may also cause alterations in pH,[51]. An added complexity would be the existence of an Na⁺/HCO₃⁻ symport, although this transporter is quite specific to renal tubular cells[96].

(3) The hindered diffusion or exclusion of macromolecules due to matrix consolidation may also account for part of the biosynthetic inhibition (Chapter II). Biosynthesis in adult cartilage appears more sensitive to compression than that in calf cartilage; a 50% biosynthetic inhibition was brought about by either a ~10% decrease in water content in adult human cartilage[10,173] or a ~50% decrease in water content in calf cartilage (Chapter II and[48], see also[49] for summary). This difference cannot be explained by age-related changes in charge density; on the contrary, adult bovine cartilage has a lower charge density than calf cartilage[62]. Alternatively, the difference may be related to the porosity of the collagen meshwork; while the cartilage from aged (five year old) pigs has a tight meshwork (uronic acid/ww of 0.029 and a dw/ww of 0.36), that from young (fetal) pigs has a relatively looser meshwork (uronic acid/ww of 0.076 and a dw/ww of 0.15[121]).

The characteristic tissue dimensions also appear important in the compression-induced biosynthetic inhibition. In experiments with calf cartilage, very thin (0.7 mm thick) strips of cartilage[75] are less sensitive to compression than 2 mm diameter disks [48], which in turn are less sensitive than 3 mm diameter disks (Chapter II); these specimens of increasing dimension exhibit a 50% reduction in proteoglycan synthesis at a stress of 3 MPa[75], 1 MPa[48], and 0.4 MPa (Figs. 2.4, 2.10), respectively. Thus, the characteristic tissue dimension as well as the porosity of the collagen meshwork appear to implicate diffusional transport.

In adult articular cartilage, the effective pore radius is computed to be 4–9 nm[101]; indeed, the partitioning of macromolecules such as albumin which has a Stokes radius of ~3.5 nm is quite sensitive to tissue hydration[101]. Molecules in the medium which may be impeded from entering cartilage include growth factors, such
as insulin-like growth factor I, which is a potent stimulator of glycosaminoglycan synthesis, as well as its serum carrier protein (Chapter II, [105]). Also, newly synthesized molecules secreted into the cartilage matrix by chondrocytes may be hindered from diffusing away from the cell surface; the inability of newly secreted proteoglycans to diffuse away would lead to an even greater local decrease in pH near the cell. It is interesting that growth factors generally exert their stimulatory effect via activation of the Na⁺/H⁺ antiport, thereby increasing pH₄ [96,112].

(4) Alterations in cell shape and the cytoskeleton may be involved in the biosynthetic effects. The effect of compression on cell shape has been observed at both the light [16] and electron microscopic [144] levels. Maintenance of the chondrocytes in the rounded shape by cell culture within agarose gels is associated with expression of the type II collagen phenotypical of cartilage chondrocytes [14]. The cytoskeleton appears to play a critical role in this phenotypical expression [13] and may act as a mechanotransducer [138]; alternatively, the cilia of chondrocytes may play a sensory role [143], as they do in other cell types such as vestibular hair cells.

Proteoglycan Maturation

Static compression leads to a dose-dependent delay in the extracellular conversion of newly synthesized ³⁵S-proteoglycans from the form with low affinity for hyaluronate to the form with high affinity (Chapter III). The t₁/₂ for conversion was prolonged from ~4.5 h in 1.25 mm control samples to 8–9 h in 0.5 mm compressed disks (Fig. 3.4A). Preliminary results suggest that the low-affinity proteoglycan may have a higher effective mobility than high-affinity proteoglycan (Appendix C).

The decrease in intratissue pH with compression appeared to completely account for this delay in conversion since incubation in acid-titrated medium without compression caused a similar delay (Fig. 3.4B). This is consistent with the catalysis of affinity conversion in solutions of purified proteoglycan by mild alkali treatment.
Protein and Proteoglycan Release

Prolonged 12- or 24-h static compression leads to a decrease in the release of $^{35}$S-proteoglycan (Figs. 4.1D-F, 4.3) and, to a lesser extent, $^3$H-protein (Figs. 4.7D-F, 4.9B-C).

*Hindered diffusion* appears to be involved since there was a sizeable decrease in $^{35}$S-proteoglycan release in disks confined between but only slightly compressed by impermeable platens (Fig. 4.1D-F, 4.3B). Decreased intratissue pH did not account for this decreased release, although increased medium pH caused an increased release of $^{35}$S-proteoglycan and $^3$H-protein (Figs. 4.2, 4.8). This would be consistent with a pH-induced stimulation of chondrocyte metabolic activity [96], including activation of catabolic enzymes. On the other hand, matrix depletion by Chondroitinase ABC apparently caused a decrease in proteoglycan loss and catabolism since net proteoglycan deposition was stimulated in the absence of prolonged biosynthetic stimulation (Appendix E).

*In vivo* Correlates

Several physiologic scenarios would cause a prolonged static compression *in vivo*, analogous to these *in vitro* experiments: (1) when a joint is maintained in a fixed position under stress for a prolonged time (e.g., sitting, standing); (2) when a joint is subjected to dynamic loading, there is a time-average static component of load that would lead to creep compression.

When cartilage in a joint is subjected to prolonged compression, it would seek to distribute stresses over a broad area of tissue (Fig. 5.1A). To accomplish this, it would seem reasonable that regions subjected to high compressional stress would need to be inhibited from growing while surrounding regions of low stress would need to be stimulated. Thus, compressed regions are inhibited from synthesizing

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Figure 5.1: Hypothesized physiological adaptation of cartilage subjected to prolonged static compression. (A) Initially, load is concentrated in one region of cartilage, while adjacent regions are unloaded. Synthesis and release of proteoglycan is inhibited in loaded region, and relatively higher in unloaded regions. (B) Later, adjacent region has grown and load is distributed over larger region of cartilage.

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matrix molecules compared to the surrounding less-compressed regions. Deposition of the newly synthesized proteoglycans may be favored in local regions of low charge density (and functionally low swelling pressure) by enhanced proteoglycan affinity conversion in these regions of relatively higher pH. At the same time, consolidation of the matrix in the more loaded regions where synthesis is inhibited serves to decrease effective pore size and hinder loss of proteoglycans. Concurrently, adjacent regions could grow, accumulate proteoglycans, and eventually share in distributing the stress (Fig. 5.1B).

5.1.2 Compression-Release and Slow Cyclic Compression

Biosynthesis

Release of a 2–12-h compression leads to a transient rebound stimulation in $^3$H-proline uptake, (Fig. 2.12, [49]). The rebound appears independent of protein synthetic processes since it was not reduced by cycloheximide-inhibition of protein synthesis (Chapter II). The effect of compression release on proteoglycan synthesis is also time-dependent; proteoglycan synthesis in disks returned to the free-swelling state is affected by previous compression lasting for 2 h (Fig. 2.12 and [49]), 12 h [49,50] or 2 d [75], occurring as much as 6 h–2 d after the compression is released. Cyclic (2-h on/2-h off) compression leads to biosynthetic effects consistent with the dosimetry and kinetics of effects of a single compression and release. Cyclic compression at moderate amplitudes (1.25 to ~1.00 mm) led to a slight increase in both proteoglycan and protein synthesis (Fig. 2.14); here, the slight increase in the proportion of $^3$H-proline converted into $^3$H-hydroxyproline may represent a slight preferential stimulation of collagen synthesis (Fig. B.5). However, high amplitude cyclic compression (1.25 to 0.5 mm) caused a marked inhibition of proteoglycan synthesis (Fig. 2.14).

Besides the mechanisms noted above relating to compression-induced alterations in charge density and matrix consolidation, fluid flow may be relevant. One
method of assessing the extent of fluid flow is to estimate the time-average volume flux. In a 2-h compression from 1.25 to 0.5 mm, the time-average rate of fluid flux is approximately \( \pi \cdot (1.5 \text{ mm})^2 \cdot (0.75 \text{ mm})/2 \text{ h} \); the corresponding time-average fluid velocity normalized to the disk area is \( \sim 0.1 \mu \text{m/s} \). Similar levels of fluid transport are predicted to occur via electroosmosis in the applied current experiments (0–10 mA/cm\(^2\) direct current, Appendix C; and 1 mA/cm\(^2\) sinusoidal current at \( f=0.1–100 \text{ Hz, [50,161]} \)); however, none of these 12-h electroosmotic stimuli elicited a stimulation in biosynthesis (Fig. C.7 and [50,161]).

Protein and Proteoglycan Release

Release of a 12- or 24-h compression leads to an increase in release of \( ^{35} \text{S-proteoglycan} \) (Figs. 4.1D-F, 4.3) and \( ^{3} \text{H-protein} \) (Figs. 4.7D-F, 4.9B-C). Cyclic (2-h on/2-h off) compression causes an amplitude-dependent enhancement of this release both during and following compression (Figs. 4.3 and 4.9). The release of \textit{in vitro} labeled \( ^{35} \text{S-proteoglycan} \) and endogenous tissue proteoglycan appear similarly affected (Figs. 4.3B, D). The release of \textit{in vitro} labeled \( ^{3} \text{H-collagen} \) residues appear particularly sensitive to compression; the medium became greatly enriched with \( ^{3} \text{H-hydroxyproline} \) residues during and following compression (Fig. 4.9D).

The \textit{removal of diffusion barriers} with unloading appears to account for the increased release from disks confined but only slightly-compressed by impermeable platens (Fig. 4.1D-F, 4.3B, the converse of the hindered diffusion discussed above).

In addition, \textit{fluid flow} appears to account for much of the cyclic accentuation of release. Similar levels of fluid transport, induced by electroosmosis (see above) caused a 25–40% increase in \( ^{35} \text{S-proteoglycan} \) and proteoglycan release from cartilage stimulated in 37°C medium (Fig. C.1). Fluid flow appeared to directly affect extracellular constituents since there was little alteration in the population of \( ^{35} \text{S-proteoglycans} \) released (Fig. C.2D-F) and the effect also occurred in an enzyme-inhibiting buffer at 4°C (Figs. C.4, C.5, C.6).

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High levels of stress also appear to affect the collagen matrix. Not only is medium enriched with collagen-derived residues (Fig. 4.9D), but also, preliminary results indicated that cartilage disks exhibit macroscopic swelling and fissuring after 48-h cyclic compression between 1.25 and 0.50 mm in the enzyme-inhibiting buffer (Appendix C).

5.1.3 Oscillatory Compression or Elongation

Biosynthesis

Oscillatory compression or elongation of chondrocyte-laden tissue at frequencies of ≥0.01 Hz and amplitudes >1% can cause a stimulation of biosynthesis. Oscillatory compression of cartilage disks at 1–5% axial displacement and frequencies of 0.01–1 Hz led to a 20–40% stimulation of proteoglycan and protein biosynthesis, as assessed by $^{35}$S-sulfate and $^3$H-proline incorporation (Fig. 2.15). Such stimuli may elicit a mild preferential stimulation of collagen synthesis since there was a slight increase in the proportion of $^3$H-proline converted into $^3$H-hydroxyproline (Fig. B.6). Cyclic 5.5% stretching of high-density chondrocytes at 0.2 Hz led to a similar stimulation of proteoglycan synthesis [31]; however, the response of this tissue was somewhat different in that protein synthesis ($^3$H-glycine incorporation) was unaffected, but DNA synthesis ($^3$H-thymidine incorporation) was stimulated. The stimulation does not appear to occur immediately with compression or stretching since synthesis was stimulated during the last 8 h or 23-h compression (Chapter II) and during the last 4 h of 24-h stretching [31], but not during the initial 4-h of stretching [31]. The stimulation in biosynthesis may be related to an increase in cAMP [31].

Several physical mechanisms by which this oscillatory compression or stretching stimulates biosynthesis are possible. To gain some insight into possible effectors, Fig. 5.2 shows the biosynthetic response to 1–2% oscillatory compression (relative sulfate incorporation, Fig. 2.15) juxtaposed with the measured dynamic stiffness
Figure 5.2: Correlation of biosynthetic response to 1–2% oscillatory compression at various frequencies with dynamic stiffness. (A) The incorporation of $^{35}$S-sulfate into cartilage disks during the last 8-h of 23-h compression was normalized to that into disks held statically (mean ± SEM, n = 12–72). Data is compiled from Tab. 2.1 and Fig. 2.15. (B) Dynamic stiffness of cartilage disks during oscillatory compression experiments. Data is from Fig. 2.8B.
amplitude (Fig. 2.8). The stimulation in biosynthesis occurs at a frequency (0.01 Hz) just where dynamic stiffness increases significantly. This suggests possible correlations between biosynthesis and hydrostatic pressure which would be maximal at the center of the disk, or dynamic strain, fluid flow, or streaming potentials which would be maximal at the radial edge of the disk [4,78].

The effects of hydrostatic pressure on biosynthesis are somewhat conflicting, with stimulatory, inhibitory, and null effects all having been reported [54,55,84,92]. It has been emphasized that the effects of hydrostatic pressurization per se must be distinguished from increases in partial pressure of gas surrounding and within the medium [84]; increased pCO₂ would lead to alteration of the medium and intratissue pH and thereby would be predicted to alter chondrocyte biosynthesis through a non-physiologic mechanism.

Fluid flow may have a role even though it is predicted to be localized at the outer annulus of the disk. An applied displacement of 1% at 0.1 Hz corresponds to an average velocity (or volume displacement normalized to area) of 1μm/s. However, this is likely to be an overestimation of the relative fluid velocity since the solid matrix also expands radially [4,17,78]. Nevertheless, this velocity is of the same order as the electroosmotic fluid velocity in the applied current experiments described above (0–10 mA/cm² direct current, Appendix C; and 1 mA/cm² sinusoidal current at f=0.1–100 Hz, [50,161]) where there was no biosynthetic stimulation. However, it is interesting that slightly stronger sinusoidal fields of ~10–30 mA/cm² at f=100 and 10,000 Hz appear to stimulate protein synthesis [95]. This suggests that streaming potentials and associated fluid velocities may have cellular effects.

Proteoglycan Maturation

Oscillatory compression of 2% amplitude at frequencies of 0.001–0.1 Hz did not markedly alter affinity conversion of newly synthesized ³⁵S-proteoglycans (Fig. 3.5).

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This is consistent with the hypothesis that intratissue pH is the mediator of proteoglycan affinity conversion, since during such low amplitude dynamic compression, there would be little dynamic alteration in intratissue pH as well as no average alteration in pH (Chapter III).

Protein and Proteoglycan Release

The effects of oscillatory compression amplitude and frequency on protein and proteoglycan release have not been assessed systematically in cartilage explants. However, low amplitude 2-h cyclic stress did not affect $^{35}$S-proteoglycan release [132].

In vivo Correlates

Several physiologic scenarios would provide dynamic loading of cartilage, although the physical phenomena which occur may be quite dependent on the joint geometry and loading patterns. During ambulation, the femoropatellar cartilage is subjected to cyclic stress as the patella traverses the femoropatellar groove [74]; the location of patellofemoral contact would vary with the degree of flexion and lead to cyclic fluid transport out from the uncovered femoropatellar cartilage surface [87]. On the other hand, the femoral head cartilage is subjected to dynamic stress with the femoral head pivoting in the acetabulum; some areas of the femoral head cartilage may remain continuously opposed to acetabular cartilage during ambulation and provide a higher resistance to fluid exudation [1,69].

When cartilage is actively used and subjected to dynamic loading, it would need to continue to remodel. To accomplish this, it would seem reasonable that biosynthesis needs to be stimulated to keep up with the increased rate of matrix loss induced by repetitive compression. Indeed, the absence of in vivo dynamic compression is both clinically and experimentally associated with a decrease in proteoglycan synthesis, a loss of glycosaminoglycan, and cartilage degeneration

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[2,27,33,128,134,154]. In contrast, increased dynamic loading in vivo leads to a maintenance or increase in proteoglycan synthesis and content [27,85,135,191], and continuous passive motion appears to stimulate cartilage growth and remodeling [124,164]. However with the abnormal loading induced by anterior cruciate ligament transection, the balance between synthesis and degeneration of matrix is altered such that despite an increase in collagen [36] and PG metabolism [25,26,168], osteoarthritis ensues [103,104,141].

5.2 Future Work

5.2.1 Assessment of Role of Specific Physical Phenomena

To elucidate the role of a specific physical phenomenon in regulating cellular metabolism, a more definitive relationship between the hypothesized extracellular effector (e.g., intratissue pH) and an intracellular regulator (e.g., pH\textsubscript{i}) must be established. Ideally, the quantitation of physical phenomena would be non-destructive and in real-time. Thus, the development of new and application of existing methodologies to analyze living cartilage would be useful.

