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Calcitriol Regulation Of Ros 17/2.8 Osteoblast-Like Cell Proteoglycans.

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CALCITRIOL REGULATION OF ROS 17/2.8 OSTEOBLAST-LIKE CELL PROTEOGLYCANS

by

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A DISSERTATION

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Bone proteoglycans comprise approximately 10% of the noncollagenous matrix in bone, a matrix which later mineralizes and undergoes subsequent remodeling. The noncollagenous extracellular matrix, distinguished from the abundant collagenous matrix, is believed to be of functional significance in the process of biomineralization. The various signals in bone remodeling are interdependent and are thought to include cell mediated factors, humeral factors, cell-matrix interactions, and the molecules of the noncollagenous matrix itself. Regulators of noncollagenous matrix molecules are currently being identified, including those that may regulate bone proteoglycan biosynthesis.

We have investigated the action of the Vitamin D metabolite, 1,25 dihydroxycholecalciferol, or calcitriol, on proteoglycan metabolism in the clonal osteoblast-like cells, ROS 17/2.8. Calcitriol is well known for its classical effects on bone mineralization and lately was shown to regulate the biosynthesis of several other bone matrix proteins so we became interested in whether it regulated bone proteoglycan metabolism.

We have found that calcitriol reduces the incorporation of [^{35}S]-sulfate into proteoglycans synthesized by ROS 17/2.8 osteoblastic cells. This action of calcitriol is dose-dependent, occurs within physiological concentrations of the hormone, and is not caused by a concurrent reduction in cell number or total protein synthesis. Calcitriol does not promote increased degradation of ROS 17/2.8 cell proteoglycans, alter the capacity of the cells to synthesize glycosaminoglycan chains or cause glycosaminoglycans of smaller relative mass to be produced. Calcitriol, in addition to reducing [^{35}S]-sulfate, reduced incorporated [^{3}H]-glucosamine by 65 to 70% in ROS 17/2.8 proteoglycans.

We found, in proteoglycans extracted from calcitriol treated cells, decreased sulfation at position 4 on the N-acetyl galactosamine residues in the glycosaminoglycan chain. This could arise from decreased activity of the specific 4-sulfotransferase or reduced synthesis of a proteoglycan core protein that contains highly 4-sulfated glycosaminoglycan chains. These data suggest that calcitriol is one regulator of bone proteoglycan metabolism and does so by decreasing the total synthesis of proteoglycans as well as by governing the sulfation pattern of these molecules.

Abstract Approved by: Committee Chairman ________

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LITERATURE REVIEW

Bone Tissue And Extracellular Matrix Molecules

Bone tissue is predominantly extracellular matrix (ECM) which is composed of organic molecules synthesized and secreted by the bone forming cells, osteoblasts. During the process of mineralization the organic matrix acquires an abundance of calcium and phosphorus in the form of hydroxyapatite. The final result is a hardened ECM in which are embedded osteocytes, blood vessels and nerve fibers. In vertebrates the skeleton provides mechanical support and it serves as a calcium reservoir. It is not a static tissue, rather, it is remodeled throughout the lifetime in a cycle that involves resorption followed by remineralization. Remineralization, like initial mineralization, involves the secretion of the organic ECM followed by deposition of mineral on and around the ECM.

Cells particular to bone include the matrix-secreting osteoblasts, the multinucleate, bone-resorbing osteoclasts, the osteocytes, and the lining cells. Lining cells cover the surface of bone, have few organelles and can differentiate into osteoblasts. The osteocytes are osteoblastic cells that have become embedded in mineral. They have numerous cytoplasmic projections which connect them within the rigid matrix so that bone is permeated by a

living cellular network. Osteoclasts are fusion cells that are derived from circulating mononucleosis cells derived from bone marrow and that seem to require the direction of osteoblasts in order to differentiate into the resorbing phenotype. The manner in which osteoclasts resorb bone is an area of intense study as are the multitude of factors that seem to control resorption (Marks and Popoff 1988). Osteoblasts synthesize and secrete the ECM which is composed of a variety of proteins, 90% of that being Type I collagen. The remaining proteins of the matrix are collectively called the non-collagenous matrix proteins or NCPs.

Many NCPs have been extracted from bone along with unidentified peptides, blood-derived products, lysosomal enzymes, collagenases and bone-derived growth factors. At least 1/3 of the NCPs in bone have not been identified or characterized (for review see Robey 1989).

Of those NCPs which have been identified, osteonectin is the NCP present in highest concentration in bone extracts. It is synthesized by both osteoblasts and fibroblasts so it is not an exclusive bone product. This protein binds to collagen and hydroxyapatite but the functional significance of this interaction is not known.

Osteocalcin, also known as bone Gla protein (BGP), is a γ-carboxyglutamic acid (GLA) containing protein that requires Vitamin K for its synthesis. This small acidic protein contains 3 Gla residues, is found in plasma and

bone, and is upregulated by calcitriol. The 15kD matrix Gla protein (MGP) is also found in bone and contains 5 Gla residues. It appears during the early stages of bone development in contrast to osteocalcin which appears later.

There are at least two sialoproteins found in bone extracts, Sialoprotein I and Sialoprotein II. Sialoprotein I is an acidic phosphorylated glycoprotein also called osteopontin. This 44kD molecule has a five residue cell attachment sequence and it binds to hydroxyapatite. It is not found exclusively in bone, in fact it seems to be induced during metastasis, suggesting a role for it in cancer promotion. Osteopontin is another ECM protein that is regulated by calcitriol (Prince and Butler 1987; Chang and Prince 1991). Bone Sialoprotein II has actually been isolated from bone and dentin. It has a sialic acid content of 5 to 13 % and a molecular mass of 57kDa. This sialoprotein also binds to hydroxyapatite and promotes cell attachment via an RGD sequence. BSP II does not show the diversity in tissue distribution that osteopontin does (Oldberg et al.1988).

The proteoglycans of bone are usually the small nonaggregating type. Many investigators agree there are two major proteoglycans extractable from bone tissue, bone organ culture systems and osteoblast-like cell cultures. These have been designated PG I or biglycan because it has two GAG chains attached to a protein core of about 40kD and PG II or

decorin which has a single GAG attached to a similarly sized core protein. Decorin is so named because it attaches to or "decorates" collagen fibrils. The core proteins are known to be separate gene products.

Bone morphogenic protein is a small glycosylated matrix protein that increases DNA synthesis and cell replication in bone organ cultures. In vivo this protein is capable of inducing synthesis of new bone. Other bone-derived growth factors have also been identified as well as glycoproteins and phosphoproteins that have not been fully characterized.

Thus, much of bone matrix has not been accounted for. Of the proteins mentioned, there is scant knowledge of their location in vivo or their metabolic regulation. Furthermore, functions of the NCPs in bone biology or mineralization have not been assigned nor is it known how they interact with each other, or with collagen, to initiate mineralization.

Theories Of Mineralization

In general, biological mineralization occurs in two phases, the first being synthesis and deposition of an organic matrix and the second being the accumulation of mineral within that matrix. The proper localization, concentration and orientation of the matrix components are necessary for mineralization to occur in a correct spatial and temporal manner. The noncollagenous proteins (NCPs) of bone are thought to govern mineralization in vertebrates because they differentiate bone osteoid from extracellular

matrix of other tissues, which may also be predominantly Type I collagen yet lack the NCPs. Since Type I collagen, the major macromolecule of bone matrix, is also found as the major protein of some unmineralized tissues, such as skin and tendon, the NCPs must have a profound influence on mineralization. Differing theories propose that these minor components either promote mineralization and, therefore, need to be synthesized prior to mineral acquisition, or they act as inhibitors and need to be degraded before mineralization occurs (Marks 1988). This activity of the NCPs is superimposed upon other regulatory processes since bone cells, which are regulated by local and humeral factors, also have a key role in mediating mineralization.

Biomineralization encompasses a wide range of activities. Under normal circumstances it can be thought of as controlled crystallization. This applies to calcium salts of vertebrate bone as well as the salts of silica, strontium, iron and barium seen in various plants, unicellular organisms, insects and other invertebrates. One of the requisites for crystal growth is nucleation, or the formation of an initial seed crystal. Thermodynamic forces favoring precipitation of solid phase crystals must overcome forces favoring dissolution. Nucleation represents attainment of sufficient energy to allow crystals to form from a supersaturated solution. In a biological environment, the energy required for nucleation can be lowered

by increasing supersaturation or by lowering interfacial energy, which is the sum of forces required at the crystal surface to allow formation of new solid phase. Supersaturation may be increased by compartmentalization of mineralization. For example, macromolecular scaffolding may allow selective ionic transport into a confined space thus creating discrete pockets of supersaturation where nucleation can occur. Similarly, matrix vesicles, which arise by budding from cell membranes, are transported to mineralization fronts in bone and may also serve as supersaturation containment areas. Interfacial energy is thought to be lowered by organic surfaces such as those found in proteins (Mann 1988). The organic surface could contain regions of high charge density for bonding of mineral nuclei, for example, regions of polyaspartic acid which are found in several phosphoproteins of bone and dentin and the polyanionic glycosaminoglycan chains of proteoglycans. Stephen Mann has put forth the idea of crystallochemical specificity stating that molecular recognition takes place between stereochemical requirements of a crystal lattice and the charged groups on a macromolecular interface. This potential complementarity renders nucleation sites having a high degree of crystal-organic surface specificity, prompting the analogy to enzyme catalytic sites and their substrates (Mann 1988). Organic polymers are believed to be organized so their surfaces

provide a topographical array of regular, repeating sites for nucleation. Studies of mollusk shell show the underlying matrix to be antiparallel ß-pleated sheets on which are aligned EDTA-soluble components in a regular fashion. In bone, hydroxyapatite nucleation may occur in the "hole zones" of Type I collagen fibrils. Phosphoprotein molecules present in these hole zones could theoretically attract calcium and phosphate ions and the specific spatial array within the hole zone would favor the structural order of the growing crystal. Nucleation on protein surfaces could also cause conformational changes in the protein which in turn favor continuing crystal growth (Simkiss et al. 1982).

The biologist's view of mineralization involves describing the locations and roles of components of the system. Osteoblasts are the matrix secreting cells of bone and so can be thought of as the bone forming cells. They secrete matrix in a remarkably polar fashion, depositing it only toward existing matrix (Sodek and Berkman 1987). This seam of matrix, or osteoid, separates the layer of osteoblasts from the calcification front. Mineral becomes more dense as one moves away from the newly secreted matrix.

The role of NCPs in mineralization is not clear. One theory is that phosphoproteins may inhibit mineralization until the proper spatial array of collagen fibrils exists to allow the growing crystal lattice to adopt the correct

conformation. Then these inhibitors would be degraded before further calcification could occur. Others believe the NCPs actually promote mineralization, perhaps by aiding supersaturation, promoting osteoblast attachment, creating zones of high charge density favoring nucleation or directing collagen conformation. In fact, it is likely that all of these mechanisms are employed by one or more of the NCPs in bone matrix.

In vitro systems have been developed to look at the effect of various NCPs on mineral induction. One system involves binding polyanionic bone matrix proteins and proteoglycans to agarose beads and incubating the beads in solutions of calcium and phosphate ions. Mineral formation on the beads is observed by scanning electron microscopy. Interestingly, Bone Gla protein, phosphoprotein, phosvitin and bone proteoglycans induced mineral deposition (Linde et al.1989). The mineral was shown to be apatitic in nature. This finding is of interest because earlier work supported the idea that cartilage proteoglycans inhibit hydroxyapatite formation in vitro (Blumenthal et al.1979).