(1) Quantitate macromolecular diffusion coefficients during varying levels of static compression and tissue hydration, and assess macromolecular transport during dynamic compression (e.g., [100]). Compression can be achieved via direct mechanical stress, or alternatively, via current induced stress [42]. Macromolecules of special interest include low- and high-affinity proteoglycans as well as radio-labeled growth factors. To differentiate enzymatic from physical mechanisms of macromolecular release, the 4°C affinity-stabilizing protease-inhibiting solution of Appendix C can be used.

(2) Quantitate intracellular and intratissue ion concentrations in different regions (pericellular, territorial, inter-territorial matrices) during different levels of static compression. Preliminary findings suggest that nuclear magnetic resonance
can assess ion content in cartilage [91].

(3) Assess cell shape and volume. Microscopy of the superficial layer of cartilage under compression confirms that regions of cartilage are deformed inhomogeneously [123], with the glycosaminoglycan-poor surface region compressed more than the underlying glycosaminoglycan-rich regions. There may be subtle biosynthetic differences between cartilage subjected to radially confined and unconfined compression especially in chondrocytes at the radial edge [50], and such differences may be related to cell shape or osmotic alterations in cell volume.

(4) Quantitate chondrocyte membrane potential. Since extracellular (intratissue) ion concentrations (and corresponding potentials) vary greatly with compression, it would be predicted that cellular transmembrane potential also would vary greatly with compression. Such alterations in transmembrane potential could in turn alter voltage-sensitive membrane channels.

5.2.2 More detailed Characterization of Cellular and Biochemical Responses

The effects of these biophysical stimuli could be benefited by more detailed spatial, biochemical and molecular characterization of the cellular and extracellular alterations. Spatial correlates of biosynthetic response, affinity change, and matrix release with physical phenomena may implicate the role of specific biophysical control mechanisms. Localization of radioactivity can be achieved by cutting tissue to isolate the regions of interest. Alternatively, localization of cells either actively producing sulfated proteoglycans can be assessed by $^{35}$S-sulfate radiolabeling and autoradiography (e.g., [168]) or generating specific mRNA can be achieved by in situ hybridization [137,166]. Spatial studies (such as comparing the central region of the cartilage disk to the annular surround) would be especially useful in assessing biosynthesis after terminating an imposed mechanical protocol; during such radiolabeling, transport of precursor would be quite uniform throughout the entire

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cartilage disk.

There are many other biochemical parameters which may be important in assessing short- or long-term chondrocyte response.

(1) Quantitate hydroxyproline in cartilage explants and medium. Methods for this indirect assessment of collagen content have been described [177,194].

(2) Assess the role of growth factors in the metabolic response of chondrocytes to physical stimuli. Biophysical stimulation experiments could be conducted in defined medium with, for example, insulin-like growth factor I [105]. Alternatively, cartilage conditioned medium could be analyzed for biologically active factors (e.g., by 35S-sulfate incorporation) as well as more specifically for individual growth factors.

(3) Assess the synthesis of hyaluronate. Since the mechanism of hyaluronate synthesis is quite different than that of protein and proteoglycan synthesis, it would be of interest to assess the possible coordinate regulation of hyaluronate and proteoglycan metabolism (e.g., [113]) during biophysical stimulation. This assessment requires the analysis of specific activity in precursor pools [114].

(4) Assess the maturation of collagen cross-links. The degree and maturation of collagen cross-links may influence the tensile properties of the extracellular matrix, and thus be a biochemical indicator of the stability of the extracellular matrix [38]. Methods to quantitate cross-links in articular cartilage have been described [35].

(5) Quantitate type IX collagen in cartilage and medium. Since type IX collagen is covalently cross-linked to type II collagen [34,190], it may have an important structural function in the cartilage matrix. It would be of interest to assess how physical stimuli affect the synthesis, deposition, and loss of this potentially critical link in the extracellular matrix.

(6) Quantitate small dermatan-sulfate proteoglycans in cartilage and medium. Two small dermatan-sulfate proteoglycans have been identified [149] in

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cartilage and extensively characterized [29,152,165]. These proteoglycans may have an important structural function in cartilage, as they have a role in regulating collagen fibrillogenesis in other connective tissues [149]. In cartilage, the content of these small proteoglycans declines somewhat with aging [165]. Thus, as with type IX collagen, it would be of interest to assess how physical stimuli affect the synthesis, deposition, and loss of small proteoglycans in the cartilage extracellular matrix.

(7) Assess the activity of collagen and proteoglycan degrading proteases. It has been observed that experimentally-induced osteoarthritis leads to an increase in neutral metalloproteoglycanases in both active and latent forms in cartilage [136]. It would be of interest to assess if physical stimuli affect matrix catabolism by induced chondrocytes to synthesize or activate these enzymes.

(8) Assess mRNA levels. Methods for extracting chondrocyte mRNA from cartilage explants and quantitating specific mRNA levels by dot blot hybridization with cDNA probes have been described [167]. Such methods have been used to assess the modulation of procollagen I, II, and III mRNA levels in chondrocyte cultures by γ-interferon and interleukin 1 [46,47]. Effects of physical stimuli could thus be assessed at an earlier molecular step in the biosynthetic process.

5.2.3 Cellular Perturbations

Growth factors and pharmacological agents may be useful in analyzing the cellular response.

(1) Assess the participation of single or multiple growth factors [130] in mediating biosynthetic responses. How do growth factors affect the pH₄ of chondrocytes in cartilage?

(2) Assess the role of specific ion channels via pharmacologic inhibitors such as amiloride, 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid (SITS), or 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) [96].

(3) Assess the role of cyclic AMP by using phosphodiesterase inhibitors such
as theophylline, cyclic AMP analogues (such as dibutryl-cyclic AMP), or forskolin which activates the catalytic subunit of adenylate cyclase [12].

(4) Assess the role of cell shape and cytoskeleton by microfilament modification with dyhydrocytochalasin B [13].

5.2.4 Extracellular Matrix Perturbations

Another tactic is to decouple tissue deformation from changes in charge density by creating a charge-depleted matrix. However, chondrocytes in such a matrix may exist under greatly different conditions. Such matrices would be more porous and be expected to allow more rapid diffusion of macromolecular growth factors as well as newly synthesized macromolecules. Indeed, a large proportion of synthesized macromolecules have been observed to escape from porous immature and matrix-depleted tissue into the medium [41,60]. Also, when such matrices are compressed, cell shape may be altered in a way different than in normal proteoglycan-rich cartilage matrices. Cells may be susceptible not only to compression but also to the matrix depletion procedure; thus, it would be critical to determine cellular viability after such a treatment. Nevertheless, several methods exist for achieving such a matrix depletion.

(1) Chondroitinase ABC can be used to achieve a fairly complete depletion of the matrix charge-containing glycosaminoglycans (Appendix E). Protease-free enzyme preparations should be used. Biosynthesis recovers to approximately control levels within 1–3 days (Fig. E.6, E.8). Preliminary results of such an experiment were difficult to interpret because a shorter duration Chondroitinase treatment was used and removed only ~40–75% of the tissue glycosaminoglycan (Appendix E and [156,157]).

(2) Trypsin has also be used to deplete the cartilage matrix [6,63]. The recovery of biosynthesis depends on the extent of the digestion [6].

(3) Cytokines such as interleukin 1 or lipopolysaccharides, possibly in

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medium without serum, can be used although matrix depletion requires fairly length treatment (∼weeks) [114,117]. During this time, explant shape may change and make subsequent physical stimulation difficult to interpret.

(4) Cartilage can be cultured in the presence of xylosides to produce a glycosaminoglycan-deficient matrix. Such a method has been used to alter the matrix formed by corneal endothelial cells and subsequently assess the effect of such matrices on these cells [147]. Since control experiments showed a slight inhibition of protein synthesis (³H-proline incorporation, Fig. B.4), more specific and potent xyloside analogues could be tried [146,148,175].

5.2.5 Characterization of Biophysical Effects During Long-term Culture

Longer term studies of the cumulative effects of physical perturbations on matrix synthesis and release could be characterized by measurement of physical properties (e.g., via electromechanical methods [42,52,90]). Biochemical correlates could include, for example, DNA (cells), glycosaminoglycan, hydroxyproline, and collagen cross-links. Such biochemical and physical correlations have demonstrated the initial formation of a mechanically function matrix in chondrocyte-laden agarose gels [21].

Long-term in vitro studies may thus allow studies on the macroscopic alteration of tissue structure which occur over a time frame similar to that in vivo during articular cartilage formation, molding, or degeneration. For example can cartilage be molded by static compression of the center of a disk (or the annulus of a disk) to inhibit growth and to allow the non-compressed region to grow relatively more? Can this molding be accomplished more readily by dynamic compression? Can an articular surface layer similar to that in vivo be generated by the appropriate shear stress? Can high amplitude static or dynamic compression lead to either a mechanical failure of the tissue or an imbalance between synthesis and loss of matrix

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components? What physical phenomena are responsible? Can osteoarthritis-like changes be simulated in a controlled mechanical environment *in vitro*, and are such changes reversible?
Appendix A

Control Studies on $^{35}\text{S}$-Sulfate, $^{3}\text{H}$-Proline, and $^{35}\text{S}$-Methionine Metabolism by Cartilage Explants

A.1 INTRODUCTION

The incorporation of radiolabeled precursors during continuous radiolabeling reflects a number of physical and biological processes (see Chapter II). Thus, the deduction of chondrocyte biosynthetic rates from radiolabel uptake requires consideration of several issues. How quickly does the isotope reach a uniform concentration within the tissue? Are intracellular and extracellular specific activities the same? How can free isotope be separated from radiolabel taken up into cells or incorporated into macromolecules? Are the biosynthetic rates steady over time? Into what molecules are the isotopes incorporated? How are the newly synthesized materials distributed between tissue and medium?

Likewise, the release of incorporated radiolabel can be used to assess the catabolism and rate of release of cartilage components. This requires characterization of the original radiolabeled material (pulsed tissue), the remaining material (pulse-chased tissue), as well as that released into the medium. What are the radiolabeled tissue and medium components? Do they form a homogeneous or heterogeneous pool? Are the release rates steady? Are the catabolized molecules salvaged by the chondrocytes and reutilized?

The objective of this study was to address a number of these issues which arise in using the isotopic precursors $^{35}\text{S}$-sulfate, $^{3}\text{H}$-proline, and $^{35}\text{S}$-methionine to assess metabolism in free-swelling calf cartilage explants maintained in medium including 10% FBS.
A.2 METHODS

Cartilage disks, 3 mm diameter x 1 mm thick were explanted and maintained in DMEM including 10% FBS, 10 mM Hepes, 0.1 mM non-essential amino acids, an additional 0.4 mM L-proline, 20 μg/ml ascorbate (Chapter II), or in modified medium formulations as noted. The medium was changed daily, and on the first day, supplemented with antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin).

A.2.1 ³H-Proline and ³⁵S-Sulfate Incorporation in Medium with Altered Concentration of Proline and Sulfate

Cartilage disks were incubated immediately after explant for 8 h in medium with either (a) 15 μCi/ml ³⁵S-sulfate and added sodium sulfate to a final concentration of 0.8, 1.2, 1.6, or 3.2 mM sulfate or (b) 20 μCi/ml ³H-proline and added proline to a final concentration of 0.02-50 mM proline. In the latter, 10% FBS contributed 0.02 mM proline, 0.1 mM non-essential amino acids contributed 0.1 mM proline (and was omitted to attain proline concentrations < 0.12 mM), and additional proline was added to attain proline concentrations > 0.12 mM. Radiolabeled disks were washed, digested, and analyzed for ³H or ³⁵S radioactivity.

A.2.2 Continuous Radiolabeling

Cartilage disks were explanted, maintained for 2 days, and then radiolabeled for 1-8 h with either (A) 5-10 μCi/ml ³⁵S-sulfate and 5-20 μCi/ml ³H-proline, or (B) 10 μCi/ml ³⁵S-methionine. After radiolabeling, each disk was serially washed six times over 90 min with PBS supplemented with 0.8 mM sodium sulfate, 1 mM proline, and 1 mM methionine at 4°C (1 ml/disk per wash) and lyophilized.
A.2.3 Pulse-Chase

Alternatively, after 8-h radiolabeling, each disk was serially washed with temperature and CO₂ equilibrated medium four times over 120 min (1 ml medium/disk per wash). Disks were then maintained for an additional 1–3 days. After this chase period, disks were washed with PBS as above and lyophilized. The daily spent medium was collected and stored at −20°C for later analysis.

A.2.4 SDS Extraction of Cartilage Disks and Quantitation of Radiolabeled Macromolecules

Dried cartilage disks were extracted with 2% SDS, 10 mM dithiothreitol, 50 mM Tris-HCl, pH 8.5 at 100°C for 5–30 min during initial control experiments, and for 15 min in all subsequent experiments. The extracted disks were washed three times over 90 min with PBS, digested with papain, and analyzed for radioactivity. The extracts were either stored at −20°C or immediately cooled to room temperature for analysis.

Portions (0.5 ml) of cartilage extracts or spent medium (made to 1% SDS by addition of 10% SDS) were separated on a PD10 column of Sephadex G-25 (MW cutoff of 1–5,000; Pharmacia, Piscataway, NJ). The elution buffer was the same as the extraction buffer, except the SDS concentration was reduced to 1%. Fractions (0.5 ml) were collected and analyzed for radioactivity. Alternatively, the macromolecular (V₀) and low molecular weight (V₁) component peaks were pooled after control studies showed that separation of radioactivity to near baseline was achieved (e.g., Fig. A.1).

In analysis of radiolabel incorporation into cartilage disks, the radioactivity in the V₀ fraction and that remaining in the disk were summed and defined to be macromolecular. The kinetics of formation of macromolecular product, P(t), during radiolabeling time t was described by fitting $P(t) = R \cdot [t + (e^{-t/\tau_p} - 1)\tau_p]$ to obtain $\tau_p$ using an iterative method of least squares [174]; $\tau_p$ is the average lifetime of a
Figure A.1: Fractionation of SDS extracts of radiolabeled cartilage disks on Sephadex G25 (PD10). Cartilage disks were radiolabeled for (●) 1 h or (■) 8 h with ^35^S-sulfate, ^3H-proline, or ^35^S-methionine, washed, and then extracted with 2% SDS, 50 mM Tris, 10 mM Dithiothreitol, pH 8.5 for 15 min at 100°C. A 0.5 ml portion of the extract was applied to a Sephadex G25 column and eluted with the same buffer, but with 1% SDS; fractions (0.5 ml) were collected and analyzed for radioactivity.

Section A.2
molecule in a single lumped intermediate pool (representing all sequential interme-
diates between the initial radiolabel added to the media and the macromolecules
produced) [83]. To allow comparison with other studies [79,109], this time constant
is converted to \( t_{1/2} = \tau_p \cdot \ln 2 \).

A.2.5 Quantitation of \(^3\)H-Hydroxyproline

Portions of medium (1 ml) or radiolabeled cartilage disks (~12 mg wet
weight) were analyzed for \(^3\)H-hydroxyproline residues essentially as described pre-
viously [178]. Samples were lyophilized, hydrolyzed with 0.2–0.4 ml 6 N HCl at
105°C in teflon-lined pyrex tubes for 16 h, diluted to 0.9 M HCl, and filtered
through a 0.22 \( \mu \)m membrane. A 0.5 ml portion of the filtrate was then run on
a 0.7×10 cm column of Dowex 50W (X8; 200–400 mesh), equilibrated and eluted
with 0.9 N HCl. Fractions (1 ml every 3 minutes) were collected and analyzed for
radioactivity. Standard samples of hydroxyproline and \(^3\)H-proline eluted at 7–11 ml
and 16–24 ml, respectively. Recovery of \(^3\)H was ~90%.
A.3 \( ^{35}\text{S}}\)-Sulfate Metabolism: Results and Discussion

A.3.1 Effect of Total Sulfate on \( ^{35}\text{S}}\)-Sulfate Incorporation

When the concentration of sulfate in the medium was increased from 0.8 to 3.2 mM while keeping the radiolabel concentration at 15 \( \mu \text{Ci/ml} \), the incorporation of \( ^{35}\text{S} \) was decreased by a factor of four (Fig. A.2). Thus, the incorporation of \( ^{35}\text{S}\)-sulfate into free swelling cartilage disks was proportional to the specific activity of sulfate in the medium (\( ^{35}\text{S}/\text{mol sulfate} \)). \( ^{35}\text{S}\)-sulfate incorporation was similarly independent of the concentration of sulfate in the medium (at 0.5–0.8 mM) for human and bovine articular cartilage \([102]\) and (at 0.8–1.6 mM) for calf epiphyseal cartilage \([48]\). The results here were consistent with (a) equilibration of intracellular and extracellular sulfate during 8-h radiolabeling, and (b) no limitation of sulfate incorporation by the concentration of sulfate in the medium. Thus, the rate of sulfate incorporation can be quantified \([99]\).

A.3.2 SDS Extractability of \( ^{35}\text{S} \)-Sulfate Labeled Cartilage Explants

To assess the distribution of radiolabel incorporated into low-MW components and macromolecules, cartilage disks were radiolabeled with \( ^{35}\text{S}\)-sulfate for 8 h and then extracted with SDS under conditions which should permeabilize cell membranes and release both intracellular and extracellular low-MW components. Control studies were performed to determine the duration of extraction sufficient to achieve maximal solubilization of low-MW \( ^{35}\text{S} \)-components. More low-MW \( ^{35}\text{S} \)-components were solubilized after extraction for 10–30 min than after extraction for 5 min (Fig. A.3). Thus, all subsequent extractions were for 15 min.

The extraction of macromolecular \( ^{35}\text{S} \)-components increased up to the longest extraction duration (30 min), when \(~1/3\) of the \( ^{35}\text{S} \)-macromolecules were released.

Section A.3
Figure A.2: Effect of sulfate concentration in the medium on $^{35}$S-sulfate incorporation by cartilage explants. Cartilage disks were radiolabeled for 8 h in medium containing 15 $\mu$Ci/ml $^{35}$S-sulfate at various concentrations of sulfate, washed, and analyzed for $^{35}$S (mean ± SEM, n = 4).
Figure A.3: Time course of SDS extraction of $^{35}$S-components from $^{35}$S-sulfate labeled cartilage explants. Cartilage disks were radiolabeled for 8 h in medium containing 5 μCi/ml $^3$H-proline and 10 μCi/ml $^{35}$S-sulfate, and washed with PBS. Disks were then extracted with 2% SDS, 50 mM Tris, 10 mM Dithiothreitol, pH 8.5 at 100°C for various durations. The extract was separated into macromolecular ($V_0$) and low-MW ($V_1$) components on Sephadex G25. Total (×) $^{35}$S-radiolabel was computed as the sum of that (▲) remaining in cartilage disks, and extracted as (●) macromolecular and (■) low-MW components (mean ± SEM, n = 4).
A.3.3 Conversion of $^{35}$S-Sulfate into $^{35}$S-Macromolecules During Continuous Radiolabeling

To assess the kinetics of $^{35}$S-sulfate incorporation into macromolecules, cartilage disks were radiolabeled for 1–8 h, washed, and analyzed for $^{35}$S-macromolecules (Fig. A.4A). Even after the 1-h radiolabel, most (>96%) of the incorporated $^{35}$S was in the form of $^{35}$S-macromolecules (Fig. A.4B). When the radiolabel duration was increased to 8 h, the proportion of incorporated $^{35}$S in macromolecules increased to >99%.

Inhibition of chondrocyte protein synthesis with cycloheximide (Fig. A.5A) reduced incorporation of $^{35}$S-sulfate into macromolecules by >97%, consistent with the dependence of the formation of $^{35}$S-glycosaminoglycans on the continued synthesis of proteoglycan core protein. Nevertheless, some core protein appeared available for addition of sulfated glycosaminoglycan since of the incorporated $^{35}$S-sulfate, a large proportion (>94%) was in the form of macromolecules (Fig. A.5C).

A.3.4 Metabolism of $^{35}$S-Sulfate During Pulse-Chase

The metabolism of $^{35}$S-sulfate was further assessed by macromolecular analysis of disks and medium after pulse (8-h) and after various durations of chase (1–3-day). The total radiolabel in cultures (disks + medium) was not altered (Fig. A.5A) by chase.

After 1-day and 3-day chase, the medium contained 3.0 ± 0.4% and 5.9 ± 1.0% (both, mean ± SEM, n = 4), respectively, of the total $^{35}$S-radiolabel (Fig. A.5B). During the first day of chase, the medium $^{35}$S was predominantly (67.1 ± 2.5%) in the form of low-MW $^{35}$S-components, whereas by the third day of chase, most (80.5 ± 1.9%) was in the form of $^{35}$S-macromolecules (Fig. A.5B, D). Thus, after 1-day and 3-day chase, the low-MW $^{35}$S-components of the spent medium accounted for 2.0 ± 0.2% and 2.9 ± 0.3%, respectively, of the total radiolabel. Of the $^{35}$S remaining in the cartilage disks (Fig. A.5C), the proportion in the

Section A.3
Figure A.4: Incorporation of \(^{35}\)S-sulfate into \(^{35}\)S-macromolecules during 1–8-h isotopic labeling of free-swelling cartilage disks. (A) Total (×) radiolabel incorporated into cartilage disks was separated into low-MW (■) and macromolecular (●) components on Sephadex G25 (mean ± SEM, n = 6). Details of cartilage extraction and fractionation are given in Materials section. Macromolecular cpm were fit to first-order kinetic model [83] to determine time to half maximal (linear) incorporation (t\(_{1/2}\)). (B) The percentage of incorporated radiolabel in the form of macromolecules (▲).
Figure A.5: Size distribution of \(^{38}\)S-constituents in cartilage and medium after \(^{38}\)S-sulfate pulse-chase or after pulse with cycloheximide. Cartilage disks were radiolabeled for 8 h; some disks were chased for 1 or 3 days. Total (x) radiolabel in culture, (A) in cartilage disks and (B) released into medium, was separated into low-MW (■, □) and macromolecular (●, ○) components on Sephadex G25 (mean ± SEM, n = 4). Details of cartilage extraction and fractionation are given in Methods section. The percentage of radiolabel in the form of macromolecules (▲, △) (C) remaining in disks and (D) released daily into the medium. Cycloheximide (100 \(\mu\)g/ml) was added to the medium of some disks (□, ○, △) in the 1.5 h before and the 8 h during radiolabeling.

Section A.3
form of $^{35}$S-macromolecules increased only slightly from 99.18 ± 0.15 immediately after 8-h label (only 0.8% of the cartilage $^{35}$S was in the form of low-MW $^{35}$S-components) to 99.36 ± 0.18 after 1-day chase and 99.43 ± 0.06 after 3-day chase. These results imply that (a) there was generation of low-MW $^{35}$S-components during chase by catabolism of $^{35}$S-macromolecules, and (b) this catabolism occurred most extensively during the first day of chase on recently synthesized $^{35}$S-proteoglycans.

The relatively high rate of degradation $^{35}$S-macromolecules during the initial day of chase may reflect that the newly synthesized $^{35}$S-proteoglycans are (a) still in low-affinity monomer form [125], perhaps not yet in link-stabilized aggregates, and thereby relatively susceptible to proteolysis, (b) still near the chondrocyte periphery where they are relatively susceptible to cell-mediated degradation [63], or (c) a heterogeneous population of $^{35}$S-proteoglycans (e.g., small and large) which have different half-lives [23]. Indeed, examination of early chase medium indicated a heterogeneous size distribution of $^{35}$S-macromolecules on Sephacryl S-1000 (see Appendix C). In addition, in contrast to the particular experiment described (Fig. A.5), the rate of release of $^{35}$S-macromolecules during the first day was usually higher than that in subsequent days (see Chapter IV).

A.3.5 $^{35}$S-Sulfate Metabolism: Conclusions

(1) The rate of $^{35}$S-sulfate uptake into calf cartilage disks can be used to quantitatively assess the rate of total sulfate incorporation into macromolecules, even for radiolabel durations as short as 1 h.

(2) The catabolism of $^{35}$S-macromolecules and release of $^{35}$S from $^{35}$S-sulfate labeled cartilage disks is high during the first day of chase; thereafter, the release rates drop and are fairly steady (~1%/day) as most of the $^{35}$S released into the medium is in the form of macromolecules. The $^{35}$S-macromolecules released are analyzed in more detail in Chapter IV and Appendix C.
A.4 \(^3\)H-PROLINE METABOLISM: RESULTS AND DISCUSSION

A.4.1 Effect of Total Proline on \(^3\)H-Proline Incorporation

When the concentration of proline in the medium was increased from 0.02 mM to 0.2 mM while keeping the radiolabel concentration at 20 \(\mu\)Ci/ml, the incorporation of \(^3\)H decreased by only 16% (Fig. A.6). Even with the proline concentration as high as 50 mM, the incorporation of \(^3\)H-proline was not proportional to the specific activity in the medium. Thus, there appeared to be either (a) non-equilibration of extracellular proline and intracellular proline pools, which has been observed in smooth muscle cells [129] or (b) an increase in proline incorporation in parallel with the proline concentration in the medium. The former is not surprising since proline is not an essential amino acid; proline can be synthesized \textit{de novo} from ornithine or glutamate and can be salvaged from degraded proteins [129]. In an attempt to maintain a relatively uniform intracellular specific activity of proline between experiments, the extracellular proline concentration was chosen to be 0.5 mM in all subsequent experiments; at 0.5 mM proline, the incorporation of \(^3\)H was approximately half of that at 0.02 mM proline.

A.4.2 SDS Extractability of \(^3\)H-Proline Labeled Cartilage Explants

To assess the distribution of \(^3\)H-proline incorporated into low-MW components and macromolecules, cartilage disks were radiolabeled with \(^3\)H-proline for 8 h, washed, and then extracted with SDS under conditions which should release intracellular and extracellular low-MW components and deacetylate \(^3\)H-prolyl-tRNA to produce a low-MW \(^3\)H-component (Chapter II). Since slightly more low-MW \(^3\)H-components were released after extraction for 10–30 min than after extraction for 5 min (Fig. A.7), all subsequent extractions were for 15 min.

The extraction from cartilage disks of macromolecular \(^3\)H-components in-
Figure A.6: Effect of proline concentration in the medium on $^3$H-proline incorporation by cartilage explants. Cartilage disks were radiolabeled for 8 h in medium containing 20 $\mu$Ci/ml $^3$H-proline at various concentrations of proline, washed, and analyzed for $^3$H (mean $\pm$ SEM, n = 4).
Figure A.7: Time course of SDS extraction of $^3$H-components from $^3$H-proline labeled cartilage explants. Cartilage disks were radiolabeled for 8 h in medium containing 5 $\mu$Ci/ml $^3$H-proline and 10 $\mu$Ci/ml $^{35}$S-sulfate, and washed with PBS. Disks were then extracted with 2% SDS, 50 mM Tris, 10 mM Dithiothreitol, pH 8.5 at 100°C for various durations. The extract was separated into macromolecular ($V_0$) and low-MW ($V_i$) components on Sephadex G25. Total ($\times$) $^3$H-radiolabel was computed as the sum of that ($\triangle$) remaining in cartilage disks, and extracted as ($\bullet$) macromolecular and ($\blacksquare$) low-MW components (mean ± SEM, n = 4).
creased up to the longest extraction duration (30 min), when ~40% of the \(^3\)H-macromolecules were solubilized.

### A.4.3 Conversion of \(^3\)H-Proline into \(^3\)H-Macromolecules and \(^3\)H-Hydroxyproline Residues During Continuous Radiolabeling

To assess the kinetics of \(^3\)H-proline incorporation into macromolecules, cartilage disks were radiolabeled for 1–8 h, washed, and analyzed for \(^3\)H-macromolecules (Fig. A.8A). After the 1-h radiolabel, most (69.5 ± 1.5%, mean ± SEM, n=4) of the incorporated \(^3\)H was in the form of low-MW \(^3\)H-components (Fig. A.8B). When the radiolabel duration was increased to 8 h, most (74.1 ± 0.6%) of the incorporated \(^3\)H was in the form of \(^3\)H-macromolecules.

Inhibition of chondrocyte protein synthesis with cycloheximide reduced incorporation of \(^3\)H-proline into \(^3\)H-macromolecules by >96% (Fig. A.9A), consistent with the \(^3\)H-macromolecules being newly synthesized \(^3\)H-proteins. The low-MW \(^3\)H-components may represent intracellular pools of \(^3\)H-proline; consistent with this, cycloheximide-treated cartilage was still able to incorporated \(^3\)H-proline into low-MW \(^3\)H-components at 66.0 ± 3.2% of low-MW \(^3\)H control levels (Fig. A.9A).

The incorporation of \(^3\)H-proline into collagen was assessed by hydrolyzing disks and isolating \(^3\)H-hydroxyproline residues on Dowex 50W-X8. Several factors suggest that the \(^3\)H-material eluting in the hydroxyproline position (Fig. A.10) was purely \(^3\)H-hydroxyproline, rather than a co-eluting metabolite (e.g., glutamate or aspartate [30]). First, the formation of such \(^3\)H-material was negligible when either (a) the radiolabel duration was very short (10 minutes, Fig. A.10A) or (b) protein synthesis was inhibited with cycloheximide during 8-h radiolabeling (Fig. A.10D). These results are consistent with the appearance of \(^3\)H-hydroxyproline being dependent on the synthesis and subsequent hydroxylation of the collagen protein.

Second, under these same conditions (a and b), a third \(^3\)H-component, eluting between \(^3\)H-hydroxyproline and \(^3\)H-proline was prominent (Fig. A.10A, D). This
Figure A.8: Incorporation of $^3$H-proline into $^3$H-macromolecules and $^3$H-hydroxyproline residues during 10-min–8-h isotopic labeling of free-swelling cartilage disks. (A) Total (×) radiolabel incorporated into cartilage disks was separated into low-MW (■) and macromolecular (●) components on Sephadex G25 (mean ± SEM, n = 6). Details of cartilage extraction and fractionation are given in Materials section. Macromolecular cpm were fit to first-order kinetic model [83] to determine time to half maximal (linear) incorporation ($t_{1/2}$). (B) The percentage of incorporated radiolabel in the form of macromolecules (▲). (C) Other radiolabeled disks were hydrolyzed (see Fig. A.10) and analyzed for (+) $^3$H-hydroxyproline residues, (●) $^3$H-proline residues, and (×) an unidentified $^3$H-component (mean ± SD, n = 2–4).

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Figure A.9: Size distribution of $^3$H-constituents in cartilage and medium after $^3$H-proline pulse-chase or after pulse with cycloheximide. Cartilage disks were radiolabeled for 8 h; some disks were chased for 1 or 3 days. Total (×) radiolabel in culture, (A) in cartilage disks and (B) released into medium, was separated into low-MW (■, □) and macromolecular (●, ○) components on Sephadex G25 (mean ± SEM, n = 4). Details of cartilage extraction and fractionation are given in Methods section. The percentage of radiolabel in the form of macromolecules (▲, △) (C) remaining in disks and (D) released daily into the medium. Disks (E) and medium (F) were hydrolyzed with 6 N HCl and separated on Dowex 50W-X8 into (+) hydroxyproline residues, (●, ○) proline residues, and (⋆, ⋆) an unidentified component (mean ± SD, n = 2–4). Cycloheximide (100 μg/ml) was added to the medium of some disks (□, ○, △, ◊, ★) in the 1.5 h before and the 8 h during radiolabeling.

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Figure A.10: Fractionation of cartilage hydrolysates on Dowex 50W-X8. Cartilage disks were radiolabeled with 5–20 μCi/ml $^3$H-proline and 5–10 μCi/ml $^{35}$S-sulfate for (A) 10 min, (B) 8 h, (C) 8 h and chased 3 days, or (D) 8 h with 100 μg/ml cycloheximide, washed, and hydrolyzed in 6 N HCl for 16 h at 106°C. Hydrolysates were made to 0.9 N HCl, and a 0.5 ml portion was applied to a Dowex 50W-X8 column and eluted with 0.9 N HCl; fractions (1 ml) were collected and analyzed for $^3$H.

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component would thus appear to be a proline metabolite, since its formation was
dependent on the presence of live cartilage, but not on the synthesis of de novo
proteins. Further, with chase, this component rapidly disappeared from the tis-
sue into the medium (Fig. A.9E, F), consistent with this component being either a
proline-derived amino acid metabolite that did not enter the amino acid pool for
protein synthesis or an amino acid metabolite that was incorporated into protein,
but rapidly metabolized.