Inhibition of mineralization has long been presumed to be the role of proteoglycans in bone. Work with genetic mutants of PG metabolism, such as the nanomelic chick and the brachymorphic mouse, have shown that specific abnormalities in PG structure and metabolism lead to disruption of normal bone formation (Heinegard and Paulsson

1984) (Pennypacker et al.1981). Other studies have implicated PG degradation as being a prerequisite for bone mineralization. However, before discussing bone proteoglycans in detail, a review of PG structure, metabolism and functions is needed to place the current information about bone proteoglycans in perspective.

Proteoglycans

Structure

Proteoglycans are a class of complex macromolecules consisting of a protein core to which one or more glycosaminoglycan (GAG) chains are covalently attached. Proteoglycans (PGs) are a heterogeneous group of molecules with PGs of even the same tissue displaying differences in protein core, GAG chains or both. The protein cores thus far described vary in mass from 30,000 to 500,000 kD. The point of covalent linkage of the GAG chain is usually a SER-GLY pair with the side group oxygen (O-) of Serine forming the glycosidic bond to the initial xylose of the GAG chain linkage region (Rodén 1980).

Substitution of protein cores by carbohydrate can occur not only by addition of GAG chains but also by the addition of N- or O-linked oligosaccharides. N-linked oligosaccharides are bound to the core protein via an ASN residue while O-linked oligosaccharides are attached to SER or THR residues. The features that set GAGs apart from oligosaccharides are that the latter are frequently

branched, short chain sugar polymers while GAGs are linear polymers of repeating disaccharides. GAGs are always negatively charged and are of higher molecular weight than oligosaccharides.

Proteoglycans can be grouped by their ability to form aggregates with hyaluronic acid. Thus, they are considered aggregating if they do bind with hyaluronic acid and nonaggregating if they do not. Within those categories, they are further grouped by size so that there are large aggregating and small aggregating proteoglycans as well as large and small non-aggregating proteoglycans. Large aggregating proteoglycans are found in cartilage, aorta and tendon, while small, aggregating PGs are found in sclera, rat brain, glial cells and glioma cells (Poole 1986). Aggregating PGs possess a hyaluronic acid binding region (HABR) which is located at the N-terminus of the core protein. An individual aggregate may contain 40 or more PG monomers bound noncovalently to hyaluronic acid, an interaction that is stabilized by small glycoproteins called link proteins (Baker and Caterson 1979). Proteoglycan aggregates occupy large hydrodynamic volumes due to their high negative charge density thus giving tissues the property of compressibility.

There is a tremendous variety of non-aggregating proteoglycans. Large chondroitin sulfate (CS) proteoglycans are found in muscle and skin. Small, non-aggregating

chondroitin sulfate proteoglycans are seen in bone and several small dermatan sulfate proteoglycans are found in cornea, cartilage (associated with type IX collagen) and in fibroblasts. Cornea contains a small keratan sulfate PG and heparan sulfate proteoglycans have been identified in glomerular basement membrane and hepatocyte plasma membrane (Poole 1986). This is not an inclusive list as proteoglycans are probably found in every tissue, their structural variability is nearly limitless, and new ones are constantly being identified.

Biosynthesis

While there are many quite different proteoglycans, the basic biosynthetic steps are common to all of them. Synthesis of core proteins is the first event and occurs in the rough endoplasmic reticulum in the same manner as for all proteins. Proteoglycans that will acquire N-linked, high mannose containing oligosaccharides have these assembled in the endoplasmic reticulum through a dolichol diphosphate intermediate. The synthesis of chondroitin sulfate, keratan sulfate and O-linked oligosaccharide chains occurs next most likely in the Golgi apparatus and the rough endoplasmic reticulum (Fransson 1987). The initiation of GAG chain synthesis seems dependent on the availability of core protein acceptor molecules. Kinetic labeling experiments have indicated that a pool of precursor protein cores exists ready to be post-translationally modified

(Kimura et al. 1984). In the Swarm rat chondrosarcoma chondrocyte culture system the half-life of the core protein pool is 90 min allowing GAG chain synthesis to continue for some time after core protein synthesis is interrupted.

GAG chain synthesis begins with the addition of xylose to the SER residue of a SER-GLY sequence on the core protein. The addition of xylose may occur in the endoplasmic reticulum as xylosyltransferase has been identified there and xylosylation precedes the next step by almost 5 min (Poole 1986). Once in the Golgi, GAG chains are rapidly completed. Using chondroitin sulfate as a general example, this involves the addition of two galactose residues by galactosyltransferases 1 and 2. Glucuronosyl-transferase 1 catalyses the transfer of the first glucuronosyl residue which is considered part of the linkage region. This tetrasaccharide then accepts the repeating disaccharides that begin with N-acetylgalactosamine, added via N-acetylgalactosaminyltransferase, followed by addition of glucuronic acid via another glucuronosyltransferase. If the GAG chain is to be dermatan sulfate, an epimerase converts glucuronic acid to iduronic acid after poly-merization. The single sugars that are added to the growing GAG chain are donated in the form of UDP sugars.

Sulfation of GAG chains also occurs in the Golgi apparatus and is concomitant with chain polymerization. Addition of sulfate is mediated by PAPS (3' phosphoadenosyl

5' phosphosulfate) and catalyzed by 4-, 6-, or N-sulfotransferases, depending on where the sulfate is to be placed (Fransson 1987). The signal for termination of chain elongation may be the addition of sulfate to carbon 4 of hexosamine residues since N-acetylgalactosamine-4-sulfate is not able to accept glucuronic acid (Rodén et al.1972). Proteoglycans are then transported out of the Golgi to the cell surface by secretory vesicles.

Habuchi and Miyashita (1982) have identified two different sulfotransferases in chick embryo cartilage, a chondroitin 4-sulfotransferase and a chondroitin 4-sulfotransferase. Another group (Inoue et al.1986) has identified four sulfotransferases in human sera, each responsible for the introduction of sulfate to different positions of chondroitin, keratan or heparan sulfate. These enzymes, located within the Golgi lumen, appear to be differentially regulated. Delfert and Conrad (1985) found the ratio of 4-sulfated to 6-sulfated glycosaminoglycan varies in cultured cells as differentiation progresses and as culture conditions are manipulated. Others have found that activities of sulfotransferases in various animal tissues were regulated differently dependent on stage of development (Sugahara et al.1987). Factors known to alter activities of sulfotransferases include pH, concentration of divalent metal ions and concentration of basic peptides and polyamines (Habuchi and Miyashita 1982) (Delfert and Conrad,

1985). Little is known of the significance or function of proteoglycan sulfation although Ribari (1991) reported the presence of oversulfated proteoglycans in otosclerotic bone.

Degradation

Degradation of proteoglycans is ongoing and the constituents are regularly recycled. Initially, extracellular proteoglycans may undergo limited cleavage of the protein core. Proteolytic fragmentation is thought to be a first step in degradation. The GAGs attached to peptides are endocytosed and digested in the lysosome to monosaccharides, sulfate, and amino acids. The peptides are degraded by cathepsins and the GAGs by endoglycosidases (Heinegård and Paulsson 1984).

Some protein cores can pass back into the Golgi and once again serve as primers for addition of GAG chain biosynthesis, thus bypassing complete degradation. This type of recycling is believed to take place, for example, in the case of the transferrin receptor (Farquhar 1985). Regulation of the degradative process is not known.

Functions

Functions of proteoglycans have not been decisively proven, yet many have been suggested. Because the large proteoglycans attract and retain water molecules, they occupy an extended hydrodynamic area and exert a swelling pressure within tissues such as cartilage, imparting the property of compressive stiffness. Studies of genetic

disorders where PG metabolism is altered have yielded information as to probable functions. Nanomelic chicks do not produce the large aggregating proteoglycans and they show skeletal defects including increased width of the epiphyseal cartilage. Examination of the skeletal defects in these chicks and the fact that proteoglycans occupy a large hydrodynamic volume has led some investigators to speculate that the proteoglycans serve as space creating material (Heinegård and Paulsson 1984). The abundant proteoglycans of growth cartilage are thought to be important not only for mechanical properties but in regulating diffusion of ions and nutrients throughout the tissue. In cartilage calcification, there is a discrepancy about whether or not proteoglycans are inhibitors of mineralization and are degraded at the mineralization front prior to mineralization. Evidence exists supporting this view as well as the view that they are merely disaggregated before mineralization (Hascall and Lowther 1982). The small proteoglycans produced by fibroblasts and osteoblasts may organize collagen fibrils during fibrillogenesis and contribute to stabilization of basement membranes. Proteoglycans may take part in cell adhesion by binding to other extracellular matrix molecules. Strength of binding seems dependent on the number of sulfate groups present indicating possible ionic interactions (Ruoslahti 1989).

Proteoglycans Of Bone

The proteoglycans embedded in the mineralized matrix of bone are usually removed by dissociative extraction in an EDTA solution. They are then isolated in a series of chromatographic steps utilizing size exclusion and anion exchange resins. In this manner the proteoglycans of the mineralized compartment are separated from components of osteoid and other soft tissues. The characterization of proteoglycans from bone has not been an easy task; since bone is a hard tissue, the amount of proteoglycans present is relatively small and the problem of contamination from surrounding soft tissue is always present. Over the past 10 years as methods have improved, the isolation and characterization of proteoglycans in bone has begun to take shape.

Prince et al. extracted one large chondroitin sulfate PG and two smaller chondroitin sulfate proteoglycans from rat bone matrix after metabolically labeling with $^{35}SO_4$ (Prince et al.1983), while Franzén and Heinegård (1984) resolved three uronic acid rich peaks following DEAE and hydroxyapatite chromatography. Chondroitin-4-sulfate proteoglycans seem to predominate in adult bovine compact bone. A single PG migrating near 90,000 Daltons on linear gradient SDS-PAGE gels, and staining positively with alcian blue, was found in adult bovine bone (Sato et al. 1985). Fisher's group and Heinegård's group noted that fetal and adult bovine bone, respectively, contained three

proteoglycans (Fisher et al.1983) (Franzén and Heinegård 1984). Three distinct chondroitin sulfate proteoglycans were isolated and characterized from fetal porcine calvaria (Goldberg et al.1988). The largest of these, with a protein core of 45,000 daltons, cross-reacts with a monoclonal antibody against a bovine skin proteoglycan, and had a high content of hydrophobic amino acid residues. The two smaller proteoglycans had protein cores of about 30,000 daltons and were enriched in acidic amino acid residues.

The two major proteoglycans that have been isolated from the mineralized compartment of bone have been named PGI, or biglycan since it contains two GAG chains, and PGII (decorin). PG II has a single GAG chain. The protein cores of PG I and PG II are similar in size, being about 40,000 daltons, leading early investigators to believe that a single protein core was simply carrying different numbers of GAG chains. The protein cores are known to be separate gene products and amino acid analysis has shown PG I to be more hydrophobic due to a higher content of leucine residues. PG II is enriched in glutamic acid and glutamine residues. There is quite a bit of sequence homology between the two proteoglycans leading to speculation that the two genes arose from gene duplication (Robey 1989). Antibodies against the core proteins of these proteoglycans do not cross react. The cDNA for the PG II core protein from adult bovine has been cloned and sequenced, revealing a 330 amino

acid protein of Mr 36,383 with three possible GAG attachment sites. This core protein has 87% nucleotide sequence homology to the PG from human embryonic fibroblasts (Day et al.1987). It was recently demonstrated by cDNA analysis that human PG I and PG II are separate gene products. Their deduced protein sequences reveal a 55% sequence homology. A striking feature was that the 368 residues of PGI include 12 tandem repeats of 24 residues, many of which are conserved leucines (Fisher et al.1989).

Biglycan and decorin are found in a variety of connective tissues including bone, skin, tendon, sclera, cornea and aorta. Little is known of regulatory factors for these PGs in various tissues, although synthesis of decorin in extracellular matrix of rat skeletal muscle is under neural control (Brandan et al.1992). Immunolocalization of biglycan and decorin in bone shows them appearing in matrix and in areas of new bone formation (Robey 1989). Functions for these two PGs in any tissues remain obscure.

The other proteoglycan in bone that has been described is larger, has more chondroitin chains and is found associated with the soft tissue compartment of developing bone (Fisher et al.1983). This larger chondroitin is immunoreactive with antibodies raised against the aggregating PG from cartilage yet was not felt to be a cartilage PG artifact.

Studies with cell culture systems have yielded differing results also. Fetal rat calvarial clonal cells were shown to produce three types of PG. One large (Mr=350,000 daltons) secreted PG was reportedly 77% chondroitin sulfate and 20% dermatan sulfate. The remaining two proteoglycans were cell layer associated, one being composed of chondroitin and dermatan sulfate chains and the other having heparan sulfate in addition to chondroitin and dermatan sulfate chains (Hunter et al.1983).

Cultured mouse osteoblasts elaborated three proteoglycans also, one large chondroitin sulfate PG and two smaller proteoglycans containing dermatan sulfate chains. The dermatan chains on the two smaller proteoglycans had an apparent Mr of 40,000 and the core proteins migrated at 45,000 daltons on SDS-PAGE. The large CS PG was a minor component of the total population of proteoglycans purified from the mouse osteoblasts and could represent contaminating material from chondrocytes (Ecarot-Charrier and Broekhuyse 1987). This group and others did notice that the GAG chains on the small proteoglycans tend to be CS when isolated from bone and DS when isolated from cell culture systems. The explanation given for this discrepancy is that post-translational modifications are somehow altered by culture conditions. This seems a highly unimaginative explanation.

The proteoglycans synthesized during mineralization in vitro by mouse MC3T3-E1 cells were shown to be chondroitin

sulfate, dermatan sulfate and heparan sulfate containing. Different proteoglycans were isolated from these cells depending on the phase of mineralization (Takeuchi et al.1990).

Cells derived from human trabecular bone produced quite an array of distinct PG species and PG degradation products. Two dermatan sulfate proteoglycans with core proteins of 49,000 and 52,000 daltons, respectively, on 4-20% gradient SDS-PAGE were identified. The protein cores differed in terms of hydrophobicity. Both proteoglycans were found in the medium and cell layer fractions. A large chondroitin sulfate proteoglycan and a possible hybrid proteoglycan, containing both heparan sulfate and chondroitin sulfate or dermatan sulfate chains were found in both media and cell layer (Beresford et al.1987). More recently, human bone cells were found to produce hyaluronic acid and four species of proteoglycan. The proteoglycans consisted of a large chondroitin sulfate proteoglycan, a heparan sulfate proteoglycan and two dermatan sulfate proteoglycans of fairly small size (Fedarko et al.1990). All are non-aggregating. The two small dermatan sulfate proteoglycans (Mr= 270,000 and 135,000) are believed to be identical to other small dermatan and/or chondroitin sulfate proteoglycans, i.e. biglycan and decorin, isolated from bone and osteoblast-like culture systems.

The consensus about which proteoglycans are truly bone proteoglycans seems to be that biglycan and decorin, as well as a large chondroitin sulfate proteoglycan, are consistently present in bone tissue preparations and in osteoblast-like cell cultures. Differences in bone proteoglycan populations are observed depending on whether the proteoglycans are isolated from bone tissue or from culture systems and vary according to species, age of animal, length of time in culture, and methods of evaluation.

Regulators Of Bone Metabolism

Bone is a dynamic tissue. In order for formation and degradation to occur at the correct location, in the proper sequence and for normal duration, a vast array of hormones, growth factors and cytokines orchestrates bone metabolism. Below I will discuss some of the major regulators of bone synthesis and degradation.

The classic action of calcitriol is the maintenance of serum calcium, a task accomplished in concert with parathyroid hormone (PTH). Some effects of calcitriol on bone are independent of PTH, such as increase in osteoclast number and subsequent bone resorption and the regulation of several matrix proteins. While Type I collagen synthesis is depressed by calcitriol, osteocalcin, osteopontin and bone Gla protein biosynthesis is increased by the hormone. These effects are observed in vivo and in vitro (Finkelman and

Butler 1985). Continuous infusion of physiological doses of calcitriol in normal mice seems to promote matrix calcification yet inhibit synthesis of new matrix. The promotion of matrix calcification is independent of alterations in serum calcium suggesting a direct effect on mineralization (Marks and Popoff 1988). In contrast, there are reports that calcitriol deficient rats show no changes in the amount of extractable bone proteoglycans, sialoprotein II, osteonectin or osteocalcin (Wientroub et al.1987a). Two dimensional gel analysis of proteins synthesized by ROS 17/2.8 cells showed that while total protein synthesis was not affected by calcitriol, altered rates of synthesis were seen for 28 different proteins (Murray et al.1990).

When isolated osteoclasts are incubated on slices of cortical bone, their ability to resorb bone is not stimulated by calcitriol. This suggests that the osteoclasts fail to resorb bone unless they are acted upon by a local factor or another cell type. One group has found that osteoblasts, under the influence of calcitriol, induce osteoclastic bone resorption (McSheey and Chambers 1987).

Alkaline phosphatase biosynthesis is increased by calcitriol in ROS 17/2.8 cells and in normal human bone cells (Robey 1989). Proteoglycans isolated from the growth cartilage of vitamin D-deficient chicks are smaller than those isolated from vitamin D replete chicks (Carrino et al.

1989). Degradation of proteoglycans in growth cartilage cells is reportedly enhanced by calcitriol (Uchida and Shimomura 1988). Osteoblastic MC3T3-E1 clonal cells showed inhibition of synthesis and increased degradation of proteoglycans when cultured in 10^{-8} M calcitriol (Takeuchi et al.1989).

In addition to calcitriol there are a number of hormones, nutrients and growth factors that affect bone constituents. Ascorbic acid has long been recognized as a necessary ingredient in primary osteoblastic cell culture systems in order to achieve mineralization. Recently, ascorbate deficiency in calcitriol replete guinea pigs resulted in lower calcium levels in serum and bone (Sergeev et al.1990). Cartilage proteoglycan synthesis, as well as collagen synthesis, is reduced in scorbutic guinea pigs (Bird et al.1986) and ascorbate also caused an increase in sulfated proteoglycan synthesis by chondrocytes and by articular cartilage slices in culture (McDevitt et al.1988) (Schwartz 1979).

Retinoic acid deficient guinea pigs have bone with an abnormal morphology and a higher glycosaminoglycan content than that of controls. Neonatal rat calvaria grown in media lacking vitamin A also had a significantly increased amount of $^{35}SO_4$ in the GAG fraction as compared to controls (Navia and Harris 1980).

Transforming growth factor-ß (TGF-ß) is now recognized as a major regulator of extracellular matrix synthesis in many tissues. TGF-ß synthesized by bone cells depresses Type I collagen synthesis and increases the expression of the small non-aggregating proteoglycans found in blood vessels, skin and sclera. The GAGs from these proteoglycans are larger than those from proteoglycans of cells not treated with TGF-ß. Additionally, it regulates the expression of fibronectin and fibronectin receptors (Bassols and Massague 1988). TGF-ß may be a primary signaling factor in bone remodeling in that it stimulates osteoblast proliferation while concurrently diminishing osteoclast activity (Komm et al.1988).

While parathyroid hormone (PTH) has long been considered a catabolic hormone, in terms of bone resorption, more recent in vivo and in vitro evidence have shown it to be both a stimulator and inhibitor of bone formation. PTH receptors are found on osteoblasts and the hormone is known to stimulate the release of many other growth factors which act on bone.

The steroid hormones estrogen and progesterone were shown to stimulate matrix-induced endochondral bone formation in rats (Burnett and Reddi 1983). Rat calvarial osteoblast-like cells showed increased cell proliferation and up-regulation of Type I collagen synthesis in response to 17-ß-estradiol (Ernst et al. 1988). High affinity

estrogen receptors have been located in normal human osteoblast-like cells and in ROS 17/2.8 osteosarcoma cells. In ROS 17/2.8 and HOS TE-85 clonal cells Type I collagen and TGF-ß mRNA levels increased when the cells were treated with estrogen (Komm et al.1988). Insulin and insulin-like growth factor are potent stimulators of proteoglycan synthesis as are platelet-derived growth factor and pituitary-derived fibroblast growth factor. Prostaglandins inhibit the synthesis of large cartilage proteoglycans while enhancing synthesis of proteoglycans in granulosa cells demonstrating that proteoglycans can be individually regulated. Within a given tissue, synthesis can be differentially regulated as in rat granulosa cells which produce two types of proteoglycan, only one of which is increased by follicle stimulating hormone (Poole 1986).

Calcitriol

Biosynthesis

1,25-dihydroxycholecalciferol, or calcitriol, is the major biologically active metabolite of the secosteroid known as Vitamin D and has been dubbed the "bone and tooth " vitamin in human nutrition. While the vitamin can be synthesized by most mammals it is also obtained by ingestion of foods such as fish, fish oils and egg yolk. Vitamin D synthesized endogenously or absorbed through the intestine must undergo metabolic activation to the active form of the vitamin. The classic actions of vitamin D have to do with

calcium and phosphorus metabolism. It, in concert with parathyroid hormone, is responsible for maintaining serum calcium levels which are critical for many vital functions. Recent discoveries pertaining to the vitamin include its mechanism of action and the regulation of events other than those in mineral metabolism.

Endogenous synthesis of vitamin D begins with 7-dehydrocholesterol in the skin which undergoes cleavage of the 9,10 bond when it interacts with ultraviolet light in a nonenzymatic reaction. The product, precholecalciferol, is thermally isomerized to cholecalciferol, or vitamin D. At this point ergocalciferol from foods and cholecalciferol are handled similarly. Vitamin D is carried to the liver by a vitamin D binding protein. A nonregulated hydroxylation of carbon 25 occurs in the liver. Since this hydroxylation is not tightly controlled, the circulating levels of 25-hydroxyvitamin D_3 are indicative of vitamin D status and are used clinically for this purpose. The kidney is the major site of the next activation step which is the hydroxylation of carbon 1. The 1-alpha hydroxylase is a mitochondrial P-450 mixed-function oxygenase that is tightly regulated by parathyroid hormone and the product, $1,25(OH)_2D_3$. Parathyroid hormone is the most potent upregulator of the kidney hydroxylase while product feedback inhibition downregulates the enzyme. Hypophosphatemia and excess estrogen are known to cause an increase in circulating

levels of $1,25(OH)_2D_3$. 1-alpha hydroxylase has been found in vitro in several cell lines that are activated by gamma interferon. Embryonic rat calvarial cells and human keratinocytes also have the active enzyme but the extrarenal production of $1,25(OH)_2D_3$ by normal individuals is not seen other than in the placenta and decidual cells during pregnancy. Other metabolites of vitamin D include $24,25(OH)_2D_3$ which is synthesized in the kidney. While the 24-hydroxylase is upregulated by $1,25(OH)_2D_3$, negative controls are not known. Whether the 24 metabolite has unique biologic activity or whether it represents a step in the elimination of D metabolites is not clear. The many other metabolites of vitamin D are thought to be transformation products associated with elimination since they involve side chain oxidations to more water soluble products many of which have been identified in the bile (for review see Reichel et al.1989).