The formation of $^3$H-collagen appeared to closely parallel the appearance of
$^3$H-macromolecules; the proportion of $^3$H residues in the form of $^3$H-hydroxyproline
increased from $11.2 \pm 3.4\%$ (mean ± range, n=2) after a 1-h radiolabel to
$27.8 \pm 5.2\%$ after an 8-h radiolabel (Fig. A.8C). After an 8-h label, assuming 45%
of the proline to be hydroxylated in type II collagen [36], $\sim 62\%$ of the total incor-
porated $^3$H-proline was in collagen molecules, and $\sim 83\%$ of the $^3$H-macromolecules
appear to be accounted for by $^3$H-collagen.

A.4.4 Metabolism of $^3$H-Proline During Pulse-Chase

The metabolism of $^3$H-proline was further assessed by macromolecular anal-
ysis of disks and medium after pulse (8-h) or pulse and chase (1–3-day). The total
radiolabel in cultures (disks + medium) was essentially unaltered (Fig. A.9A) by
chase ($^3$H-cpm in 1-day or 3-day chase cultures were 11–14% lower than that in
pulse samples, p=0.05-0.10).

After 1-day and 3-day chase, the medium contained $21.5 \pm 1.4\%$ and
$27.7 \pm 2.3\%$ (both, mean ± SEM, n = 4), respectively, of the total $^3$H-radiolabel
(Fig. A.9B). During the first day of chase, little ($23.0 \pm 1.3\%$) of the medium $^3$H
was in the form of $^3$H-macromolecules (Fig. A.9B, D); by the third day of chase,
$^3$H-macromolecules constituted $37.6 \pm 4.9\%$ of the medium $^3$H. Thus, after 1-day
and 3-day chase, the low-MW $^3$H-components of the spent medium contained a sig-
nificant portion ($16.7 \pm 1.1\%$ and $20.5 \pm 2.2\%$, respectively) of the total radiolabel.
Of the $^3$H remaining in the cartilage disks (Fig. A.9C), the proportion in the form of $^3$H-macromolecules increased from 67.7 $\pm$ 1.2% immediately after 8-h label to 97.2 $\pm$ 0.3 after 1-day chase and 98.9 $\pm$ 0.1 after 3-day chase. This increase in the proportion of $^3$H-macromolecules in cartilage disks appeared to be paralleled by an increase in the proportion of cartilage $^3$H in the form of $^3$H-collagen molecules as the proportion of $^3$H-hydroxyproline, increased from 27.8 $\pm$ 5.2% after an 8-h radiolabel to 34.8 $\pm$ 1.6% after 1-day chase and 36.2 $\pm$ 0.5% after 3-day chase (Fig. A.9E). At the same time, the proportion of medium $^3$H in the form of $^3$H-hydroxyproline, increased from 15.5 $\pm$ 2.1% in the first day of chase to 22.7 $\pm$ 5.9 in the second day (Fig. A.9F).

Assuming 45% of the proline to be hydroxylated in type II collagen [36], $\sim$77% and 80% of the $^3$H-radiolabel after 1-day and 3-day chase, respectively, were in type II collagen molecules. In the medium, 34% and 50% of the $^3$H released during the first and second day of chase, respectively, were derived from collagen molecules. Since the proportion of $^3$H-hydroxyproline released into the medium is less than that in the cartilage, the cartilage disks became even more enriched in $^3$H-collagen components during chase. This would be expected since collagen has a long half-life, compared to most proteins.

A.4.5 $^3$H-Proline Metabolism: Conclusions

(1) The rate of $^3$H-proline incorporation into calf cartilage disks during short 1-hr radiolabel durations predominantly reflects $^3$H-proline uptake into chondrocytes.

(2) During longer 8-hr radiolabel durations, $^3$H-proline uptake predominantly reflects incorporation into macromolecular $^3$H-proteins; by $^3$H-hydroxyproline analysis, $^3$H-collagen appears to account for $\sim$60% of the total $^3$H incorporated and $\sim$80% of the $^3$H-protein.

(3) In the first day of chase, low-MW $^3$H-components remaining after ra-
diolabeling, are processed into $^3$H-proteins or released into the medium so that >97% of the $^3$H remaining in cartilage disks after 1-day chase are in the form of $^3$H-macromolecules.

(4) By $^3$H-hydroxyproline analysis, the majority (~77–80%) of the $^3$H remaining in $^3$H-proline labeled cartilage disks after 1-day chase are in collagen molecules.
A.5 \textit{\textsuperscript{35}S-METHIONINE METABOLISM: RESULTS AND DISCUSSION}

A.5.1 Conversion of \textit{\textsuperscript{35}S-Methionine into \textit{\textsuperscript{35}S-Macromolecules During Continuous Radiolabeling}

To assess the kinetics of \textsuperscript{35}S-methionine incorporation into macromolecules, cartilage disks were radiolabeled for 1–8 h (Fig. A.11A), washed, and analyzed for \textsuperscript{35}S-macromolecules. After the 1-h radiolabel, \textasciitilde50\% of the incorporated \textsuperscript{35}S was in the form of \textsuperscript{35}S-macromolecules (Fig. A.4B). When the radiolabel duration was increased to 4 h, the proportion of incorporated \textsuperscript{35}S in macromolecules increased to \textasciitilde75\%. The relatively rapid \textit{t}_{1/2} (12 min) may reflect the dependence of cells on methionine in the medium since methionine is an essential amino acid.

A.5.2 \textit{\textsuperscript{35}S-Methionine Metabolism: Conclusions}

The incorporation of \textsuperscript{35}S-methionine into calf cartilage disks during radiolabel durations of 4–8 h predominantly reflects \textsuperscript{35}S-methionine incorporation into \textsuperscript{35}S-proteins.
Figure A.11: Incorporation of ^35^S-methionine into macromolecules during 1–8-h isotopic labeling of free-swelling cartilage disks. (A) Total (x) radiolabel incorporated into cartilage disks was separated into low-MW (■) and macromolecular (○) components on Sephadex G25. Details of cartilage extraction and fractionation are given in Materials section. All points are mean ± SEM (n = 6). Macromolecular cpm were fit to first-order kinetic model [83] to determine time to half maximal (linear) incorporation (t_{1/2}). (B) The percentage of incorporated radiolabel in the form of macromolecules (▲).
Appendix B

Biophysical Mechanisms of Altered Biosynthesis During Cartilage Compression

B.1 INTRODUCTION

The biological and physical mechanisms by which static and dynamic compression alter biosynthesis are unclear. More detailed assessments of both the metabolic responses to compression as well as possible physical regulatory mechanisms may provide clues about the transduction pathway(s) by which compression alters cartilage metabolism.

Since static compression causes an inhibition of both glycosaminoglycan synthesis (^35S-sulfate incorporation, Chapter II, [10,48,49,75,173]) as well as protein synthesis (^3H-proline incorporation, Chapter II, [48,49]), it is possible that inhibition of glycosaminoglycan synthesis is simply secondary to a limitation of proteoglycan core protein synthesis. In serum-depleted bovine cartilage explants [62] as well as cycloheximide-treated chondrosarcoma cell culture [108,109], β-xyloside relieved the limitation of glycosaminoglycan synthesis due to inhibition of protein synthesis and was used to assess the maximal capacity of the cells to synthesize chondroitin sulfate glycosaminoglycans. Together, these studies suggest that the effects of static compression on glycosaminoglycan biosynthesis can be studied independently of the possible limiting formation of proteoglycan core protein by addition of β-xyloside.

Static and dynamic compression may have specific effects on the synthesis of particular proteins such as collagen. Since ^3H-proline incorporation into collagen is accompanied by hydroxylation of ^3H-proline residues [36], the ^3H-hydroxyproline content in ^3H-proline labeled cartilage is an indirect measurement of collagen biosynthesis. The effect of static and dynamic compression on collagen synthesis can thus be assessed by analyzing compressed and control sample groups from previous ex-
periments (Chapter II) for $^3$H-hydroxyproline.

One mechanism by which static compression appears to inhibit biosynthesis is through a physicochemical acidification of the intratissue environment [48]; in these experiments, epiphyseal cartilage was cultured with 0.1% Nuserum, conditions that maintained biosynthesis at steady state rates [48]. In calf femoropatellar cartilage maintained in medium with 10% FBS, static compression also appeared to exert metabolic effects through a physicochemical mechanism; the extracellular processing of proteoglycan in cartilage was slowed when the intratissue pH was lowered either by compression or incubation in acidic pH without compression (Chapter III). Since static compression also inhibited biosynthesis in these calf cartilage explants (Chapter II), it is of interest to determine the role of intratissue pH in mediating the effects of static compression on biosynthesis in this culture system.

The objectives of this study were thus to characterize the biosynthetic effects of static and dynamic compression in more detail and assess the regulatory role of pH in the alteration of biosynthesis during static compression.

B.2 MATERIALS AND METHODS

Materials for the culture and analysis of cartilage explants were obtained as described previously (Chapter II). In addition, p-Nitrophenyl $\beta$-D-xylopyranoside and shark chondroitin sulfate were from Sigma, St. Louis, MO.

B.2.1 Glycosaminoglycan Synthesis in the Presence of $\beta$-Xyloside

Cartilage slices, 1 mm thick, were explanted and pooled (six fragments totaling $\sim$60 mg wet weight per sample), and incubated in DMEM with 0.1 mM non-essential amino acids, 0.4 mM proline, 20 $\mu$g/ml ascorbate, 10 mM HEPES and 10% FBS (Hyclone, Logan, UT). The medium was changed daily, and on the first day, supplemented with antibiotics (100 U/ml penicillin and 100 $\mu$g/ml streptomycin). After two days of culture, groups of disks were preincubated for 4 h in
medium containing 0–2 mM β-xyloside (prepared using a stock solution of 20 mM β-xyloside in medium), and then further incubated for 24 h in medium (1 ml/sample) with β-xyloside and 5 μCi/ml ³⁵S-sulfate.

B.2.2 Biosynthesis During Static Compression in the Presence of β-Xyloside

Cartilage disks, 1 mm thick × 3 mm diameter were explanted and cultured for 2–3 days as above. Disks were then preincubated free-swelling for 4 h in medium containing 0 or 1 mM β-xyloside and then subjected to 12-h static compression while bathed in fresh medium containing the same concentration of β-xyloside as well as 10 μCi/ml ³H-proline and 5 μCi/ml ³⁵S-sulfate.

B.2.3 Biosynthesis at Altered pH

Cartilage disks were explanted and cultured for 2–3 days as above. Free-swelling disks were then incubated for 12-h while in medium that was titrated slightly acidic with 1 N HCl or slightly basic with 1 N NaOH (Chapter III) and included 20 μCi/ml ³H-proline and 10 μCi/ml ³⁵S-sulfate.

B.2.4 Analysis of Radiolabeled Disks and Medium

Radiolabeled cartilage was washed with PBS at 4°C over 90 min (three changes of PBS in xyloside experiments and six changes in pH experiments). Some disks were digested with papain, and analyzed for ³H, ³⁵S, and DNA; other disks were extracted with SDS and analyzed by chromatography on Sephadex-G25 (PD10) to quantitate the distribution of radiolabel in low-MW and macromolecular components (Chapter II). The wash and radiolabel medium were pooled and analyzed for radiolabeled macromolecules by chromatography on Sephadex-G25 columns equilibrated and eluted with 2 M guanidinium-HCl, 0.5 M Na acetate, 0.02% Na₂ azide, pH 6.8.
B.2.5 Gel Permeation Chromatography

The size distribution of shark chondroitin sulfate, tissue glycosaminoglycan, tissue $^{35}$S-glycosaminoglycans, and medium $^{35}$S-glycosaminoglycans were assessed by analytical gel permeation chromatography. Papain digests of tissue and medium were prepared by heating samples to 100°C for 15 min to inactivate the papain.

Some samples (~0.5 ml) were analyzed on a column (95 x 0.65 cm) of Sepharose CL-6B, equilibrated and eluted at 1.5-2 ml/h with 2 M guanidinium-HCl, 0.5 M Na acetate, 0.02% Na$_2$ azide, pH 6.8. Carrier shark chondroitin sulfate (250 µg) was added to medium samples, whereas tissue digests contained enough endogenous glycosaminoglycan to obviate the need for added carrier. Bovine nasal proteoglycan (A1D1D1) was added to all samples as a $V_0$ marker. Fractions (~0.6 ml) were collected, weighed, and assayed for glycosaminoglycan [40] and $^{35}$S.

Other samples were passed through a 0.45 µm filter; 0.25 ml portions were analyzed on a column (30 x 0.7 cm) of TSK PW-XL 40 in tandem with a TSK PW-XL guard column, which were equilibrated and eluted at 1.0 ml/min on an Hewlett Packard 1040 High Pressure Liquid Chromatograph with 0.2 M NaCl, 0.01 M Na phosphate, pH 7.0. Carrier shark chondroitin sulfate (100 µg) was added to medium digests, whereas tissue digest contained enough endogenous glycosaminoglycan to obviate the need for carrier. $^{3}$H$_2$O was added to all samples as a $V_1$ marker. Fractions (8 drops ~ 0.4 ml) were collected, weighed, and assayed for glycosaminoglycan [40] and radiolabel.

B.2.6 Quantitation of $^3$H-Hydroxyproline

Papain digests of cartilage disks from control and experimentally-compressed groups (Chapter II) were pooled, hydrolyzed, and analyzed for for $^3$H-hydroxyproline residues essentially as described previously ([178] and Appendix A).

Section B.2
B.3 RESULTS

B.3.1 Glycosaminoglycan Synthesis in the Presence of β-Xyloside

Control studies were performed to assess the ability of β-xyloside to act as an artificial acceptor of newly synthesized sulfated glycosaminoglycan chains. When cartilage samples were radiolabeled with $^{35}$S-sulfate in the presence of β-xyloside, the distribution of $^{35}$S-macromolecules between tissue and medium was altered (Fig. B.1), consistent with the formation of xyloside-initiated glycosaminoglycan chains. With 0.3–1 mM β-xyloside, >50% of the $^{35}$S-macromolecules were recovered in the medium, whereas without xyloside, only ~1% were in the medium. Addition of xyloside did not alter the already high rate of sulfate incorporation (~0.13 μmol/gm wet wt·hr, Chapter II) into macromolecules in our calf cartilage. In contrast, when β-xyloside was added to adult bovine cartilage, sulfated glycosaminoglycan synthesis was stimulated by +70% [62].

The glycosaminoglycans released by papain digestion of tissue eluted with a peak $K_{av}$ of ~0.56 ($V_e$ ~22.8 ml) on Sepharose CL-6B and were well discriminated from commercial shark chondroitin sulfate with a peak $K_{av}$ of ~0.37 ($V_e$ ~19.0 ml, Fig. B.2A, B). The size of newly synthesized $^{35}$S-glycosaminoglycan chains both in the tissue (Fig. B.2C, D) and released into the medium (Fig. B.2E, F) were decreased by increasing concentration of xyloside, consistent with the hypothesis that the size of newly synthesized chondroitin sulfate chains is inversely related to the number of glycosaminoglycan acceptors (core protein or exogenous xyloside) [108].

Alternatively, the relative size of $^{35}$S-glycosaminoglycan chains could be assessed by HPLC on TSK PW-XL 40. Tissue glycosaminoglycans eluted with a peak $V_e$ of ~10.3 ml on TSK PW-XL 40 (Fig. B.3) and were well discriminated from commercial shark chondroitin sulfate with a peak $V_e$ of ~9.3 ml. However due to the column dimensions, the total volume of solvent within the column gel

Section B.3
Figure B.1: Effect of β-xyloside on incorporation of $^{35}$S-sulfate. Cartilage slices were preincubated for 4 h in medium including 0–2 mM β-xyloside, and then incubated in medium with $^{35}$S-sulfate and β-xyloside for 24 h. The cartilage tissue was washed, papain digested, and assayed for $^{35}$S and DNA. The wash and medium were combined and analyzed for $^{35}$S-macromolecules by PD-10 chromatography or dialysis. The $^{35}$S in the tissue (bottom solid bar) and $^{35}$S-macromolecules in the medium (top empty bar) are plotted normalized to DNA content (mean ± range of duplicate cultures of pooled cartilage).