Classical Action of Calcitriol

The classic vitamin D endocrine system is the regulator of calcium metabolism and its elaborate nature attests to the fact that calcium is required for so many important functions including nerve conductance, muscle contraction, second messenger functions, blood coagulation, and enzymatic activity to name a few. When the plasma calcium level drops to below 10 mg/dL, PTH is released from the parathyroid glands, which causes calcitriol to be synthesized in the

kidneys. Calcitriol independently causes increased intestinal absorption of calcium by upregulation of calbindin D or calcium binding protein. In concert with PTH, calcitriol mobilizes calcium from bone, causes increased renal retention of calcium and inhibits phosphorus retention. If dietary calcium is inadequate or if vitamin D levels are low, bone can become depleted of calcium, as the skeleton is the major reserve of calcium for vertebrates. So, while calcitriol does cause mobilization of calcium from bone, it also protects against skeletal mineral loss by ensuring optimal calcium absorption from the intestine and restricting calcium excretion when necessary. If calcium concentration rises above 10 mg/dL, calcitonin inhibits calcium mobilization from bone. The vitamin D deficiency disease, rickets, is caused by failure of the organic matrix of bone to mineralize due to low serum calcium and phosphorus. Administration of vitamin D will cause an increase in calcium absorption restoring the calcium-phosphorus ratio to a level that allows mineralization to occur.

Receptor and Genomic Effects

From the early work of William Bayliss and Earnest Starling in 1904 the following concepts about hormones emerged: hormones are molecules synthesized and secreted by specific glands directly into the bloodstream, then transported to the site of action where they specifically

alter the activities of responsive tissues (target organs) (Stryer 1988). The fact that calcitriol is actually a hormone is supported by the fact that the terminal step in its synthesis occurs by the action of kidney 1-α-hydroxylase and then calcitriol is transported to the target tissues (intestine, kidney, bone, etc.). It also has the basic steroid nucleus as do many hormones derived from cholesterol. Like other steroid hormones calcitriol diffuses through the cellular membrane then binds noncovalently to a specific cytosolic receptor which translocates into the nucleus (Reichel et al. 1989) (Finkelman and Butler 1985). There the hormone-receptor complex causes alteration of specific gene expression. Genes known to be regulated by calcitriol include calcitonin, fibronectin, Type 1 collagen, interleukin-2, prolactin, calbindin, interferon, and several bone matrix proteins to mention a few.

Calcitriol receptors have been found in almost all tissues studied. The receptors are acidic proteins of 50 to 60 kilodaltons that bind to calcitriol with high affinity. Other vitamin D metabolites have reduced biological activity, reflecting binding to the receptor with less affinity. The DNA binding domain of the calcitriol receptor displays a strong structural homology to the DNA binding region of the receptors of the other steroid hormones, suggesting that the calcitriol receptor is part of the

supergene family of steroid receptors (Pike 1985). While much of the work on the receptor has been performed with the avian receptor, it is known that mammalian receptors are similar in almost every respect to the avian proteins except for a slightly reduced size, 54kd as opposed to 60kd (Haussler 1986). Ligand binding to the steroid supergene family of receptors allows the receptors to bind to hormone responsive elements on DNA that are close to the target genes. In the case of retinoic acid, the receptor may already be bound to DNA but ligand binding is required for activation of the responsive element (Blomhoff et al.1990). Calcitriol has been shown to act through a cis acting element on DNA. Recently the calcitriol response elements in the promoters for the osteocalcin and osteopontin genes have been described (Demay et al.1989) (Noda 1989).

Effects on Non Mineralized Tissues

Since the receptors for calcitriol have been found in nearly all tissues, interest into other possible functions for this vitamin/hormone have emerged. Calcitriol halts proliferation and induces differentiation in hematopoietic cells and induces fusion of cells of the monocyte-macrophage line. Polyamines are intracellular mediators of calcitriol's induction of cell fusion, a process that also requires calcium. Calcitriol increases the number of osteoclast like multinucleated giant cells in vitro, suggesting that one of

its mechanistic functions in skeletal resorption is to cause osteoclast proliferation (Suda 1989).

Receptors for calcitriol have been located in cells of the immune system, including lymphocytes, thymocytes, blood monocytes and tissue macrophages. There is in vitro evidence that the hormone exerts a powerful influence on immune function. For example, calcitriol decreases the proliferative response of leukocytes to mitogens under specific conditions and causes activated peripheral blood mononucleosis cells (PBMCs) to produce less interleukin-2 (Manologas et al. 1989). Human and bovine PBMCs, T cells, T and B lymphocytes, and murine thymocytes display a calcitriol-induced inhibition of the proliferative response to a variety of activators and mitogens. Calcitriol also inhibits immunoglobulin production by B lymphocytes and activates macrophage cytotoxicity. Enhanced phagocytosis by human monocytes treated with calcitriol has been observed (Rigby 1988).

Clinical observations support the hypothesis that calcitriol may stimulate immune function. There is epidemiological data showing a positive association between Vitamin D status and incidence of tuberculosis. Furthermore, calcitriol has anti tuberculosis activity in vitro. In end-stage renal disease, where 1-alpha hydroxylase activity is low or absent, cellular immunity is

severely impaired. 1-alpha hydroxy D_3 given to renal patients restores immune responses of lymphocytes.

Calcitriol may regulate PTH gene expression, thus influencing PTH synthesis. Bovine parathyroid cells incubated with increasing doses of calcitriol have a dose-dependent decrease in pre-pro-PTH mRNA levels. This effect was noted first at 6 to 8 h but reached maximal levels at 24 to 48 h (Sherwood and Russel 1989). This group has also shown the same effect occurs in vivo.

Human and animal cancer cell lines also possess the specific receptor for calcitriol (Eisman et al.1989). Calcitriol seems to regulate cell replication in these cancer cell lines. Human breast cancer cells in vitro were stimulated by calcitriol at doses of 10^{-12} M to 10^{-10} M, yet showed decreased replication at doses above 10^{-9} M. Calcitriol at high doses increases survival time of mice injected with leukemic cells and slows the growth of human malignant melanoma and colon cancer cells. The effects of calcitriol on cancer cell replication are thought to occur via induced alterations in cell cycle kinetics and perhaps the down regulation of growth factor receptors. Accumulation of cells in the G2 and M phase of the cell cycle has been observed in the human breast cancer cell line,T 47D, suggesting an increase in cell cycle length. T 47D cells show a dose and time dependent reduction in EGF receptor levels in response to calcitriol (Eisman et

al.1989). Some cell lines respond to physiological levels of calcitriol by increasing fibronectin synthesis, which results in enhanced cell adhesion and a change in cell morphology. Transformed cells, generally having a rounded morphology, will flatten when induced to synthesize fibronectin, thus adopting a nontransformed phenotype (Franceschi et al.1987).

Lastly, calcitriol affects pituitary gland function by causing an influx of extracellular calcium via depolarization of voltage-operated calcium channels and activation of Na^{+}/Ca^{++} exchange mechanisms. As intracellular calcium increases, pituitary hormone release is affected. Thus, calcitriol modulates pituitary hormone release by regulating intracellular calcium concentration (Tornquist and Tashjian 1989).

Experimental Design

For all experiments, we have chosen to use the osteoblastlike, clonal osteosarcoma cell line, ROS 17/2.8. This subclone was derived from the parent cells, ROS 17/2, originally cloned from a transplantable rat osteosarcoma, R3559/52A (Majeska et al. 1980). Clonal cells typically display stable phenotypes over time in culture. For example, ROS 2/3 displayed stability for 2.5 yr after initial cloning and ROS 17/2 was stable 10 months after cloning. Because primary cells are not stable when cultured for any length of time, this property makes cloned cells

especially valuable for the study of metabolism in culture conditions.

Osteoblasts are responsible for synthesizing the mineralizable extracellular matrix of bone. Biochemical properties typical of osteoblasts include high alkaline phosphatase activity, synthesis of collagen Type I, ability to form bone in diffusion chambers in vivo, ability to form bonelike extracellular matrix in vitro, PTH responsive increase in adenylate cyclase, possession of calcitriol receptors and increased production of specific extracellular matrix proteins in response to calcitriol (Jilka and Cohn 1984). Primary osteoblastlike rat calvarial cells display these characteristics, also making them a good model for the study of bone, yet because they are not stable in culture for longer than two weeks, long-term experiments are difficult.

For cloned cells to be useful for metabolic experiments, they must display characteristics like those of the primary cell lines and of normal tissue. Like primary bone cells and osteoblastlike calvarial cells, ROS 17/2.8 cells are PTH responsive, synthesize Type I collagen and other bone matrix proteins, possess alkaline phosphatase activity and show calcitriol responsiveness (Gronowicz et al. 1986). These cells can produce mineralized tumors in host animals and respond to calcitriol in much the same manner as primary cultures of rat osteoblasts. Thus ROS

17/2.8 cells represent a good model for the osteoblast and will be the cells of choice for these studies.

Development Of Hypothesis

Based on preliminary results, I decided to investigate the effects of calcitriol on ROS 17/2.8 cell proteoglycans. My hypothesis is that calcitriol does, in fact regulate bone cell proteoglycan metabolism. This hypothesis is based on several observations by other investigators as well as our preliminary results. Calcitriol is known to regulate the synthesis of Type I collagen and several noncollagenous matrix proteins including osteopontin and osteocalcin. It also modulates the activity of bone alkaline phosphatase, enhances osteoclast formation and stimulates bone resorption. The mechanism of bone resorption is not known in detail, but it is postulated to involve bone extracellular matrix molecules. While roles for these molecules have not been assigned with certainty, it is thought that several factors, including calcitriol, may govern their metabolism and thereby regulate this process. The bone proteoglycans are major constituents of the extracellular matrix, yet little is known of the function or regulation of metabolism of these molecules. We have preliminary evidence that calcitriol may regulate the metabolism of bone cell PGs.

EXPERIMENTAL DESIGNS AND METHODS

Specific Aim 1

Determine that the sulfated macromolecules synthesized by ROS 17/2.8 cells are predominantly proteoglycans.

Rationale

The first specific aim of this work was to determine if the sulfated material produced by the rat osteosarcoma clonal cell line, ROS 17/2.8, was predominantly proteoglycan. While it is not likely that another macromolecules would incorporate [^{35}S]-sulfate to the degree that the proteoglycans do, we wanted experimental evidence that we were working with proteoglycans and not some other macromolecular species. The sulfated glycosaminoglycan (GAG) chains, chondroitin and dermatin sulfate, can be digested with the enzyme chondroitinase ABC which is specific for these substrates. Digestion yields the constituent disaccharide units which can be separated from macromolecules by size exclusion chromatography. [^{35}S]-labeled disaccharide units elute at the total volume (V_t) of the size exclusion column.

Methods

To accomplish this aim we cultured ROS 17/2.8 cells in 75 cm^2 flasks in Dulbecco's Modified Essential medium(DMEM) with 10% fetal bovine serum (FBS), 2% L-glutamine and 1%

antibiotic/antimycotic. At approximately 80% confluency, cell cultures were labeled with 50 μCi/mL [^{35}S]-sulfate for 24 h. Cells were cultured to confluency, then the media and cell layer were separated and extracted in 4 M guanidine hydrochloride (Gdn. HCl)/50 mM Tris-HCl (2-amino 2-[hydroxymethyl] -1,3-propanediol hydrochloride) at 4°C. The samples were concentrated to approximately 2 mL using Ambition Centriprep concentrators with molecular weight cutoff of 10,000 in a Sorvall benchtop centrifuge run at 3500 rpm and refrigerated to 10°C. When the desired sample volume was reached, the media and cell layer samples were separately chromatographed on 1.5 by 30 cm columns packed with BioRad P-30 size exclusion gel. Sample were loaded onto the columns and eluted with 0.2 M NH_4HCO_3. Twenty-five fractions were collected from each column run. Fifty microliters per fraction were removed and analyzed for [^{35}S]-sulfate by liquid scintillation counting using a Beckman LS 8000. This first chromatographic step separated unincorporated sulfate label from sulfated macromolecules which eluted at the void volume of the column. The fractions comprising the void volume were pooled and lyophilized.

Following lyophilization, samples were dissolved in 2 mL of 0.02 M Tris/Acetate/BSA (bovine serum albumin), pH 8.0 prior to digestion with 0.5 units of chondroitinase ABC, an enzyme that specifically cleaves the glycosaminoglycan chains of chondroitin and dermatan sulfate proteoglycans into constituent disaccharide units. Chondroitinase ABC

digestion was carried out for 5 h at 37°C. The reaction was terminated by the addition of 2 mLs of 8 M guanidine HCl.

The samples, now digested and in solution in 4 mLs of buffer, were loaded directly onto 1.5 cm X 30 cm columns of BioRad P-30 size exclusion gel for a second desalting run. Columns were eluted with 4M Gdn HCl/ 50 mM Tris, pH 7.4. Twenty-five fractions were collected and each was monitored for radioactivity by liquid scintillation counting as before.

Specific Aim 2

The second specific aim was to determine that the effect of calcitriol on ROS 17/2.8 proteoglycans (a reduction in [^{35}S]-sulfate incorporation) was dose-dependent and metabolite specific.

Rationale

Preliminary evidence from our laboratory indicated that calcitriol did cause a reduction in the amount of incorporated, or macromolecular, [^{35}S]-sulfate taken up by ROS 17/2.8 cells, thus subsequent experiments were aimed at further explaining the nature of this effect. Once we had shown that we were indeed working with a population of proteoglycans we needed to answer some basic questions concerning calcitriol's mode of action. If the decrease in [^{35}S]-sulfate incorporation is physiologically important then it should be caused by physiological doses of calcitriol.

Calcitriol, or the 1,25 hydroxy metabolite, is well known for numerous effects on bone tissue and other tissues as well. The other two metabolites, being similar in structure to calcitriol, may bind to the calcitriol receptor with more or less affinity and may actually cause a measurable response. The 24,25 metabolite has been found concentrated in fetal bone and injured, healing bone leading to the speculation that it may be necessary for normal bone formation. With this in mind we decided to test 24,25 $(OH)_2$ D3 and 25 (OH) D_3 for metabolic activity in the ROS 17/2.8 cells. We accomplished the objectives set forth here in two separate experiments.

Methods

We chose five different concentrations of calcitriol based on reported physiological ranges plus or minus two orders of magnitude. Human blood concentrations of 1, 25 dihydroxycholecalciferol are in the range of 10^{-10} M, so the concentrations used for the dose response experiments were 10^{-8} M to 10^{-12} M. We decided that the initial dose-response experiments needed to include a variable time factor in them so cells were treated with calcitriol for 6, 12, 18, 24, and 48 h. In later experiments we deleted the 6 h time point and added a 72 h time point. These experiments included four replicates of each dose at each time point, thus allowing statistical analysis of the data.

ROS 17/2.8 cells were maintained at 37°C in a humidified atmosphere of 5% CO2. Cells were routinely cultured in DMEM

supplemented with 10% FBS, 2% L-glutamine, and 1% antibiotic/antimycotic mixture. Media were changed three times weekly, subculturing performed as necessary and monitoring for mycoplasma contamination was done once monthly. For all dose-response experiments, ROS 17/2.8 cells were seeded at a density of 10,000/cm^2 into 24-well plastic culture dishes. Two milliliters of medium were added and cells were grown to approximately 80% confluence. The media was then changed to HAM'S F-12 containing 100 μg/mL of bovine serum albumin (BSA), 2% L-glutamine and 1% antibiotic/ antimycotic plus 100 μCi/mL of [^{35}S]-sulfate and the various doses of calcitriol. Each well received 0.5 mL of this media and cells were allowed to grow for predetermined lengths of time. Experiments were terminated by removing the media and combining it with 1/2 mL of 8M Gdn HCl. To the cell layers was added 1/2 mL of 4M Gdn HCl/0.5% Triton X-100 plus a cocktail of protease inhibitors (5 mg/L pepstatin, 10mM PMSF, 1 mg/L Trypsin inhibitor, 5 mM levamisole, 5 mM benzamidine). Both fractions were extracted overnight at 4°C and stored frozen at 0°C.

The amount of [^{35}S]-sulfate incorporated into macromolecules was determined by chromatographing 500 μL of each sample on 10 mL BioGel P-30 size exclusion columns. Twelve one milliliter fractions per sample were collected and analyzed for radioactivity by liquid scintillation counting. The macromolecular portion of each sample eluted at the void volume of the 10 mL columns. The four replicates

of each sample were averaged, and the multiple means were analyzed for differences by Analysis of Variance and the Student-Newman-Keuls test for multiple means comparison.

The second part of this specific aim, to determine if the response to calcitriol was metabolite-specific, was achieved in another experiment using a similar experimental design. Three metabolites, 1,25 dihydroxycholecalciferol, 24,25- dihydroxycholecalciferol and 25-hydroxy-cholecalciferol, were tested. Cells were cultured as previously described. Based on data obtained in the dose-response experiments, we decided to expose cells to the various metabolites for 48 hours at doses of 10^{-8} M and 10^{-10} M. Four replicates per concentration of each metabolite were obtained. Fifty microcuries per milliliter of [^{35}S]-sulfate were used for metabolic labeling during the final 6 h of the experiment. Harvest procedures, quantitation of incorporated radioactivity and statistical analysis were done as previously described.

Specific Aim 3

Specific aim three involved determining whether the reduction in sulfate incorporation was due to a decrease in cell proliferation or a decrease in total protein synthesis.

Rationale

The effect we have observed could be due to a generalized toxic response to the hormone so we decided to monitor cell proliferation and total protein synthesis as measures of cell viability. In addition, there was the

possibility that calcitriol down-regulated protein synthesis in general thus its action would not be specific to the proteoglycans. Concurrent cell counts allowed us to evaluate how the different doses, metabolites and treatment times affected cell proliferation. Quantitation of macromolecular [^{3}H]- leucine incorporation was used to evaluate total protein synthesis.

Methods

Concurrent with both of the above experiments we evaluated the effects of the various doses and metabolites on cell proliferation. Thus, duplicate plates were treated as already described. No metabolic label was applied to these plates. Instead they were processed for cell counting at the end of each treatment time. Cell counting procedures consisted of trypsinization to release cells from plates, then counting a small volume of the cell suspension in isotonic diluent in a Coulter counter. These data were analyzed as previously described. In addition, ongoing visual inspection by phase contrast microscopy of cell morphology gave additional information on whether treatments were affecting cells adversely.

To assess total protein synthesis we designed a dose-response experiment using five concentrations of calcitriol applied for five different lengths of time as described in the previous section. Calcitriol at 10^{-8} M to 10^{-12} M was applied for 12, 18, 24, 48, and 72 h. Concurrent with

calcitriol treatment 10μCi/mL of [^{3}H]-leucine was added to allow all synthesized proteins to be labeled.

As in prior dose-response experiments, we cultured the ROS 17/2.8 cells to 80% confluency in DMEM with 10% FBS, 2% L-glutamine and 1% antibiotic/antimycotic added. Medium was changed to HAMS F-12 with 100 μg/ml BSA, 2% L-glutamine, 1% antibiotic/antimycotic plus the hormone and [^{3}H]-leucine. At the end of the treatment period, media and cell layer were combined and extracted in 4M Gdn HCl/50mM Tris, pH 7.2, plus protease inhibitors. Samples were then frozen at -70°C until further processing.

For analysis of macromolecular [^{3}H]-leucine, 500 uL of each sample were chromatographed on a 10 mL column of Bio-gel P-30 eluted with 4 M Gdn HCl/50mM Tris, pH 7.2. After sample application to the column, twelve 1 mL fractions were collected and analyzed for [^{3}H]-leucine by liquid scintillation counting. Radioactivity eluting at the void volume of these columns represented the total macromolecular counts and thus indicate the amount of protein synthesized. The multiple means were analyzed statistically by Analysis of Variance.

Specific Aim 4

The forth specific aim was to determine if the action of calcitriol was specific to one population of proteoglycan or if it affected all proteoglycans synthesized by this cell line.

Rationale

The ROS 17/2.8 cell line synthesizes more than one subpopulation of proteoglycan. Our data from previous experiments do not allow us to evaluate if calcitriol differentially regulates these molecules. To determine whether the decrease in sulfate incorporation could be occurring in one or more proteoglycan populations synthesized by ROS 17/2.8 cells, we designed an experiment to ascertain if the effect of calcitriol was specific to certain proteoglycan subpopulations or if it was generalized.

Methods

ROS 17/2.8 cells were cultured to 80% confluency in DMEM containing 10% FBS, 2% L-glutamine and 1% antibiotics. Media were changed to HAMS F-12 with 100 ug/mL BSA, 2% L-glutamine and 1% antibiotic. Cells were then labeled with 50 μCi/mL [^{35}S]-sulfate and half the cultures were treated with 10^{-8} M calcitriol. After 24 h the media and cell layer were separated and extracted in 4 M Gdn HCl/50mM Tris at 4°C overnight. Samples were concentrated (Ambition Centriprep) and desalted on Biogel P-30 gel equilibrated and run in 50% formamide, 0.1 M KCl, 0.04 M KH_2PO_4, pH 6.0. An aliquot of each sample containing 4000 CPM was analyzed by SDS-PAGE (sodium dodecyl sulfate- polyacrylamide gel electrophoresis) using a Laemmli system with a 3% stacking gel and a 5 to 15% polyacrylamide gradient resolving gel. Gels were processed for fluorography using 22% PPO (2,5-diphenyloxazole) in DMSO

(dimethyl sulfoxide). Since we corrected for the difference in absolute quantity of radioactivity by loading equal amounts per lane, a difference between the control sample and the calcitriol-treated sample would be evident only if there were a difference in the distribution of proteoglycan species within each sample. Fluorograms were further analyzed by densitometry using a Hoeffer GS 300 densitometer.

Specific Aim 5

We wanted to determine if the reduction in sulfate incorporation arose from a decreased ability of the calcitriol treated cells to synthesize glycosaminoglycans (GAGS).

Rationale

One of the possible explanations for the observed decrease in sulfate incorporation caused by calcitriol is that calcitriol causes an overall decrease in the capacity of ROS 17/2.8 cells to synthesize glycosaminoglycan (GAG) chains. This would indicate that one or more of the enzymes involved in the synthesis of GAG chains was regulated by calcitriol. By using an exogenous acceptor for the synthesis of GAG chains, we designed an experiment to examine this possible mechanism of calcitriol's action. P-Nitrophenyl ß-D-xylopyranoside is a substrate for galactosyltransferase I, the enzyme that catalyzes the transfer of galactose to the xylose residue attached to the proteoglycan core protein. P-Nitrophenyl ß-D-xyloside with an attached galactose residue

can function as a substrate for the subsequent enzymes in GAG synthesis. Thus a GAG chain is synthesized on a molecule of P-nitrophenyl ß-D-xyloside instead of on a protein backbone. GAG synthesis is limited by the availability of xylosylated protein cores in the normal situation. Since the xylosylated organic molecule can be easily added in excess to culture media one can test the maximum capacity of a population of cells to produce GAGs.

Methods

For this experiment, ROS 17/2.8 cells were cultured to 80% confluency in two 24-well plastic culture dishes in DMEM with 10% FBS, 2% L-glutamine and 1% antibiotics. In one plate the medium was changed to HAMS F-12 containing 100 ug/mL BSA, 2% L-glutamine, and 1% antibiotic plus 10^{-9} M calcitriol for the remaining 48 h of the experiment. The control plate underwent a media change to HAMS F-12 containing the above additives minus the calcitriol. After 46 h, fifty microcuries per milliliter of [^{35}S]-sulfate were added to all wells and half of the wells in each plate received xyloside at 1.0 mM 0.1 mM or 0.001 mM (4 wells/dose). As in previous experiments we set up duplicate plates to monitor the effects of calcitriol plus the xlyopyranoside on cellular proliferation. The experiment was terminated at 48 h.