Section B.3
Figure B.2: Size distribution on Sepharose CL-6B of standard glycosaminoglycans and $^{35}$S-glycosaminoglycans from cartilage radiolabeled with $^{35}$S-sulfate in the presence of $\beta$-xyloside. Samples containing ~250 $\mu$g glycosaminoglycan and ~100 $\mu$g proteoglycan (as a $V_0$ marker) were fractionated on a Sepharose CL-6B column (95 x 0.65 cm) eluted with 2 M guanidinium-HCl, 0.5 M Na acetate, 0.02% Na$_2$ azide, pH 6.8; fractions were assayed for glycosaminoglycan (●) and $^{35}$S ([●], ▲). Samples were (A) proteoglycan and shark chondroitin sulfate, (B) proteoglycan and papain-digested cartilage, (C) and (D) papain-digested cartilage, radiolabeled with $^{35}$S-sulfate in the presence of 0.1 mM and 1.0 mM $\beta$-xyloside, respectively (see Fig. B.1), (E) and (F) papain-digested medium from cartilage cultures radiolabeled in the presence of 0.05 mM and 0.3 mM $\beta$-xyloside (profiles omit radiolabel eluting near the total column volume).
Figure B.3: Size distribution on TSK PW-XL 40 of standard glycosaminoglycans and $^{35}$S-glycosaminoglycans from cartilage radiolabeled with $^{35}$S-sulfate in the presence of $\beta$-xyloside. Samples containing 100–200 $\mu$g glycosaminoglycan and $^3$H$_2$O were fractionated on a TSK PW-XL 40 column (30 × 0.7 cm) in tandem with a TSK PW-XL guard column eluted with 0.2 M NaCl, 0.01 M Na phosphate, pH 7.0; fractions were assayed for glycosaminoglycan (●), $^{35}$S (■), and $^3$H (○). Samples were (A) shark chondroitin sulfate, (B) papain-digested cartilage, and (C–E) papain-digested cartilage, radiolabeled with $^{35}$S-sulfate in the presence of 0.05, 0.3, or 2.0 mM $\beta$-xyloside, respectively (see Fig. B.1).
available to small molecules \(V_0-V_f\) is smaller in the 30×0.7 cm HPLC column compared to that in the 100×0.65 cm CL-6B column. Thus, elution profiles are spread over a smaller volume on the former column, and it was not surprising that \(^{35}\text{S}-\text{glycosaminoglycan}\) chains from papain digests of \(\beta\)-xyloside treated cartilage eluted only slightly later with increasing \(\beta\)-xyloside concentration (Fig. B.3C–E). The sensitivity of this chromatography system could be increased by adding an additional column in series [151].

B.3.2 The Effect of Static Compression on Biosynthesis in the Presence of \(\beta\)-Xyloside

To determine the effect of static compression on glycosaminoglycan synthesis in the presence of excess glycosaminoglycan chain initiator, biosynthesis was assessed during compression in the absence or presence of \(\beta\)-xyloside. In cartilage disks compressed to a thickness of 1.00 mm, treatment with 1.0 mM \(\beta\)-xyloside caused a diversion of >50% of the \(^{35}\text{S}\)-macromolecules to the medium, as compared to <5% in no-xyloside controls, without affecting the total \(^{35}\text{S}\)-sulfate incorporation into macromolecules (Fig. B.4A). This xyloside treatment during mild compression also had little effect on \(^{3}\text{H}\)-proline incorporation or distribution of \(^{3}\text{H}\)-macromolecules between tissue and medium (Fig. B.4B).

With increasing levels of compression, the total incorporation of \(^{35}\text{S}\)-sulfate was similarly depressed in the absence or presence of \(\beta\)-xyloside (Fig. B.4A). Thus, static compression appears to inhibit glycosaminoglycan synthesis through a general cellular metabolic effect, rather than through a limitation of proteoglycan core protein. The incorporation of \(^{3}\text{H}\)-proline into macromolecules was also generally depressed by compression with or without \(\beta\)-xyloside, although the extent during the largest amplitude compression tested (to 0.63 mm) was slightly greater in the presence of \(\beta\)-xyloside (Fig. B.4B).
Figure B.4: Effect of static compression and β-xyloside on incorporation of $^{35}$S-sulfate and $^3$H-proline. Cartilage disks were preincubated for 4 h in medium including 0 (−) or 1 (+) mM β-xyloside, and the incubated in medium with $^{35}$S-sulfate, $^3$H-proline, and 0 or 1 mM β-xyloside during 12-h compression. The cartilage disks were washed, papain digested, and assayed for $^3$H and $^{35}$S (bottom solid bars). The wash and medium were combined and analyzed for radiolabeled macromolecules (top empty box) by PD-10 chromatography. Each sample consisted of the pooled cartilage and medium from four disks (mean ± SEM, n = 3).
B.3.3 The Effect of Static and Dynamic Compression on Conversion of $^3$H-Proline into $^3$H-Hydroxyproline

To examine the possibility that compression may have specific effects on the conversion of $^3$H-proline into $^3$H-collagen molecules, radiolabeled samples were analyzed for $^3$H-hydroxyproline. Static compression diminished the proportion of incorporated $^3$H in the form of $^3$H-hydroxyproline from 24.9 ± 1.6% (mean ± range, n=2 pooled groups of 6 disks each) at 1.25 mm to 18.4 ± 0.7% at 0.56 mm. This is consistent with the finding that static compression not only diminished the total incorporation of $^3$H-proline, but also slightly decreased the proportion of $^3$H in the form of macromolecules (Chapter II).

In contrast, cyclic compression and release (2-h on/2-h off) between 1.25 mm and 1.125 to 0.875 mm appeared to cause an enhancement of $^3$H-proline conversion into collagenous molecules, with an increase both in the proportion of $^3$H in the form of $^3$H-hydroxyproline (Fig. B.5A) as well as the total $^3$H-hydroxyproline formed (Fig. B.5B). This magnitude of cyclic compression also caused an increase in total $^3$H-proline and $^{35}$S-sulfate uptake (Chapter II). Together, these results are consistent with a stimulation of the sequence of reactions involved in protein synthesis rather than a specific accentuation of $^3$H-proline uptake (into low-MW protein precursors).

Similarly, oscillatory compression appeared to cause an acceleration of conversion of $^3$H-proline into collagenous molecules; both the proportion of $^3$H in the form of $^3$H-hydroxyproline (Fig. B.6A) and the total $^3$H-hydroxyproline formed (Fig. B.6B) were increased. Interestingly, the magnitude of the later increase (∼ +80% at f=0.01 Hz and 2–4% strain) was somewhat greater than the stimulation in total $^3$H-proline uptake (∼ +40%, Chapter II). These results are also consistent with a general biosynthetic stimulation by oscillatory compression rather than a specific effect on $^3$H-proline uptake.

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Figure B.5: Effect of cyclic compression/release on formation of $^3$H-hydroxyproline. Control disks were statically compressed to 1.25 mm; experimental disks were cyclically compressed between 1.25 and 0.88-1.13 mm. Disks were radiolabeled with $^3$H-proline during the last 8 h of 23-h cyclic compression, and control or experimental groups of 12 disks were pooled for $^3$H-hydroxyproline analysis. (A) % of total incorporated $^3$H in the form of $^3$H-hydroxyproline. Line segments connect values for control (1.25 mm) and cyclically compressed (to 1.13–0.88 mm) samples within an experiment. (B) Ratio of total $^3$H-hydroxyproline in compressed samples to that in controls.
Figure B.6: Effect of oscillatory compression on formation of $^3$H-hydroxyproline. Control disks were statically compressed to 1 mm; experimental disks were compressed to 1 mm with a superimposed oscillatory strain of up to ~5% at frequencies of ≤0.001 Hz (A, B), 0.01 Hz (C, D), and ≥0.1 Hz (E, F). Disks were radiolabeled with $^3$H-proline during the last 8 h of 23-h oscillatory compression, and control or experimental groups of 12 disks were pooled for $^3$H-hydroxyproline analysis. (A, C, E) % of total incorporated $^3$H in the form of $^3$H-hydroxyproline. Line segments connect values for static control (0% strain) and oscillatory experimental (0.6–4.4% strain) samples within an experiment. (B, D, F) Ratio of total $^3$H-hydroxyproline in oscillatory samples to that in controls.
B.3.4 The Effect of Altered Medium pH on Biosynthesis

While the biochemical mechanisms leading to altered biosynthesis during static and dynamic compression are unclear, previous studies [48] have suggested that altered intratissue pH may be the physicochemical regulatory signal by which static compression causes a reduction in $^{35}$S-sulfate and $^3$H-proline incorporation. There, similar decreases in intratissue pH are achieved by either 50% tissue compression in normal pH medium, or no compression in medium titrated acidic by ~0.4 pH units.

In our culture system with calf femoropatellar cartilage in medium with 10% FBS, the role of intratissue pH in modulating biosynthesis was assessed. In two experiments, the effects of altered medium pH on sulfated glycosaminoglycan synthesis were slightly different (Fig B.7A), although there appeared to be a consistent trend of a 15–20% increase in glycosaminoglycan synthesis from low pH (~7.0) to high pH (~7.8). The magnitude of this increase in the pH 7.0–7.8 range is consistent with that found in previous studies [48].

The effects of altered medium pH on $^3$H-proline incorporation were more consistent (Fig B.7B), with a +50–90% increase in $^3$H-proline incorporation with pH, which was most marked from pH~7.5 to pH~7.8. This increase is somewhat larger than that observed in previous studies in the pH 7.0–7.8 range [48].

The medium contained only a small proportion of the total radiolabeled macromolecules. However, the the percentage of macromolecules in the medium decreased with increasing pH for both $^3$H (4.5%, 4.1%, 4.1%, and 3.2% of $^3$H-macromolecules were in the medium during radiolabeling at pH 6.99, 7.20, 7.50, and 7.77, respectively) and $^{35}$S (0.37%, 0.25%, 0.26%, and 0.23% of $^{35}$S-macromolecules were in the medium during radiolabeling at pH 6.99, 7.20, 7.50, and 7.77, respectively). This trend would be consistent with several possible mechanisms of increased deposition of newly synthesized molecules at high pH such as increased aggregability and assembly (Chapter III). A less likely mechanism is a pH retarda-
Figure B.7: Effect of altered medium pH on incorporation of $^{35}$S-sulfate and $^3$H-proline. Cartilage disks were incubated for 12 h in pH adjusted medium containing $^{35}$S-sulfate and $^3$H-proline. Radiolabeled disks were washed, papain digested, and assayed for $^3$H and $^{35}$S. The rates of (A) sulfate and (B) proline incorporation were computed (mean ± SEM, n = 12) for each of two experiments (○, □) and (●, ■).
tion of catabolic processes since increased pH was found to cause a slight increase in the rate of matrix release (Chapter IV).

The radiolabeled constituents within the washed cartilage disks were predominantly in the form of macromolecules. The distribution between macromolecules and low-MW components was not greatly altered by medium pH, with 68 ± 3%, 76 ± 3%, 78 ± 2%, and 74 ± 3% (mean ± SEM, n=4) of tissue $^3$H in the form of $^3$H-macromolecules at pH 6.99, 7.20, 7.50, and 7.77, respectively, and >99.5% of tissue $^{35}$S in $^{35}$S-macromolecules at all pH.

Analysis of pooled papain digests showed no marked difference in the size distribution of newly synthesized glycosaminoglycans on TSK PW-XL 40; the $^{35}$S-glycosaminoglycans synthesized at pH 7.70 appeared to be only slightly smaller (eluting ~0.1 ml later) than both the $^{35}$S-glycosaminoglycans synthesized at pH 6.99 and endogenous tissue glycosaminoglycans (data not shown).

B.4 DISCUSSION

Static compression inhibited glycosaminoglycan synthesis even in the presence of β-xyloside, which was sufficient in quantity to divert ~50% of the $^{35}$S-macromolecules to the medium. Thus, the observed inhibition of sulfated glycosaminoglycan synthesis by static compression (Chapter II, [10,48,49,75,173]) does not appear to be secondary to an inhibition of proteoglycan core protein synthesis.

The use of β-xyloside allows molecular manipulation of newly synthesized proteoglycans in cultures of calf cartilage explants maintained with medium including 10% FBS, without marked effects on protein synthesis (as assessed by $^3$H-proline incorporation into macromolecules). By diverting ~50% of newly synthesized glycosaminoglycans to the medium, β-xylosides may allow formation of a charge deficient matrix in long-term cartilage cultures. By reducing the deposition of newly synthesized sulfated glycosaminoglycan in cartilage from 3%/day to 1.5% day, β-xyloside would be predicted to cause measurably significant differences

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in glycosaminoglycan content by \( \sim 7 \) days of treatment. In addition, \( \beta \)-xyloside causes the formation of smaller tissue glycosaminoglycans (and hence smaller proteoglycans). The effects of glycosaminoglycan size on proteoglycan aggregation and turnover could be thus investigated. Such studies may be relevant to changes in articular cartilage with aging since the proteoglycans synthesized in cartilage from aged (but non-arthritic) cows have shorter glycosaminoglycan chains than those in cartilage from young calves [62]. Alternatively, such studies may be geared toward repair mechanisms since the proteoglycans synthesized in osteoarthritic cartilage are larger than those synthesized in normal aged cartilage [26].

The static and dynamic compression protocols each altered (increased or decreased) the proportion of \(^3\)H-proline incorporated into \(^3\)H-hydroxyproline in an manner consistent with the observed effect (increase or decrease) in total radiolabel incorporation (Chapter II). These trends give further support to the ability of of static and dynamic compression to elicit general cellular metabolic effects. The changes in \(^3\)H-hydroxyproline formation (both relative to the total \(^3\)H incorporated and absolute) could reflect alterations in the rate of collagen synthesis relative to other proteins as well as changes in the kinetics of collagen synthesis as assessed by \(^3\)H-hydroxyproline formation (Fig A.8C).

Altered intratissue pH does not appear to completely account for the inhibition of biosynthesis by compression; however, its is difficult to make a quantitative comparison without accurate measurements of intratissue pH during compression of explants and incubation of non-compressed explants in acidic pH medium. Further, there may be spatial gradients in pH, especially near the chondrocyte surface.

Interestingly, high pH (\( \sim 7.8 \)) caused a more marked stimulation of \(^3\)H-proline incorporation than \(^35\)S-sulfate incorporation. If this was associated with a relatively higher increase in the synthetic rate of proteoglycan core protein than in that of glycosaminoglycans, a decrease in \(^35\)S-glycosaminoglycan chain size would be expected. However, preliminary studies showed only a small decrease in \(^35\)S-glycosaminoglycan

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chain size at pH~7.8 relative to that at pH~7.0. This then suggests that high pH may alter the spectrum of proteins synthesized. However, preliminary results in epiphyseal cartilage have indicated that the lowering of intratissue pH by static compression does not qualitatively alter the spectrum of proteins synthesized [50].

These preliminary results suggest that by a combination of physical, biochemical, and pharmacological methods, it should be possible to further deduce the biophysical mechanisms by which compression alters cartilage metabolism.
Appendix C

Effect of Applied Current on Proteoglycan Release from Cartilage Explants

C.1 INTRODUCTION

The release of $^{35}$S-proteoglycan from cartilage explants in vitro is increased by one or multiple cycles of compression and release (Chapter IV). Several physical phenomena, including deformation of matrix and chondrocytes, fluid flow, streaming potentials, and physicochemical changes occur during compression; any of these phenomena may trigger the release of proteoglycan either through a direct physical effect on the extracellular matrix, or through an indirect stimulation of cell-mediated catabolism. To begin to separate and identify the role of specific physical forces, one can utilize the natural electrokinetic coupling that occurs in cartilage [42,43]; an applied electrical current would result in electrophoretic forces on charged molecules, as well as electroosmotic fluid flow [50,161] and entrainment of mobile molecules. In a configuration where the cartilage surfaces are free-draining, applied currents could cause significant fluid flow without tissue consolidation.

Proteoglycans exist in a variety of forms and types in articular cartilage [64,66]. It is clear that newly synthesized large proteoglycans initially have a low binding affinity for hyaluronate, as assessed by their ability to form aggregates on chromatography with limiting amounts of hyaluronate [8,9,106,125,170]; these proteoglycans appear to undergo a maturation process whereby they attain a high binding affinity for hyaluronate, characteristic of endogenous tissue proteoglycans. Other large proteoglycans exist within cartilage as proteolytically cleaved products and are not able to form aggregates even in the presence of excess hyaluronate and link protein (Chapter III, [23,59]). In addition, several small cartilage proteoglycans have been identified [149,150]. While the large proteoglycans endow cartilage with
a high swelling pressure, the function of small proteoglycans is not clear. Physical forces may differentially modulate the release of these proteoglycan species.

The objectives of these studies were to (1) characterize the effect of applied electric currents on the release of newly synthesized proteoglycans and on specific proteoglycan species and (2) distinguish between cellular and non-cellular mechanisms by which applied currents may affect proteoglycan metabolism.

C.2 METHODS

Cartilage disks, 1 mm thick × 3 mm diameter, were explanted, inserted into circumferential teflon holders [183], and incubated in DMEM with 0.1 mM non-essential amino acids, 0.4 mM proline, 20 μg/ml ascorbate, 10 mM HEPES and 10% FBS (Hyclone, Logan, UT). The medium (0.5 ml/disk) was changed daily, and on the first day, supplemented with antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Disks were maintained in culture for three or four days before radiolabel and electrical stimulation protocols, described below.