The media and cell layer were not separated at harvest, instead 0.5 mL of 8 M Gdn HCl was added to well and the macromolecules extracted overnight at 4°C. Incorporated

radioactivity quantified by size exclusion chromatography on a 10 mL column of Biogel P-30 eluted with 4 M Gdn HCl/50 mM Tris, 0.5% Triton X-100, pH 7.2. The data were analyzed by Analysis of Variance. The plates set up for counting cells were also harvested at 48 hours and the cells released by trypsinization and counted in a Coulter counter.

Specific Aim 6

The sixth specific aim was to determine whether the reduction in sulfate incorporation was due to an increased rate of degradation of newly synthesized proteoglycans.

Rationale

Another possible explanation for the reduction in incorporated [^{35}S]-sulfate into ROS 17/2.8 proteoglycans is that degradation of proteoglycans occurs at a faster rate in calcitriol-treated cells. Factors governing the activities of the many enzymes responsible for proteoglycan catabolism are not known to any great extent. If proteoglycans were degraded at a faster rate in calcitriol treated cultures, an apparent reduction in incorporated sulfate would result because of fewer intact proteoglycans.

The overall approach to this experiment was to add partially purified [^{35}S]-sulfate labeled proteoglycans to ROS 17/2.8 cell cultures and monitor the extent to which the labeled proteoglycans were degraded under varying conditions of time and calcitriol treatment.

Methods

The partially purified, [^{35}S]-sulfate labeled proteoglycans were prepared by labeling several 150 cm^2 culture flasks of ROS 17/2.8 cells with [^{35}S]-sulfate, removing and concentrating the media, then passing the media over a BioGel P-30 column equilibrated in phosphate buffered saline (PBS) pH 7.2. The macromolecular fraction eluting at the void volume was collected, concentrated, filter sterilized and a known quantity of radioactivity diluted in 2x DMEM prior to being added to cell cultures.

ROS 17/ 2.8 cells were seeded into 4-well plastic culture dishes at 10,000 cells per cm^2 in DMEM supplemented with 10% FBS, 2% L-glutamine, 1% antibiotics. Cells were grown to 80% confluency then medium was changed to HAMS F-12 with 100 ug/mL BSA, 2% L-glutamine, and 1% antibiotics. Half of the cells were treated with 10^{-8} M calcitriol and all of the wells received the exogenous, labeled proteoglycans (70,000 CPM per well). At 2 h, 6 h, 12 h, 24 h, and 48 h after addition of exogenous proteoglycans the plates were processed. Autodegradation was accounted for by including a culture plate, terminated at 48 h, with no cells but which contained media and exogenous, labeled proteoglycans. In each 4-well plate there were two wells treated with calcitriol and two control wells. These were separately harvested by removing the 1/2 mL of media, washing the wells twice with 250 uL of sterile PBS and adding this to the medium giving a 1 mL sample. The cell layer was extracted

with 0.5 mL of 4 M Gdn HCl/50 mM Tris, 0.5% Triton X-100. Duplicate samples were combined in order to have enough radioactivity for the subsequent chromatography.

Calcitriol-treated and control samples of media from each time point were separately chromatographed on a 1 cm X 120 cm column packed with Sepharose CL6B and eluted with 4 M Gdn HCl, 0.05 M Tris, 1% Triton X-100, pH 7.4 at a flow rate of 20 mL per h. Equal sample volumes were loaded onto the column, and the precise amount of radioactivity loaded was quantitated. Prior to the samples being run on this column, it was calibrated with Blue Dextran and [^{35}S]-sulfate. The V_t was 69 mL and the V_o was 26 mL. Seventy-five 1 mL fractions were collected and radioactivity per fraction was monitored by liquid scintillation counting. The intact proteoglycans could thus be separated from the proteoglycan degradation products by molecular sieving. There was minimal radioactivity in cell layer samples.

Specific Aim 7

Specific aim seven involved determining if the reduction in sulfate incorporation was related to a decrease in proteoglycan core protein synthesis.

Rationale

A reduction in sulfate incorporation may result from a decreased availability of the core proteins upon which to construct the sulfated GAG chains. We will attempt to quantitate the proteoglycan core proteins using two techniques, immunoprecipitation and HPLC.

Methods

For immunoprecipitation a protocol developed in our laboratory was followed. We received four polyclonal antibodies from Larry W. Fisher's lab, one against a mixture of rat bone proteoglycans, one against human bone PG I and PG II, one against a synthetic peptide of human biglycan and one against a synthetic peptide of human decorin. Joachim Sasse's lab supplied us with three polyclonal antibodies, one against bovine PG I, one against bovine PG II, and one against the bovine aggregating cartilage PG. To determine if any of the antibodies cross-react with PGs from the ROS 17/2.8 cells, we first obtained [^{35}S]-sulfate labeled PGs from the media of untreated cells. The lyophilized PG sample was dissolved in 50mM Tris HCl, pH 8.0, and diluted to yield approximately 50,000 CPM/20uL. Aliquots of sample were incubated for 2 h at 4°C with 50 uL of 10% Pansorbin to which nonimmune serum was adsorbed. After centrifugation, 20 uL of antibody solution was added to the supernatant and incubated for 4 h followed by an overnight incubation after addition of 100 uL of 10% Pansorbin. The samples were centrifuged and the pellets washed three times with washing buffer (0.25% deoxycholate, 0.1% Nonidet P-40, 0.51M NaCl, 0.1M Tris HCl, pH 7.4), twice with PBS and once with glass distilled water. Reducing electrophoresis sample buffer was added and the pellets were heated at 90°C for 5 minutes then centrifuged prior to electrophoresis. Pre-immune sera and Pansorbin alone served as negative controls.

For HPLC separation of ROS 17/2.8 proteoglycans we planned to use a modification of a method by Fedarko et al. (1990) for separating human bone cell proteoglycans. Proteoglycans synthesized by control and calcitriol-treated cells were labeled and extracted in 4 M Gdn HCl/50mM Tris. Samples were concentrated then chromatographed on a Bio-Gel P-30 column equilibrated in 50% deionized formamide, 0.1M KCl, pH 6.0. The multistep HPLC approach first involved ion exchange on a Nucleogen-4000-10 DEAE column followed by size exclusion on a 1 X 50 cm Omnifit glass column packed with TSK-GEL ToyoPearl HW 75(F). [^{35}S]-Sulfate labeled peaks were identified by scintillation counting and SDS-PAGE fluorography.

Specific Aim 8

The eighth specific aim was to determine if glycosaminoglycan chain size or degree of sulfation was affected by calcitriol.

Rationale

Since calcitriol could cause alterations in the size of the GAG chains or the degree to which they are sulfated we designed experiments to test these possibilities. Size of GAG chain is of functional significance and may be a site of hormonal control. Such control could be exerted by regulation of one or more of the enzymes responsible for chain elongation. Regulation of the degree of sulfation can be achieved by controlling the intracellular concentration of cations, alteration of one or more of the sulfotrans-

ferases, the rate of sulfate transport or formation of PAPS, the active sulfate donor.

Methods

To test the hypothesis that calcitriol may be causing reduced size of GAG chains we first prepared [^{3}H]-glucosamine-labeled, partially purified proteoglycans. One hundred fifty cm^2 flasks of ROS 17/2.8 cells were cultured in DMEM with 10% FBS, 2% L-glutamine and 1% antibiotics. At about 80% confluency, the medium was changed to HAMS F-12 with 100 ug/mL BSA, 2% L-glutamine, and 1% antibiotics. Half of the flasks were treated with 10^{-8} M calcitriol and cells in all the flasks were labeled with 20 μCi/mL [^{3}H]-glucosamine. Labeling and calcitriol treatment continued for 48 h. The medium and cell layer were separately harvested, then extracted overnight at 4°C in 4 M Gdn HCl/50 mM Tris, including protease inhibitors, pH 7.2. Samples were concentrated to approximately 5 mLs in Ambition Centriprep concentrators and then desalted on 1.5 cm X 30 cm columns of BioGel P-30 eluted with 0.2 M NH_4HCO_3. Fractions were collected, monitored for radioactivity by scintillation counting, and the macromolecular fraction, eluting at the void volume, was collected, pooled and lyophilized.

Lyophilized samples were dissolved in 4 mLs of 6 M Urea/50 mM Tris, pH 7.51. 3.5 milliliters were loaded onto a column of DEAE (diethylaminoethyl) Sepharose equilibrated in the same buffer. The sample was washed onto the column with 25 mL of buffer. Then a 200 mL gradient of 0 to 1 M NaCl in

the same buffer was used to elute the column. One hundred two milliliter fractions were collected and monitored for [^{3}H]- glucosamine by scintillation counting. Generation of the salt gradient was monitored by conductivity measurements. The proteoglycan peak was identified by its characteristic elution at approximately 0.6 M NaCl. This was the last of three peaks which eluted from the column.

The proteoglycan peaks were pooled, concentrated, desalted then lyophilized. Lyophilized material was dissolved in 1 mL of 0.05 M acetate buffer, pH 5.6, containing 0.2 M EDTA (ethylenediaminetetraacetic acid) and 5 mM cysteine hydrochloride. Proteoglycan core proteins were digested by adding 1 to 3 mg of papain per mL of sample and letting the reaction proceed for 24 h at 56°C. The reaction was terminated by freezing.

Papain-digested proteoglycans (900 uL/sample) were chromatographed on a 1 cm X 120 cm column of Sepharose CL6B size exclusion gel equilibrated in 4 M Gdn HCl/50mM Tris, pH 7.06. Fractions were collected and analyzed for [^{3}H]-glucosamine by scintillation counting. K_{av} for media and cell layer control and calcitriol- treated samples were calculated.

Partially purified, [^{3}H]-glucosamine labeled medium proteoglycans, obtained essentially as described above except they were labeled for only 24 h, were also used to examine the degree of sulfation of control and calcitriol

treated samples. The media and cell layer were separately harvested in 4 M Gdn HCl/50 mM Tris.

Lyophilized samples were dissolved in 500 uL of Tris HCl, pH 8.0 , and 0.05 to 0.10 units of chondroitinase ABC were added and the samples digested at 37°C for 12 h when reaction was terminated by freezing. The [^{3}H]-glucosamine labeled disaccharides liberated by chondroitinase ABC digestion were analyzed for degree of sulfation by chromatography on an Alltech Licrosorb amino column eluted with a 20 minute gradient of 20% to 80% 0.8 M phosphate buffer. Nonsulfated, four-sulfated, and six-sulfated disaccharide standards were well separated under these chromatographic conditions. Elution of disaccharides generated from ROS 17/2.8 cell proteoglycans was monitored by collecting 65 fractions per run and then analyzing for radioactivity by scintillation counting. Sample peaks were identified by comparison to standards.

Specific Aim 9

Specific aim nine involved determining whether calcitriol decreases GAG synthesis and whether cell density effects the incorporation of [^{3}H]-glucosamine into macromolecules synthesized by calcitriol-treated or control cells.

Rationale

Calcitriol could be causing fewer GAGs to be synthesized, resulting in less [^{35}S]-sulfate incorporation. In order to differentiate between effects on sulfation alone versus effects on GAG synthesis in general we designed an

experiment to test the effect of calcitriol on [^{3}H]-glucosamine incorporation into ROS 17/2.8 cell macromolecules.

Because of earlier observations that calcitriol's action may be cell density dependent we designed this experiment to also test the hypothesis that the response to calcitriol varies with cell density at the time of treatment.

Methods

Twelve-well plates were seeded at 10,000 cells per cm^2 in 2 mL of DMEM with 10% FBS, 2% L-glutamine, 1% antibiotics. The following day one-third of the wells in the treatment group received 10^{-8} M calcitriol in HAMS F-12 media with 100 μg/mL BSA, 2% L-glutamine, and 1% antibiotics. Two days postseeding another third of the wells received this treatment and on day 3 the last third of the cells received this treatment. All calcitriol treatment lasted 48 h. Twenty-four hours after receiving calcitriol, cells were labeled with 10 μCi/mL [^{3}H]-glucosamine for the final 24 h of the experiment. Controls underwent identical procedures except that they were not treated with calcitriol.

Media were harvested at the end of the 48 h treatment time by removing it to separate vials and extracting overnight in 4 M Gdn HCl/50 mM Tris at 4°C. Each medium sample was separately chromatographed on a column of BioGel P-30 to remove unincorporated radioactivity. The void volume was collected, concentrated, and chromatographed on a column

of DEAE Sepharose equilibrated in 6 M urea/50 mM Tris, pH 7.5 eluted with a step-wise gradient of NaCl. A 4 mL wash at 0.15 M NaCl was followed by 10 mL of 0.37 M NaCl and another 10 mL of 0.68 M NaCl. One milliliter fractions were collected and monitored for tritium by scintillation counting.

RESULTS

The first specific aim of this work was to demonstrate that the sulfated macromolecules synthesized by ROS 17/2.8 cells were predominantly proteoglycan. Sulfated macromolecules in the media and cell layer fractions were desalted, digested with chondroitinase ABC and subjected to a second desalting run on the same columns. We found consistently good recovery in the first chromatographic step with the media fraction and were able to enzymatically digest this fraction with little difficulty. The second chromatographic step with the digested media samples produced a V_o peak and a V_t peak containing 43% and 57% of the recovered radioactivity, respectively. We re-digested and redesalted the V_o peak from this run recovered 19% and 81% of the [^{35}S]-sulfate at the V_o and the V_t, respectively. These results show that the majority of the [^{35}S]-sulfate labeled macromolecules in the media are susceptible to chondroitinase ABC digestion and are, therefore, proteoglycans.

The cell layer fraction proved to be troublesome to work with. The desalted cell layer sample either did not digest well or gave a very poor recovery. Following digestion with chondroitinase ABC, only 25% of the radioactivity recovered after the first desalting step remained. We did not find an explanation for the loss

of radioactivity during digestion. When the digested cell layer sample was desalted only 66% of total radioactivity loaded onto the column was recovered with 9% eluting at V_t and 91% eluting at the V_o. We repooled the Vo fraction from this run and subjected it to chon- chondroitinase ABC for a longer period of time. After this second digestion there was little radioactivity remaining in the sample. After attempting to work with the cell layer fraction twice and not achieving good results, we tried simply visualizing the proteoglycans on a fluorogram. This approach would at least allow us to observe the labeled cell layer molecules and speculate whether or not they were proteoglycans. The fluorograms we prepared did show labeled cell layer macromolecules that were typical of proteoglycans in appearance, and had apparent molecular weights in the range of those previously observed for ROS 17/2.8 cell layer proteoglycans. No other sulfated molecules appeared in the cell layer fluorograms which was what was expected.

The next several experiments dealt with the questions of dose responsiveness, length of treatment, and the effect of calcitriol on cell proliferation.

In the first dose-response experiment, ROS 17/2.8 cells were treated with calcitriol, from 10^{-8} M to 10^{-12} M, for 2 to 48 h. After 48 h of exposure to calcitriol, both the media and cell layer had a dose-dependent reduction in the amount of [^{35}S]-sulfate incorporated into V_o macromolecules as compared to controls. In the medium fraction the

reduction was seen at all doses at 48 hours but was significant at 10^{-8} M ($p=.009$). At the other lengths of treatment we did not observe a drop in incorporated sulfate in the medium samples. To correct for cell number, we calculated CPM per cell and found the trend toward reduced [^{35}S]-sulfate to be consistent. In the cell layer samples all doses produced a downward shift in [^{35}S]-sulfate at 48 h with 10^{-8} M giving a significant response. At 18 and 12 h but not at 24 h the cell layer samples were also reduced with respect to [^{35}S]-sulfate, however these reductions were not significant. We do not have an explanation for the result at 24 h.

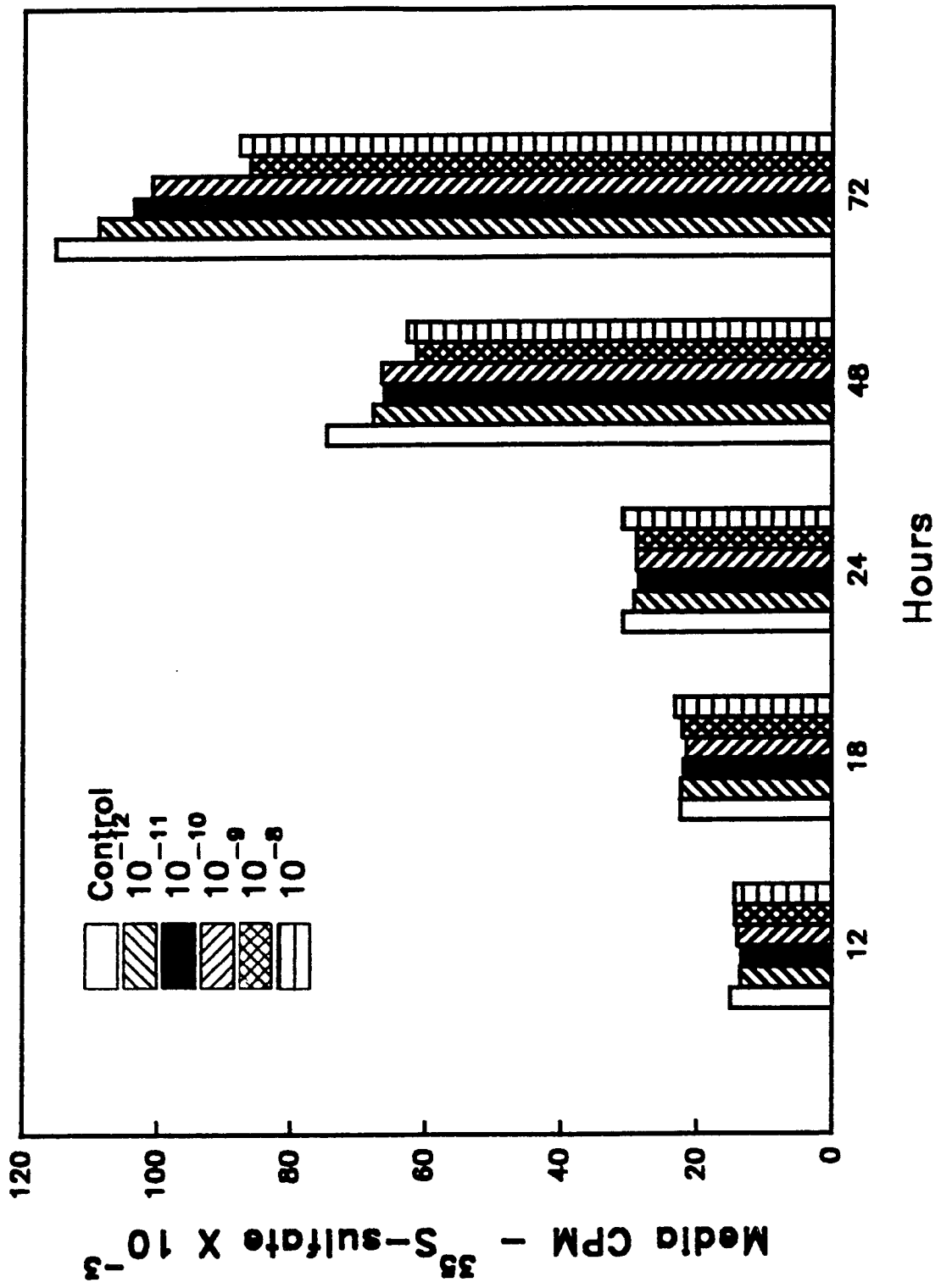
At every dose, for each time point, we counted the number of cells in four wells in order to monitor the effect of calcitriol on cellular proliferation. After 48 h of exposure to calcitriol we found no difference in the number of cells at any dose compared to the controls. At the 24 h exposure time we did observe some differences in cell number but they were not systematic.

The results from this first experiment were revealing in a number of ways. The experimental design and methods worked well and could be used for many of the subsequent studies. We identified a range of effective doses of calcitriol and we knew how long to expose cells to the hormone in order to see a reduction in [^{35}S]-sulfate incorporation.

Based on the results of the first experiment, we decided to change the lengths of treatment in future experiments. Because we did not observe significant decreases in sulfate at 6 and 12 h we decided to delete the 6 h exposure time and add a 72 h exposure time in the follow-up experiment. This second dose-response experiment utilized the same design as the first dose-response experiment in that five concentrations of calcitriol were used for five varying amounts of time. Lengths of treatment were 12, 18, 24, 48, and 72 h. Hormone treatment, labeling, and harvesting of media and cell layer were carried out as described in the preceding section. The primary objective of this experiment was to see if the dose-dependent reduction in [^{35}S]-sulfate was reproducible and to test the effect of calcitriol on cell proliferation.

In the media samples, while no significant changes were noted at 12, 18, or 24 h, the incorporated sulfate was significantly reduced after 48 h of exposure to all doses of calcitriol (see figure 1). After 72 h of exposure to calcitriol, media samples showed significant reductions in [^{35}S]-sulfate incorporation at all doses except 10^{-12} M. The macromolecular radioactivity in the media fraction at 48 hours were 83%, 82%, 89%, 89% and 91% of controls for 10^{-8} M, 10^{-9} M, 10^{-10} M, 10^{-11} M and 10^{-12} M, respectively. Macromolecular radioactivity in the media fraction after a 72 h treatment time with calcitriol were 76%, 74%, 87%, 89%, and 94% at 10^{-8} M , 10^{-9} M, 10^{-10} M, 10^{-11} M and 10^{-12} M,