C.2.1 Proteoglycan Release during Applied Current

Disks were pulse radiolabeled for 1.5–3 h with medium including 200 μCi/ml 35S-sulfate (1 ml/4 disks) before use in various chase protocols described below.

Current Application in 37°C Culture Medium. Pulse-labeled disks were washed three times with culture medium (1 ml/disk) over a total of 0.5 h on a shaker within the incubator at 60 rpm. Some radiolabeled and washed disks were then placed in the electrical stimulation chamber and subjected to current densities of 0–5 mA/cm² during 0–3-h of chase. Groups of 6–16 disks were isolated in the chamber from each other by ~1 cm long barriers of agarose:medium (5% w/v), and from the platinum electrodes by >16 cm long agarose bridges. The medium (0.5 ml/disk) was collected, pooled, and stored at −20°C. Other radiolabeled and washed disks were further incubated for 24 h in 37°C medium before transfer to the

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electrical chamber and application of current.

**Current Application in 4°C Protease Inhibitor Solution.** Some pulse-labeled disks were washed (1 ml/disk) once with 37°C medium on the shaker, and then twice by gentle rocking in a phosphate buffered saline solution (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 7H₂O, 1.5 mM KH₂PO₄) at 4°C including protease inhibitors (0.1 M 6-aminohexanoic acid, 10 mM Na₂·EDTA, 10 mM benzamidine-HCl, 10 mM N-ethylmaleimide, 1 mM phenylmethanesulfonyl chloride, 1 µM peptatin, and 1 µM leupeptin) [67] at pH 7.0 (PBSI). These washed disks were then subjected to electrical current densities of 0–10 mA/cm² during 0–48-h of chase in 4°C PBSI. The conditioned PBSI (0.5 ml/disk) was collected, pooled, and stored at −20°C. Other radiolabeled disks were washed in 37°C medium and further incubated in medium for 48 h before being washed with 4°C PBSI, transferred to the electrical chamber, and subjected to current in 4°C PBSI.

**Analysis of Cartilage.** Some cartilage disks were digested with papain and analyzed for ³⁵S and glycosaminoglycan (Chapter II). Other disks were cryostat sectioned, extracted with 4 M guanidinium, and fractionated by cesium chloride density gradient centrifugation and portions were analyzed for ³⁵S and glycosaminoglycan (Chapter III). The affinity of D1 proteoglycans for hyaluronate was assessed by mixing samples with 1.6% (w/w) hyaluronate and chromatography on Sephacryl S-1000 under associative conditions (Chapter III). Column fractions were analyzed for glycosaminoglycan by one of two methods: (1) pipetting a 100 µl portion of each column fraction to a disposable polystyrene cuvet, adding 1 ml dimethylmethylen blue dye (DMB) solution [40], and immediately measuring the A₅₂₅ nm on a Perkin Elmer λ3 spectrophotometer; (2) pipetting 20 µl portions of each column fraction in duplicate to a 96-well assay plate (Nunc), quickly (in ~30 sec) adding 200 µl DMB solution per well using a 12-place pipettor, and rapidly (in ~5 sec) measuring the A₅₂₅ nm on a Molecular Devices v_max plate reader. In the latter method, the duplicate samples were located in the dish and the DMB added in such a way.

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that the sum total time that duplicate sample portions were mixed with DMB was approximately the same for all samples.

**Analysis of Chase Solutions.** Protease inhibitors were added to all medium and PBSI solutions. Chase solutions were then analyzed for glycosaminoglycan by reaction with dimethylmethylene blue (Chapter IV, [40]) and $^{35}$S-macromolecules by Sephadex G-25 (PD10) chromatography (Chapter IV). The total $^{35}$S-macromolecules in each culture were calculated as the radioactivity remaining in the cartilage disks at the termination of the experiment plus the summed total macromolecular radioactivity released into the collected medium and PBSI solutions. From this total, the rate of release of $^{35}$S-macromolecules was computed. The rate of sulfated glycosaminoglycan release was similarly computed.

**Aggregability of Medium $^{35}$S-proteoglycans with Hyaluronate and Link Protein.** To assess the aggregability of $^{35}$S-macromolecules released into the medium, portions (3 ml) were mixed with 280 $\mu$g of bovine nasal cartilage proteoglycan monomer, concentrated and buffer exchanged with a Centricon-10 microconcentrator (MWCO 10,000) first into 1 ml 0.1 M Tris, 0.05 M Na acetate, pH 8.6 for 24 h, then into 1 ml 0.15 M Na acetate, pH 6.8 for 24 h [172], and finally into 1 ml 4 M guanidinium-HCl (Chapter IV). Dissociated samples were then reacted with 5% (w/w) hyaluronate and 5% (w/w) link protein for 2 h at 4°C, dialyzed into 0.15 M Na acetate, pH 6.8, stored at −20°C, and later analyzed by Sephacryl S-1000 chromatography. All concentration and dialysis procedures were at 4°C and with protease inhibitors.

**C.2.2 $^{35}$S-Sulfate Incorporation During Applied Current**

Disks were incubated in the electrical chamber for 9 h with medium including 20 $\mu$Ci/ml $^{35}$S-sulfate (0.5 ml/disk) while subjected to current densities of 0–10 mA/cm². Disks were then washed and analyzed for radioactivity (Chapter II). Medium and wash solutions were analyzed for $^{35}$S-macromolecules by Sephadex G-25 chromatography as above. The rate of sulfate incorporation was computed using

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the specific activity of the medium at the termination of the experiment, assuming
a sulfate concentration of 0.8 mM.

C.3 RESULTS

C.3.1 The Effect of Applied Current on Proteoglycan Release from Cartilage Explants in Culture

Previous experiments have shown that the release of $^{35}$S-macromolecules from $^{35}$S-sulfate labeled cartilage disks during chase in 37°C medium is accelerated by applied current densities of $J = 2-10$ mA/cm² [183]. To further characterize the released $^{35}$S-macromolecules, the medium from large treatment groups (16 cartilage disks) were pooled for analysis. The dependence of $^{35}$S-macromolecule release on chase time and applied current were consistent with previous findings (Fig. A.5 and [183]). The rate of release of $^{35}$S-macromolecules without applied current (Fig. C.1) was higher during early chase times (1.49%/day during 0–3-h chase) than during later chase times (∼1.25%/day during 0–24-h and 24–27-h chase). A current density of 5 mA/cm² during both 0–3-h and 24–27-h of chase increased the rate of release of $^{35}$S-macromolecules to 1.83 and 1.85%/day, respectively. A lesser current density of 2 mA/cm² caused also caused a marked stimulation at 24–27-h of chase, but had little effect at 0–3-h.

To determine the type and distribution of $^{35}$S-macromolecules in the medium, aggregating $^{35}$S-proteoglycans were separated by reacting portions of medium (and added carrier proteoglycan) with excess (5% w/w) hyaluronate and excess (5% w/w) link protein, dialysis to remove low-MW components, and then chromatography on Sephacryl S-1000 under associative conditions (Fig. C.2). Three components of $^{35}$S macromolecules were thus resolved: (1) aggregating $^{35}$S-proteoglycans, eluting at the void volume, $V_0$ ∼14 ml, (2) large non-aggregating $^{35}$S-proteoglycan, eluting at $V_e$ ∼23 ml, and (3) small $^{35}$S-macromolecules, eluting at $V_e$ ∼30 ml.

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Figure C.1: Effect of applied current on release of $^{35}$S-macromolecules during 0–3-h or 24–27-h of chase in 37°C culture medium. Cartilage disks were pulse (3-h) radiolabeled with $^{35}$S-sulfate and washed (30 min) with medium. Some disks were subjected to a current of 0, 2, or 5 mA/cm² during 0–3-h chase (left panel). Other disks were chased in medium for 24-h (center panel) before being subjected to current during 24–27-h chase (right panel). Chase medium was analyzed for $^{35}$S-macromolecules, and the rate of $^{35}$S-macromolecular release was determined. Each point represents a sample group of 16 pooled cartilage disks.
Figure C.2: Aggregability with excess hyaluronate and link protein of $^{35}$S-macromolecules released into the medium during an applied current density of 0–5 mA/cm$^2$. Cartilage disks were pulse radiolabeled with $^{35}$S-sulfate, chased for 0 (A–C) or 24 h (D–F) in culture medium, and then subjected to a current of 0 (A, D), 2 (B, E), or 5 (C, F) mA/cm$^2$ during an additional 3 h of chase (Fig. C.1). Medium from the current treatment groups (each of 16 pooled cartilage disks) were mixed with carrier proteoglycan and protease inhibitors, concentrated, dialyzed to pH 8.6, mixed with 5% (w/w) hyaluronate and 5% (w/w) link protein under dissociative conditions, dialyzed to associative conditions, and then eluted on Sephacryl S-1000 under associative conditions. Fractions were analyzed for glycosaminoglycan (−) and $^{35}$S (●). The percentage of $^{35}$S eluting in the delineated size distribution was computed.

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The latter component elutes with a peak $K_{av}$ of $\sim 0.8-0.9$, and may represent small $^{35}S$-proteoglycans or fragments of catabolized large $^{35}S$-proteoglycans such as free $^{35}S$-glycosaminoglycan chains. This component comprised 48% of the medium $^{35}S$-macromolecules during 0–3-h of chase without current but only $\sim 11\%$ during 24–27-h of chase (Fig. C.2A and D). Thus, it appeared to account for the rate of release of $^{35}S$-macromolecules being higher during early 0–3-h chase than late 24–27-h chase.

In 0–3-h and 24–27-h chase medium without applied current, aggregatable $^{35}S$-proteoglycan accounted for 13.6% and 16.0%, respectively, of the released $^{35}S$-macromolecules (Fig. C.2A and D). Taking into account the slightly higher rate of release of total $^{35}S$-macromolecules at 0–3-h, the release rates of aggregatable $^{35}S$-proteoglycan were essentially identical at 0–3-h and 24–27-h chase. Thus, low affinity $^{35}S$-proteoglycans (existing during early 0–3-h chase) do not appear to be preferentially released from free-swelling cartilage disks over high affinity $^{35}S$-proteoglycans (existing during later 24–27-h chase).

The application of current ($J = 2$ or 5 mA/cm$^2$) appeared to cause a relatively proportional increase in the rate of release of large and small $^{35}S$-macromolecules during early (0–3-h) and late (24–27-h) chase times (Fig. C.2B, C, E and F). However, closer inspection of the data reveals differences in the ratio of aggregating large $^{35}S$-proteoglycan to the total large (aggregating and non-aggregating) $^{35}S$-proteoglycan. In the absence of current, this ratio decreased from 0.26 during 0–3-h chase to 0.18 during 24–27-h chase. This ratio was increased slightly by current application during 0–3-h of chase, from 0.26 at $J = 0$ and 2 mA/cm$^2$ to 0.31 at $J = 5$ mA/cm$^2$; yet, during 24–27-h of chase, this ratio was not altered by applied current (0.18, 0.17, and 0.19 at $J = 0$, 2, and 5 mA/cm$^2$, respectively). This relatively higher rate of release of newly synthesized aggregatable $^{35}S$-proteoglycan with applied current during early chase would be consistent with a higher mobility of low affinity $^{35}S$-proteoglycans than high affinity $^{35}S$-proteoglycans when

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subjected to electrokinetic stress. However, it is also possible that the current application resulted in more complex effects; for example, an enrichment of medium with aggregatable $^{35}$S-proteoglycans would also probably result if the applied current inhibited the catabolism of newly synthesized proteoglycans and thus reduced the tissue pool of non-aggregatable $^{35}$S-proteoglycans.

C.3.2 The Effect of Applied Current on Proteoglycan Maturation in and Release from Enzymatically-Inhibited Cartilage Explants

Since the previous experiments with applied electric currents were performed on explants laden with viable chondrocytes in 37°C culture medium, the stimulated release of $^{35}$S-macromolecules may be due to either a direct electromechanical effect of current on extracellular macromolecules, or an indirect effect of altered proteoglycan catabolism mediated by chondrocytes. To assess the isolated physical effect, the application of current to pulse or pulse-chased cartilage disks was performed in a 4°C bath of PBS with protease inhibitors (including N-ethylmaleimide). Such a bath would be predicted to slow enzymatic activity as well as the extracellular maturation of the newly synthesized $^{35}$S-proteoglycans which may involve a rearrangement or formation of disulfide bonds [8,106].

Indeed, even during a lengthy 48-h of chase, the 4°C PBSI bath maintained the newly synthesized $^{35}$S-proteoglycans in the low affinity form; when D1 proteoglycans were mixed with limiting (1.6% w/w) hyaluronate and fractionated on Sephacryl S-1000, only 8% of $^{35}$S-proteoglycans eluted as aggregates compared to 57% of tissue proteoglycan (Fig. C.3A). As expected, after 48-h of chase in 37°C medium (and a further 48-h of chase in the 4°C PBSI bath), newly synthesized proteoglycans were converted to the high affinity form, with ~60% of both $^{35}$S-proteoglycans and tissue proteoglycans eluting as aggregates (Fig. C.3C). These binding properties of pulse and pulse-chased samples were not affected by 48-h application of 10 mA/cm$^2$ current (Fig. C.3B, D). Thus, the 4°C PBSI bath maintained the large $^{35}$S-proteoglycan

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in stable functionally distinct (low and high binding affinity) forms.

The rate of release of $^{35}$S-macromolecules from cartilage disks during 0–48-h chase without applied current (Fig. C.4) was higher in 37°C medium (~1%/day) than in 4°C PBSI (0.33%/day). During the same period and incubation conditions, the rate of release of sulfated glycosaminoglycans from cartilage (Fig. C.5) was similarly higher in 37°C medium (~1%/day) than in 4°C PBSI (0.14%/day). These differences may be due to an biochemical inhibition of proteases, as well as a biophysical decrease in diffusivity of macromolecules at the lower temperature.

The rate of release of $^{35}$S-macromolecules from explants in 4°C PBSI without applied current was lower during late 48–96-h chase (0.15%/day) than early 0–48-h chase (0.33%/day, Fig. C.4), consistent with the decrease in the rate of release of $^{35}$S-macromolecules from early (0–3-h) to later (24–27-h) chase in 37°C medium (Fig. C.1). As expected, there was no difference in the rate of release of total sulfated glycosaminoglycan without applied current during these different chase times (Fig. C.5). Thus, the specific activity of released material was higher (1,300 $^{35}$S cpm/μg glycosaminoglycan) during 0–48-h chase in 4°C PBSI than that (600 $^{35}$S cpm/μg glycosaminoglycan) during 0–48-h chase in 37°C medium or that (600 $^{35}$S cpm/μg glycosaminoglycan) during 48–96-h chase in in 4°C PBSI (Fig. C.6). These findings would be consistent with the differential release of several populations of sulfated-glycosaminoglycan containing molecules with different specific activities (e.g., small $^{35}$S-proteoglycans released rapidly and with a higher specific activity than large proteoglycans).

Applied current increased the rate of release of $^{35}$S-macromolecules from cartilage disks in 4°C PBSI in a dose-dependent manner (Fig. C.4). The maximal release rate with a current of $J = 10$ mA/cm$^2$ was similar (~0.4%/day) during 0–48-h and 48–96-h chase, although the relative increase was greater during later 48–96-h chase than early 0–48-h chase. Applied current also caused a stimulation of total sulfated glycosaminoglycan release, which exhibited a similar dosimetry

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Figure C.3: Effect of applied current on the hyaluronate binding properties of proteoglycan monomers from cartilage disks at various times after radiolabeling with $^{35}$S-sulfate. Cartilage disks were pulse (1.5-h) radiolabeled with $^{35}$S-sulfate, chased in 37°C medium for 0 h (A, B) or 48 h (C, D), and then subjected to a current of 0 mA/cm² (A, C) or 10 mA/cm² (B, D) for 48-h while in PBS with 10 mM N-ethylmaleimide and protease inhibitors at 4°C. Proteoglycan monomers were D1 purified from cartilage disks. Portions of monomer samples were reacted with 1.6% (w/w) hyaluronate and fractionated on Sephacryl S-1000; fractions were assayed for proteoglycan (-) and $^{35}$S (○).
Figure C.4: Effect of applied current on release of $^{35}$S-macromolecules during 0–48 h or 48–96 h of chase at 4°C. Cartilage disks were explanted, cultured for three days, and then pulse (1.5-h) radiolabeled with 200 μCi/ml $^{35}$S-sulfate. Some disks were washed with medium at 37°C followed by PBS with 10 mM N-ethylmaleimide and protease inhibitors at 4°C, and then subjected to a current of 0, 5, or 10 mA/cm² during 0–48-h chase (left panel) in the same 4°C solution. Other disks were chased in medium for 48-h (center panel) before being washed in 4°C solution and subjected to current during 48–96-h chase (right panel). Chase medium was analyzed for $^{35}$S-macromolecules, and the rate of $^{35}$S-macromolecular release was determined (mean ± range of duplicate groups of six pooled cartilage disks).
Figure C.5: Effect of applied current on release of glycosaminoglycans during 0-48 h or 48-96 h of chase at 4°C. Cartilage disks were pulse (1.5-h) radiolabeled with $^{35}$S-sulfate. Some disks were washed with medium at 37°C followed by PBS with 10 mM N-ethylmaleimide and protease inhibitors at 4°C, and then subjected to a current of 0, 5, or 10 mA/cm² during 0-48-h chase (left panel) in the same 4°C solution. Other disks were chased in medium for 48-h (center panel) before being washed in 4°C solution and subjected to current during 48-96-h chase (right panel). Tissue and chase medium were analyzed for sulfated glycosaminoglycans, and the rate of glycosaminoglycan release was determined (mean ± range of duplicate groups of six pooled cartilage disks).
Figure C.6: Effect of applied current on specific activity of released glycosaminoglycans during 0–48 h or 48–96 h of chase at 4°C. Cartilage disks were pulse (1.5-h) radiolabeled with $^{35}$S-sulfate. Some disks were washed with medium at 37°C followed by PBS with 10 mM N-ethylmaleimide and protease inhibitors at 4°C, and then subjected to a current of 0, 5, or 10 mA/cm² during 0–48-h chase (left panel) in the same 4°C solution. Other disks were chased in medium for 48-h (center panel) before being washed in 4°C solution and subjected to current during 48–96-h chase (right panel). Tissue and chase medium were analyzed for $^{35}$S-macromolecules and sulfated glycosaminoglycans, and the specific activity of released glycosaminoglycan was determined (mean ± range of duplicate groups of six pooled cartilage disks).
during 0–48-h and 48–96-h chase (Fig. C.5). Thus, during early 0–48-h chase, the specific activity of released sulfated glycosaminoglycans with \( J = 0-5 \) mA/cm\(^2\) was high, while the specific activity with \( J = 10 \) mA/cm\(^2\) was lower and approached that of material released during 0–48-h chase in 37°C medium and 48–96-h chase in 4°C PBSI (Fig. C.6). The similarly low specific activities would be consistent with a current-induced enrichment during early chase of the solution with large mobile (low hyaluronate binding affinity) \(^{35}\)S-proteoglycan with a relatively low specific activity.

Indeed, in the extracts of cartilage disks fractionated by cesium chloride equilibrium density gradient centrifugation, there was a disappearance of \(^{35}\)S from the D4 fraction with chase and with application of current. The D4 fraction of pulse-labeled disks (radiolabeled 3-h and soaked for 48-h in PBSI at 4°C) accounted for 1.21% of the total radioactivity. The D4 fraction of pulse samples subjected to 48-h current (10 mA/cm\(^2\) in PBSI at 4°C) was reduced to 1.01%. The D4 fraction of pulse and 48-h chased disks subjected to either 0 or 10 mA/cm\(^2\) for 48-h (during the 48–96-h after radiolabeling) in PBSI at 4°C was even less and accounted for only 0.76 and 0.77%, respectively, of the total radioactivity. This would be consistent with the rapid release of a small D4 \(^{35}\)S-macromolecule from the tissue to the medium.

C.3.3 The Effect of Applied Current on Proteoglycan Biosynthesis

To assess the cellular sensitivity to applied current, the incorporation of \(^{35}\)S-sulfate into \(^{35}\)S-macromolecules during application of current was examined. The rate of sulfate incorporation into zero current controls (≈1.4 nmol/hr-disk) was consistent with previously reported rates (Chapter III). There was a slight decrease (−10%) in the rate of sulfate incorporation with \( J = 5-10 \) mA/cm\(^2\) compared to \( J = 0-2 \) mA/cm\(^2\) (Fig. C.7). Thus, there appeared to be a minimal cellular response to these levels of current. This insensitivity of cartilage biosynthesis to applied current are consistent with previous studies where sinusoidal current at frequencies
of 0.1–100 Hz and magnitudes up to 1 mA/cm² were applied [50,159,161].

However, there was a measurable physical electrokinetic effect; the concentration of $^{35}$S-sulfate in the radiolabeling medium decreased with current in a dose-dependent manner (requiring sulfate incorporation rates to be computed using the specific activity of the medium for each current level). This would be consistent with an electroosmotic mixing of the isotope-free medium (originating within the agarose bridges) with the isotope-containing radiolabeling medium. Also, there was a trend toward an increase in the proportion of the newly formed $^{35}$S-macromolecules in the medium ($5.2 \pm 1.5\%$, $5.6 \pm 0.3\%$, and $7.6 \pm 0.3\%$ at $J = 0, 5$, and $10$ mA/cm²).

C.4 DISCUSSION

Currents of 2–5 mA/cm² stimulate the release of both $^{35}$S-proteoglycans and tissue proteoglycans from cartilage explants in culture. Aggregation of medium proteoglycans with excess hyaluronate and link protein before chromatography allowed identification of three macromolecular $^{35}$S-components: aggregating $^{35}$S-proteoglycans, non-aggregating $^{35}$S-proteoglycans, and a smaller $^{35}$S-macromolecule which may be the proteoglycan which is rapidly turned over in bovine cartilage explants [23] or rabbit articular cartilage explants [171]. This rapidly released $^{35}$S-component constituted $\sim 50\%$ of the $^{35}$S-macromolecules released during the 0–3-h of chase, but only $\sim 11\%$ during 24–27-h of chase.

The proportion of large $^{35}$S-proteoglycan in the medium which was able to aggregate with excess hyaluronate and link protein was higher during early 0–3-h chase than late 24–27-h chase; the application of current densities of 5 mA/cm² during 0–3-h chase but not 24–27-h chase caused a slight enrichment of the medium with aggregatable $^{35}$S-proteoglycan. Since most $^{35}$S-proteoglycans in pulse samples chased for 3-h still have a low binding affinity for hyaluronate, whereas those in 24-h chased tissue have a high binding affinity (Chapter III), the low affinity $^{35}$S-proteoglycans may be slightly more sensitive to electromechanical effects than high

Section C.4
Figure C.7: Effect of DC electric field on incorporation of $^{35}$S-sulfate into $^{35}$S-macromolecules. Cartilage disks were incubated for 9 h with $^{35}$S-sulfate while subjected to a current of 0–10 mA/cm$^2$. Disks were washed, papain digested, and analyzed for $^{35}$S. The wash and radiolabel medium were analyzed for $^{35}$S-macromolecules by Sephadex G-25 (PD10) chromatography. The rate of sulfate incorporation into macromolecules was computed from the sum of tissue $^{35}$S and medium $^{35}$S-macromolecules (mean ± range of duplicate groups of six pooled cartilage disks).

Section C.4
affinity $^{35}\text{S}$-proteoglycans.

The mechanism of electrically stimulated proteoglycan release was examined by applying a current to $^{35}\text{S}$-sulfate pulse labeled or pulse-chased disks bathed in PBS with protease inhibitors and N-ethylmaleimide at $4^\circ\text{C}$. Under these conditions, the newly synthesized $^{35}\text{S}$-proteoglycan were maintained in the low affinity state for 48-h; this is in marked contrast to the rapid conversion during incubation in $37^\circ\text{C}$ medium of newly synthesized proteoglycan to the high affinity form ($t_{1/2} \sim 4.5$ h, Chapter III). Possible functional differences between the low and high affinity forms of proteoglycan could be studied with the $^{35}\text{S}$-proteoglycan macromolecules thus maintained in a stable state in situ.

In the $4^\circ\text{C}$ bath, currents of 5–10 mA/cm$^2$ still enhanced the release of $^{35}\text{S}$-proteoglycans and tissue proteoglycans. Thus, the mechanism of stimulated release appears to be at least in part electromechanical and independent of enzymatic reactions. In this experimental configuration, electroosmosis occurred and may be a stimulatory physical mechanism, although electrophoresis of the highly charged proteoglycan could also be responsible. Consistent with a direct physical effect of current on extracellular matrix release rather than a chondrocyte-mediated effect, the same current densities had minimal effects on glycosaminoglycan synthesis. It would be important in future experiments to determine whether the $^{35}\text{S}$-macromolecules released in $4^\circ\text{C}$ PBSI had a similar distribution to those released in $37^\circ\text{C}$ medium.

Physical processes appear to play an important role in regulating the rate of proteoglycan release from cartilage explants. The rate of $^{35}\text{S}$-proteoglycan release is affected by the characteristic distance from tissue to the medium, and is inversely related to the thickness of the cartilage slice [183]. In addition, preliminary experiments indicate that slow cyclic compression of radiolabeled explants can still stimulate $^{35}\text{S}$-proteoglycan release when disks are bathed in the $4^\circ\text{C}$ enzyme-inhibiting solution (data not shown).

While in the present studies, applied electric fields induced release of macro-
molecules, applied electric fields could potentially be used to inject (e.g., electrophorese or electroosmose) molecules (such as growth factors) with the proper function, size, and charge into cartilage. Thus, electrokinetic phenomena within live cartilage may have useful therapeutic applications.
Appendix D

Effect of Serum on Biosynthesis and Matrix Accumulation in Cartilage Explants

D.1 INTRODUCTION

A control study was carried out to determine if a DMEM-based medium formulation would maintain explant cultures of calf cartilage disks during a two week period at steady state levels of protein and proteoglycan biosynthesis, as assessed by $^3$H-proline and $^{35}$S-sulfate incorporation.

D.2 METHODS

Cartilage disks, 3 mm diameter x 1 mm thick were explanted (Chapter II), and incubated in one of five medium formulations, adapted from that described by Hascall et al. [62]. The media were DMEM with 1%, 3%, 5%, or 20% NuSerum (Collaborative Research, Waltham, MA) or DMEM with 0.1 mM non-essential amino acids, 0.4 mM proline, 20 µg/ml ascorbate and 10 mM HEPES and 10% FBS (HyClone, Logan, UT). The medium was changed daily, and on the first day, supplemented with antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). After 1–13 days of culture, groups of disks were radiolabeled for 16 h in medium containing 5 µCi/ml $^3$H-proline and 10 µCi/ml $^{35}$S-sulfate (1 ml/disk), and then washed and analyzed for incorporated radiolabel and total GAG (Chapter II). Eight disks from a single core were maintained in each culture condition, and duplicate samples were analyzed at each time point.

D.3 RESULTS AND DISCUSSION

Appendix D
Figure D.1: Effect of serum on biosynthesis in cartilage explants. Cartilage disks were explanted and maintained free swelling in medium including 1% (●), 3% (▲), 5% (★), or 20% (■) NuSerum, or 10% FBS (×). At various times after explant, biosynthesis was assessed by radiolabeling disks for 16 h with both (A) $^3$H-proline and (B) $^{35}$S-sulfate. The mean and range are plotted for duplicate cultures at each time point.
The incorporation of $^{35}$S-sulfate and $^3$H-proline (Fig. D.1) increased between day 1 and 5 in 1%, 3%, 5%, and 20% NuSerum; however, even at 5% and 20% NuSerum, incorporation levels fell from day 9 to day 13. In contrast, in 10% FBS (Hyclone), the incorporation levels remained fairly steady through day 13. The stability of biosynthesis with 10% FBS is similar to that of slices of adult bovine cartilage in media supplemented with 10% FBS [62].

Total glycosaminoglycan content (Fig. D.2) increased at a rate of 16, 9, 21, and 44 µg/day in 1%, 3%, 5%, and 20% NuSerum, respectively, and 17 µg/day in 10% FBS. Relative to the glycosaminoglycan content on day 1, the rate of increase was 3.1, 1.7, 3.0, and 5.9%/day in 1%, 3%, 5%, and 20% NuSerum, respectively, and 3.1%/day in 10% FBS. This is somewhat different than the glycosaminoglycan (uronate) content of slices of 1–6 month old calf cartilage in 20% FBS, which was constant over 21 days of culture [116]. The difference may be due to the dependence of the rate of proteoglycan release on the ratio of surface area to volume, which is likely to be higher in thin slices [116] than our disks, or due to a slightly higher rate of catabolism in older animals (1–6 month [116] versus 1–2 week (Chapter II).

**D.4 CONCLUSIONS**

When calf cartilage disks were cultured in media with 10% FBS over ~two weeks, biosynthesis of protein and proteoglycan were fairly steady and glycosaminoglycan accumulated at ~3%/day.

*Section D.4*
Figure D.2: Effect of serum on glycosaminoglycan accumulation in cartilage explants. Cartilage disks were explanted and maintained free swelling in medium including 1% (●), 3% (▲), 5% (★), or 20% (■) NuSerum, or 10% FBS (×). At various times after explant, disks were solubilized with papain and the glycosaminoglycan content was determined. The mean and range are plotted for duplicate cultures at each time point.
Appendix E

Effect of Chondroitinase ABC on Biosynthesis and Matrix Accumulation in Cartilage Explants

E.1 INTRODUCTION

The highly charged proteoglycan content of the cartilage extracellular matrix not only endows the tissue with its load-bearing properties, but also governs the intratissue physicochemical environment \[52,100\]. The physicochemical environment may in turn influence cellular processes. Static compression of cartilage increases the concentration of negatively charged glycosaminoglycans, leading to an alteration in the physicochemical environment. It has been proposed that the compression leads to an inhibition in chondrocyte biosynthesis via an acidification of the intratissue environment \[48\], an increase in the sodium concentration \[187\], or an increase in the transmembrane pressure \[173\].

In the absence of compression, alterations in the proteoglycan content of the extracellular matrix may have cellular consequences. Matrix depletion may be achieved by treatment with enzymes which directly excise matrix components. Enzymes studied include the proteases papain \[15\], trypsin \[6,63,107,142\], and leukocyte elastase \[7\], as well as the somewhat more specific glycosidases Streptomyces hyaluronidase \[24\] and testicular hyaluronidase \[41,60\]. It is not clear if the source or maturity of the cartilage explants or the culture conditions influenced the biosynthetic response. Even so, moderate treatment with papain \[15\], trypsin \[6,107\], or hyaluronidase \[41,60\] led to a stimulation in biosynthesis. Proteoglycan was more sensitive to trypsinization than link protein, especially in the pericellular region, although both were partially replenished in all regions during subsequent culture \[142\]. In contrast, mild or severe treatment with trypsin \[6\] or leukocyte elastase \[7\] led to a decrease in biosynthesis. Thus, the relationship between the specific
molecules excised and the cellular response is unclear.

Matrix depletion has also been achieved via chondrocyte-dependent mechanisms using agents such as lipopolysaccharides, retinoic acid, interleukin 1, and tumor necrosis factor (see Chapter IV). Since these agents tend to alter cellular metabolism and inhibit chondrocyte biosynthetic processes, it is difficult to ascertain how the matrix depletion per se effects chondrocyte biosynthesis.

Alternatively, the extracellular milieu can be altered by modifying the proteoglycan content in the bathing medium. When proteoglycans levels in the bathing medium were raised, chondrocyte synthesis of proteoglycan was inhibited [56,169]. Here, the mechanism of action may be complex, as the polyanionic nature of the glycosaminoglycans may competitively bind growth factors present in the medium, or directly affect the chondrocytes [66,153]. Also, if the concentration of osmotically active molecules in the medium becomes great enough, osmotic forces will act to compact the cartilage [10,173].

A major difficulty in these studies is in differentiating between cellular responses to an altered matrix versus the direct cellular effects on chondrocytes of the applied agent. In the present study, Chondroitinase ABC was used in an attempt to fairly specifically deplete the extracellular matrix of charged glycosaminoglycan with minimal proteolysis. The effects of Chondroitinase ABC treatment on $^3$H-protein and $^35$S-proteoglycan excision, proteoglycan content and replenishment, and protein and proteoglycan biosynthesis were examined.

E.2 MATERIALS AND METHODS

Chondroitinase ABC (5 U/vial) was from Seikagaku Kogyo, Tokyo.

Cartilage disks, 3 mm diameter x 1 mm thick were explanted and maintained in DMEM including 10% FBS as described previously (Chapter II), except where noted. The medium was changed daily, and on the first day, supplemented with antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin).

Section E.2
E.2.1 Extractability of Cartilage Disks with Chondroitinase ABC

On the day of explant, cartilage disks were radiolabeled for 24 h with medium including 40 μCi/ml $^3$H-proline and 20 μCi/ml $^{35}$S-sulfate (1 ml/4 disks), and washed with 6 changes of medium (1–2 ml/4 disks), over 2 h. Radiolabeled disks were then incubated (0.25 ml/disk) with medium containing 0.0, 0.1, 0.2, or 0.4 U Chondroitinase/ml or 0.1 M Tris and enzyme inhibitors [44], pH 8.0 with 0.1 U Chondroitinase/ml for 1, 2, or 3 days. After enzyme treatment, disks were further incubated in the same medium or buffer without enzyme until day 3. Cartilage disks were digested with papain, and solutions and tissue digests were analyzed for radiolabel and sulfated glycosaminoglycan (Chapter II). The percentage of $^3$H and $^{35}$S remaining in the disks at each time point were determined for each culture relative to the total radiolabel released into the medium and remaining in the tissue. The percentage of glycosaminoglycan remaining in the disks after enzyme treatment was determined as the ratio of glycosaminoglycan in enzyme treated disks relative to that in untreated controls.

E.2.2 Effect of Chondroitinase ABC on Biosynthesis and Proteoglycan Accumulation in Cartilage Disks

Cartilage disks were treated for 3 days beginning on the day of explant or the following day with daily changes of medium (1 ml/4 disks) containing 0.0 or 0.1–0.2 U/ml Chondroitinase ABC. Disks were then washed with medium as above. After 0–13 additional days of culture, groups of disks were radiolabeled for 4 h in medium (1 ml/disk) containing 5–20 μCi/ml $^3$H-proline and 10–20 μCi/ml $^{35}$S-sulfate, washed, and analyzed for incorporated radiolabel and total sulfated glycosaminoglycan.
E.2.3 Assay of Chondroitinase ABC Activity

A stock solution of Chondroitinase ABC was prepared for addition to cultures by injecting ~3 ml medium (including 10% FBS) into the enzyme-containing vial, allowing the mixture to dissolve (~15 minutes), and passing the solution through a 0.22 μm filter pretreated with 2 ml of 10 mg/ml bovine serum albumin. This stock solution was stored at 4°C and used within one week; small portions were taken for assay of enzyme activity [181].

E.3 RESULTS

E.3.1 Extractability of Cartilage Disks with Chondroitinase ABC

The depletion of sulfated glycosaminoglycans and 35S-glycosaminoglycans from cartilage disks by incubation with Chondroitinase ABC was dependent on the concentration of enzyme in the medium and the duration of treatment (Fig. E.1). For example, after 1 day at 0.1 U or 0.2–0.4 U Chondroitinase/ml, ~65% or ~75%, respectively, of the sulfated glycosaminoglycans and ~40% or ~60% of the 35S-glycosaminoglycans were extracted. By 3 days of digestion with 0.1–0.4 U Chondroitinase/ml, ~90% of the sulfated glycosaminoglycans and ~75% of the 35S-glycosaminoglycans were extracted.

In partial 1- and 2-day digests, there was a large variability in the degree of 35S and glycosaminoglycan extraction, similar to that observed in Chondroitinase digests of bovine cartilage disks [44]. This variability may be related to differences in water content, as extraction occurred more rapidly in the more swollen disks both (1) from near the articular surface than from disks 1 mm deeper and (2) after 1–6 days post explant than immediately after explant (data not shown). This would be consistent with the proposed steric hinderance [118] of diffusion of Chondroitinase ABC within cartilage, which is a relatively large enzyme of 150 kD [195].

In order to assess the effects of Chondroitinase-induced matrix depletion

Section E.3
Figure E.1: Effect of Chondroitinase ABC on release of radiolabel and glycosaminoglycan from $^3$H-proline and $^{35}$S-sulfate labeled cartilage explants in media with 10% FBS. Cartilage disks, radiolabeled with $^3$H-proline and $^{35}$S-sulfate, were incubated in medium with 0.0 (○), 0.1 (▲), 0.2 (■), or 0.4 (★) U Chondroitinase/ml or in 0.1 M Tris and enzyme inhibitors, pH 8.0 with 0.1 U Chondroitinase/ml (×) for 1, 2, or 3 days. The percentage of (A) glycosaminoglycan, (B) $^{35}$S, (C) and $^3$H remaining in the disks after digestion were determined (mean ± SEM, n = 3).
Figure E.2: Effect of 1-day treatment with Chondroitinase ABC on release of radiolabel from $^3$H-proline and $^{35}$S-sulfate labeled cartilage explants in media with 10% FBS. Cartilage disks, radiolabeled with $^3$H-proline and $^{35}$S-sulfate, were incubated in medium with 0.0 (●), 0.1 (▲), 0.2 (■), or 0.4 (★) U Chondroitinase/ml or in 0.1 M Tris and enzyme inhibitors, pH 8.0 with 0.1 U Chondroitinase/ml (×) for 1 day, washed and further incubated without enzyme until day 3. Medium and tissue were analyzed for radiolabel, and the percentage of (A) $^{35}$S and (B) $^3$H remaining in the disks were determined (mean ± SEM, n = 3).
Figure E.3: Effect of 2-day treatment with Chondroitinase ABC on release of radiolabel from $^3$H-proline and $^{35}$S-sulfate labeled cartilage explants in media with 10% FBS. Cartilage disks, radiolabeled with $^3$H-proline and $^{35}$S-sulfate, were incubated in medium with 0.0 (●), 0.1 (△), 0.2 (■), or 0.4 (★) U Chondroitinase/ml or in 0.1 M Tris and enzyme inhibitors, pH 8.0 with 0.1 U Chondroitinase/ml (×) for 1 day, washed and further incubated without enzyme until day 3. Medium and tissue were analyzed for radiolabel, and the percentage of (A) $^{35}$S and (B) $^3$H remaining in the disks were determined (mean ± SEM, n = 3).

Section E.3
on subsequent cartilage metabolism, it was essential to first determine if enzyme could be washed out of the cartilage after treatment. Rinsing disks with media over 2 h appeared sufficient to remove essentially all Chondroitinase activity since disks which were digested for 1 or 2 days showed a return of $^{35}$S-sulfate release to untreated levels (Figs. E.2A and E.3A).

The specificity of the Chondroitinase preparation was assessed by also radio-labeling disks with $^3$H-proline and monitoring the release of $^3$H. There appeared to be a slight but dose-dependent release of $^3$H-proline labeled material with increasing Chondroitinase (Figs. E.1C, E.2B, and E.3B). Since the majority of the $^3$H-proline is incorporated into collagen (Appendix A) which is relatively resistant to proteolysis, even slight increases in $^3$H release may indicate significant non-collagen proteolysis. Several mechanisms may account for the $^3$H release. (1) Chondroitinase ABC has some hyaluronidase activity, especially at the culture pH of $\sim 7.4$ [195]; thus, proteoglycan monomers (including $^3$H-proline in the protein core) could be released. (2) The Chondroitinase ABC preparation used was not protease free; however, the addition of protease inhibitors often used with Chondroitinase [126,127] would probably have had deleterious effects on cellular metabolism.

E.3.2 Effect of Chondroitinase ABC on Biosynthesis and Proteoglycan Accumulation in Cartilage Disks

After disks were treated extensively (0.2 U/ml, 3 days) with Chondroitinase, both the wet weight and dry weight of digested disks were decreased compared to undigested controls (Fig. E.4A, B). The decrease in dry weight was approximately what would be predicted from the independently measured decrease in glycosaminoglycan content (Figs. E.4B and E.5). After treatment, the digested disks were initially more hydrated than undigested controls (Fig. E.4C). This seems paradoxical since the state of cartilage hydration is determined by a balance between the proteoglycan swelling pressure and the restraining tensile forces of collagen [98]; thus,
water loss should accompany the proteoglycan loss, leaving behind the majority of the collagenous tissue mass, leading to a decrease in hydration. One explanation for this may be an inability of the collagen framework to contract and reorganize quickly in response to the rapid matrix depletion; indeed, nine days after explant, both control and digested disks appeared to reach a maximum in hydration and subsequently become less hydrated (Fig. E.4C).

This extensive Chondroitinase treatment removed 96% of the sulfated glycosaminoglycan (24 ± 11 versus 650 ± 68 μg/disk, mean ± SEM, n = 6, Fig. E.5). After Chondroitinase treatment, glycosaminoglycan appeared to accumulate at a faster rate in the digested cartilage disks than the untreated controls (linear fit of day 3–12, where \( T \) = days after treatment, was 21.5 + 26.1 \( T \) in digested disks and 648.0 + 15.8 \( T \) in controls). As a percentage of total tissue glycosaminoglycan on day 3, the rate of glycosaminoglycan accumulation was 2.4%/day in control disks compared to 4.0%/day in Chondroitinase treated disks.

However, this accumulation did not appear to be the result of stimulated bioynthesis, since both sulfate and proline incorporation were inhibited or unaltered by Chondroitinase treatment (Fig. E.6). This suggests that the rate of catabolism of sulfated glycosaminoglycan must have been slowed. A similar conclusion was also reached in studies of trypsin treated bovine cartilage [63].

To more definitively determine the effects of Chondroitinase depletion of matrix, another experiment designed to have a smaller standard error (twelve disks per time point instead of six) was performed. The effect of stimulated glycosaminoglycan replenishment in disks depleted with Chondroitinase (0.1 U/ml, 3 days) was quite reproducible (Fig. E.7). Here, the Chondroitinase treatment removed 82% of the sulfated glycosaminoglycan (688 ± 21 versus 121 ± 26 μg/disk, mean ± SEM, n = 12). After Chondroitinase treatment, glycosaminoglycan accumulated at a faster rate in the digested cartilage disks than the untreated controls (linear fit of day 4–9, where \( T \) = days after treatment, was 117.4 + 36.5 \( T \) in digested disks and

Section E.3
Figure E.4: Effect of 3-day 0.2 U Chondroitinase ABC on mass of cartilage explants in media with 10% FBS. Cartilage disks were explanted and treated with 0.0 (■) or 0.2 (○) U Chondroitinase/ml for 3 days. At various times, cultures were terminated and the (A) wet weight, (B) dry weight, and (C) dry weight/wet weight were determined (mean ± SEM, n = 6).
Figure E.5: Effect of 3-day 0.2 U Chondroitinase ABC on glycosaminoglycan accumulation in cartilage explants in media with 10% FBS. Cartilage disks were explanted and treated with 0.0 (■) or 0.2 (●) U Chondroitinase/ml for 3 days. At various times, cultures were terminated and the glycosaminoglycan content was determined (mean ± SEM, n = 6).
Figure E.6: Effect of 3-day 0.2 U Chondroitinase ABC on $^3$H-proline and $^{35}$S-sulfate incorporation into cartilage explants in media with 10% FBS. Cartilage disks were explanted and treated with 0.0 (■) or 0.2 (○) U Chondroitinase/ml for 3 days. At various times, biosynthesis was assessed by radiolabeling disks for 16 h with both (A) $^3$H-proline and (B) $^{35}$S-sulfate (mean ± SEM, n = 6).
Figure E.7: Effect of 3-day 0.1 U Chondroitinase ABC on glycosaminoglycan accumulation in cartilage explants in media with 10% FBS. Cartilage disks were explanted and treated with 0.0 (■) or 0.1 (●) U Chondroitinase/ml for 3 days. At various times, cultures were terminated and the glycosaminoglycan content was determined (mean ± SEM, n = 12).
685.2 + 14.8 T in controls). As a percentage of total tissue glycosaminoglycan on day 3, the rate of glycosaminoglycan accumulation was 2.2%/day in control disks compared to 5.3%/day in Chondroitinase treated disks.

Here, the effects of Chondroitinase on biosynthesis were time-dependent (Fig. E.8). Immediately after treatment, digestion caused a reduction (~30%) in 35S-sulfate incorporation; however, three days after treatment, 35S-sulfate incorporation was increased (+41%) in digested disks. In contrast, 3H-proline incorporation was unaffected until 3 and 5 days after treatment, when incorporation was reduced by ~5.3% and ~21.4%, respectively.

In several experiments under low serum (0.1% NuSerum) conditions, a less extensive extraction with Chondroitinase had a variable effect on glycosaminoglycan replenishment (Figs. E.9 and E.10 [156]). For example, the rate of glycosaminoglycan accumulation was 0.7%/day in control disks and 0.3%/day in Chondroitinase treated disks (Fig. E.9), while in another experiment (Fig. E.10 [156]), glycosaminoglycan accumulation appeared stimulated (0.2%/day in controls versus 2.9%/day in treated disks).

Incorporation of 35S-sulfate and 3H-proline were either not altered (Fig. E.11) or inhibited ~25% (Fig. E.12 [156]). Thus, under low serum conditions, it also appears that increased deposition of sulfated glycosaminoglycan is predominantly a result of decreased proteoglycan catabolism, rather than enhanced biosynthesis.

### E.4 DISCUSSION and CONCLUSIONS

(1) Three day treatment with Chondroitinase ABC at 0.1–0.2 U/ml medium resulted in a fairly complete (~90%) depletion of glycosaminoglycan from 1 mm thick cartilage disks. The metabolic effects of less complete digestion may be difficult to interpret since the extraction may not be homogeneous.

(2) Glycosaminoglycan deposition was more rapid in enzymatically-depleted
Figure E.8: Effect of 3-day 0.1 U Chondroitinase ABC on $^3$H-proline and $^{35}$S-sulfate incorporation into cartilage explants in media with 10% FBS. Cartilage disks were explanted and treated with 0.0 (■) or 0.2 (●) U Chondroitinase/ml for 3 days. At various times, biosynthesis was assessed by radiolabeling disks for 4 h with both (A) $^3$H-proline and (B) $^{35}$S-sulfate (mean ± SEM, n = 12).
Figure E.9: Effect of Chondroitinase ABC on glycosaminoglycan accumulation in cartilage explants in media with 0.1% NuSerum (Experiment A). Cartilage disks were explanted and treated with 0.0 (■) or 0.2 (●) U Chondroitinase/ml for 24 h. At various times, cultures were terminated and the glycosaminoglycan content was determined (mean ± SEM, n = 5).
Figure E.10: Effect of Chondroitinase ABC on glycosaminoglycan accumulation in cartilage explants in media with 0.1% NuSerum (Experiment B). Cartilage disks were explanted and treated with 0.0 (■) or 0.2 (●) U Chondroitinase/ml for 24 h. At various times, cultures were terminated and the glycosaminoglycan content was determined (mean ± SEM, n = 6).

Section E.4
Figure E.11: Effect of Chondroitinase ABC on $^3$H-proline and $^{35}$S-sulfate incorporation into cartilage explants in media with 0.1% NuSerum (Experiment A). Cartilage disks were explanted and treated with 0.0 (■) or 0.2 (●) U Chondroitinase/ml for 24 h. At various times, biosynthesis was assessed by radiolabeling disks for 16 h with both (A) $^3$H-proline and (B) $^{35}$S-sulfate (mean ± SEM, n = 5).
Figure E.12: Effect of Chondroitinase ABC on $^3$H-proline and $^{35}$S-sulfate incorporation into cartilage explants in media with 0.1%NuSerum (Experiment B). Cartilage disks were explanted and treated with 0.0 (■) or 0.2 (○) U Chondroitinase/ml for 24 h. At various times, biosynthesis was assessed by radiolabeling disks for 12 h with both (A) $^3$H-proline and (B) $^{35}$S-sulfate (mean ± SEM; n = 6).
cartilage disks than untreated control disks.

(3) The increased deposition was not explained by a biosynthetic response, since incorporation of $^{35}$S-sulfate into depleted samples was initially depressed and subsequently only transiently increased. Although medium fractions were not analyzed for radiolabeled macromolecules, it is not clear if newly synthesized material may have been preferentially released into the medium of matrix-depleted samples. Nevertheless, the incorporation rates suggest that decreased catabolism and loss of newly synthesized proteoglycans was the major factor leading to increased deposition.

(4) It is difficult to differentiate between the response of chondrocytes to (A) excision of extracellular proteoglycans by Chondroitinase ABC (e.g., through a possible physicochemical effect) and (B) possible non-specific proteolysis of cellular or extracellular components by contaminating proteases which exist in the Chondroitinase ABC preparation [127]. It should be noted that a protease-free Chondroitinase ABC preparation has recently become commercially available (Seikagaku Kogyo or Boehringer Manhiem).
Bibliography


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