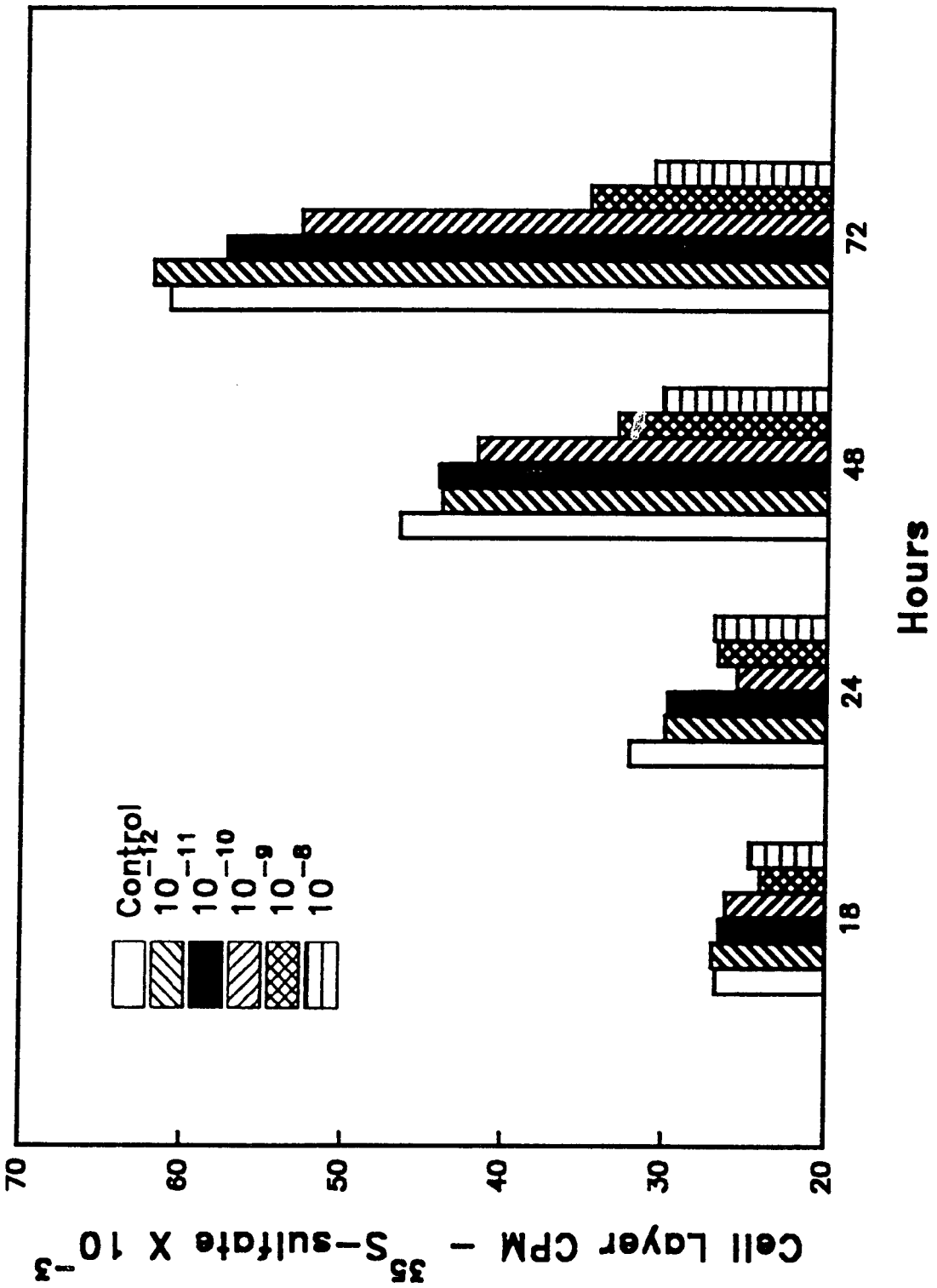
Figure 1. Effect of calcitriol on [^{35}S]-sulfate incorporation into ROS 17/2.8 cell proteoglycans extracted from the culture media. In this experiment [^{35}S]-sulfate and calcitriol were applied for the variable lengths of time shown on the X axis. Y axis - [^{35}S]-sulfate CPM X 10^{-3}. Various doses of calcitriol are shown in the insert.


Media CPM - ^{35}S-sulfate X 10^{-3}
120
100
80
60
40
20
0
12
18
24
48
72
Hours
Control
10^{-12}
10^{-11}
10^{-10}
10^{-9}
10^{-8}

respectively. Macromolecules harvested from the cell layer showed similar reductions in the amount of [^{35}S]-sulfate incorporated (see figure 2). Dose-dependent reductions in sulfate were seen at all concentrations as early as 18 h after exposure to calcitriol, and significant reductions in the amount of incorporated sulfate were seen after 48 h at 10^{-8} M , 10^{-9} M and 10^{-10} M. After 72 h of exposure to calcitriol the cell layer samples were significantly reduced with respect to sulfate at 10^{-8} M , 10^{-9} M and 10^{-10} M. All statistical analysis were by One-Way Analysis of Variance ($p=0.05$ or less).

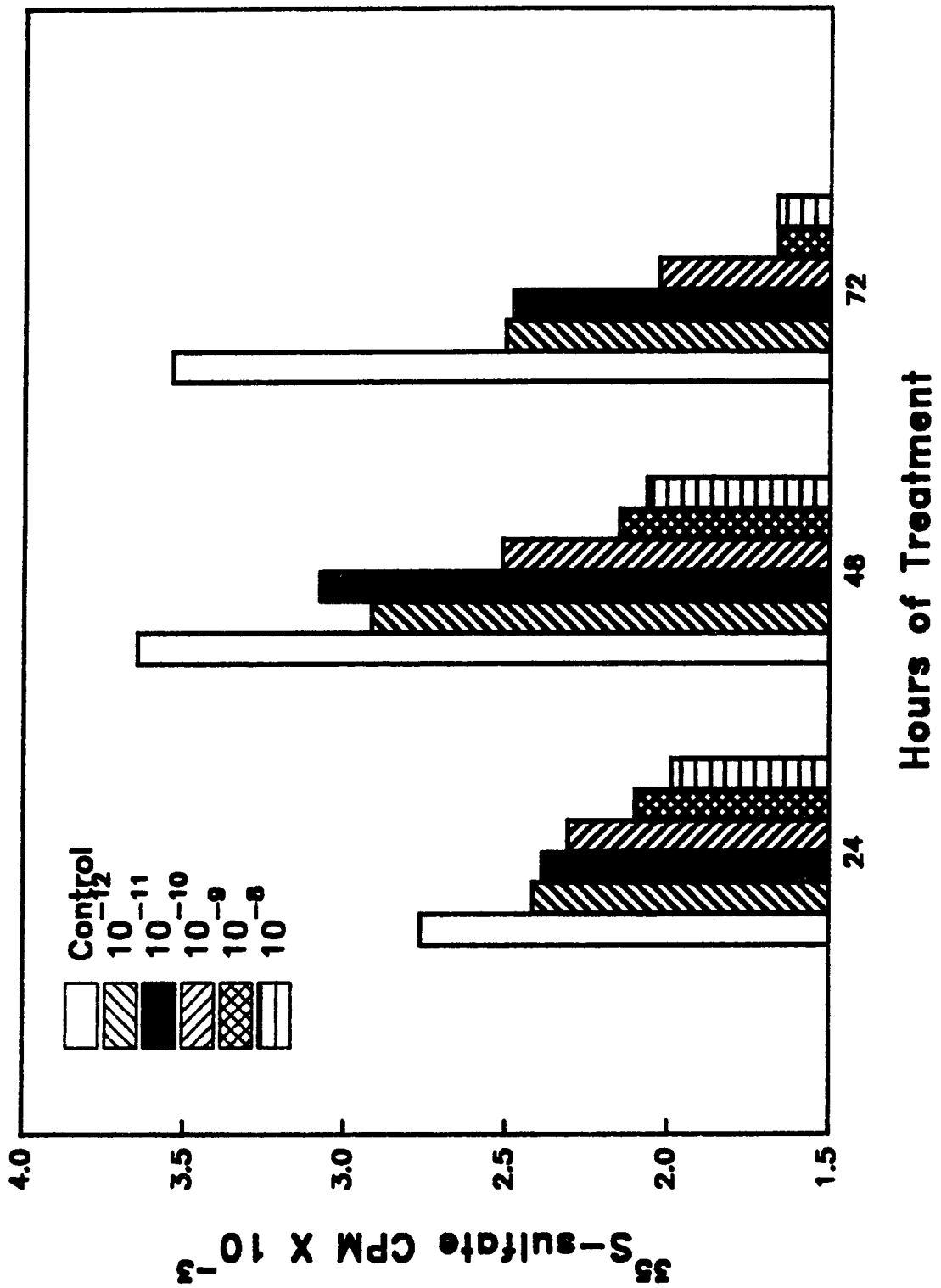
Numbers of cells were counted at every time point except 72 h for each dose and the results analyzed by One-Way Analysis of Variance. Some significant differences appeared at 12 and 18 h but not at 24 h or 48 h. In analyzing the multiple means however it was apparent that the differences occurred in a random rather than a systematic fashion indicating possible random error or imprecision in cell counting. For example, after 12 h of treatment with calcitriol, there was a significant difference between means yet the highest dose of the hormone was associated with the highest number of cells, the lowest dose of calcitriol was associated with the lowest number of cells and the controls were associated with an intermediate number of cells as were the remaining concentrations of calcitriol. Another random pattern was seen after 18 h of treatment with calcitriol. The highest dose of hormone, 10^{-8}

Figure 2. Effect of calcitriol on [^{35}S]-sulfate incorporation into ROS 17/2.8 cell proteoglycans extracted from the cell layer. [^{35}S]-sulfate and calcitriol were applied for the various lengths of time shown on the X axis. Y axis - [^{35}S]-sulfate CPM X 10^{-3}. The doses of calcitriol are depicted by the bars and shown in the insert.


Cell Layer CPM - ^{35}S-sulfate X 10^{-3}
70
60
50
40
30
20
18
24
48
72
Hours
Control
10^{-12}
10^{-11}
10^{-10}
10^{-9}
10^{-8}

M, and the control group were associated with the lowest number of cells , the greatest number of cells was seen at 10^{-9} M while intermediate numbers of cells were noted at the other doses. Despite the fact that differences between some of these means were significant, the actual numerical values were not greatly different, and the means varied in a random rather than a dose-dependent fashion indicating again that the differences in these numbers probably arose because of random error (see figure 4). If the number of cells had increased in a dose-responsive manner we would have speculated that calcitriol did have a proliferative effect on the ROS 17/2.8 cells. On the other hand,if we noted a dose-dependent decrease in cell number it would be an indication that calcitriol was inhibiting cell growth and this would provide a possible explanation for the reduction in [^{35}S]-sulfate incorporation. It was interesting that at the longer exposure times of 24 and 48 h there were no differences between cell number means. Based on these results we decided to continue monitoring cell number in future dose-response experiments. Generating more data with more replicates would reveal whether or not random error was involved and would also improve the odds of overcoming the imprecision inherent in cell counting. Some amount of imprecision can be introduced while seeding cells, during harvest of cells and a small degree of systematic error is expected in the use of a coulter counter to count cells. We found that increasing replication at every step during the

Figure 3. The effect of calcitriol on [^{35}S]-sulfate incorporation into ROS 17/2.8 cell proteoglycans extracted from the culture media. Cells were treated with the various doses of calcitriol shown in the insert for 24, 48 and 72 h. [^{35}S]-sulfate was added to the culture media for the final 6 h of treatment time. Bars represent the means of four replicates at each dose per treatment time.


Control
10⁻¹¹
10⁻¹⁰
10⁻⁹
10⁻⁸
4.0
3.5
3.0
2.5
2.0
1.5
24
48
72
Hours of Treatment
³⁵S-sulfate CPM X 10⁻³

cell counting process increased the likelihood of obtaining accurate numbers.

The last two experiments we conducted to observe the effect of dose on the synthesis of proteoglycans by ROS 17/ 2.8 cells involved some minor changes in the experimental design. For one experiment we decided to label with [^{35}S]-sulfate for the final 6 h of the experiment rather than for 24 h. Cells would be treated with calcitriol for 24, 48 or 72 h and then would receive the metabolic label for the shorter period of time in order to eliminate the large number of [^{35}S]-sulfate labeled macromolecules that build up during a long labeling time. Some of these molecules are undoubtedly synthesized before calcitriol may take effect yet are in the pool of labeled proteoglycans that are harvested possibly obscuring the number of proteoglycans actually altered by the hormone. In labeling for a short amount of time after calcitriol had taken effect, we hoped to enhance the observed effect of calcitriol. The results of this experiment are shown in figure 3 and table 1. Again, for each exposure time we used five doses of calcitriol as detailed in the methods section. With this experimental design a clear-cut dose response was seen as early as 24 h. While the means at 24 h did show a trend toward a dose-responsive decrease in sulfate incorporation these means were not significantly different from one another. At 48 and 72 h there were significant differences between means in those groups ($p=0.0001$ for 48 h and $p=0.0003$ for 72 h) .

Figure 4. Effect of calcitriol on ROS 17/2.8 cell proliferation as determined by the number of cells counted at various time points after treatment with calcitriol at different doses. Y axis - number of cells X 10^{-3}. X axis - hours of treatment with calcitriol. Insert - Various doses of calcitriol. Four replicates at each dose per time point were averaged. Means are represented by the bars.

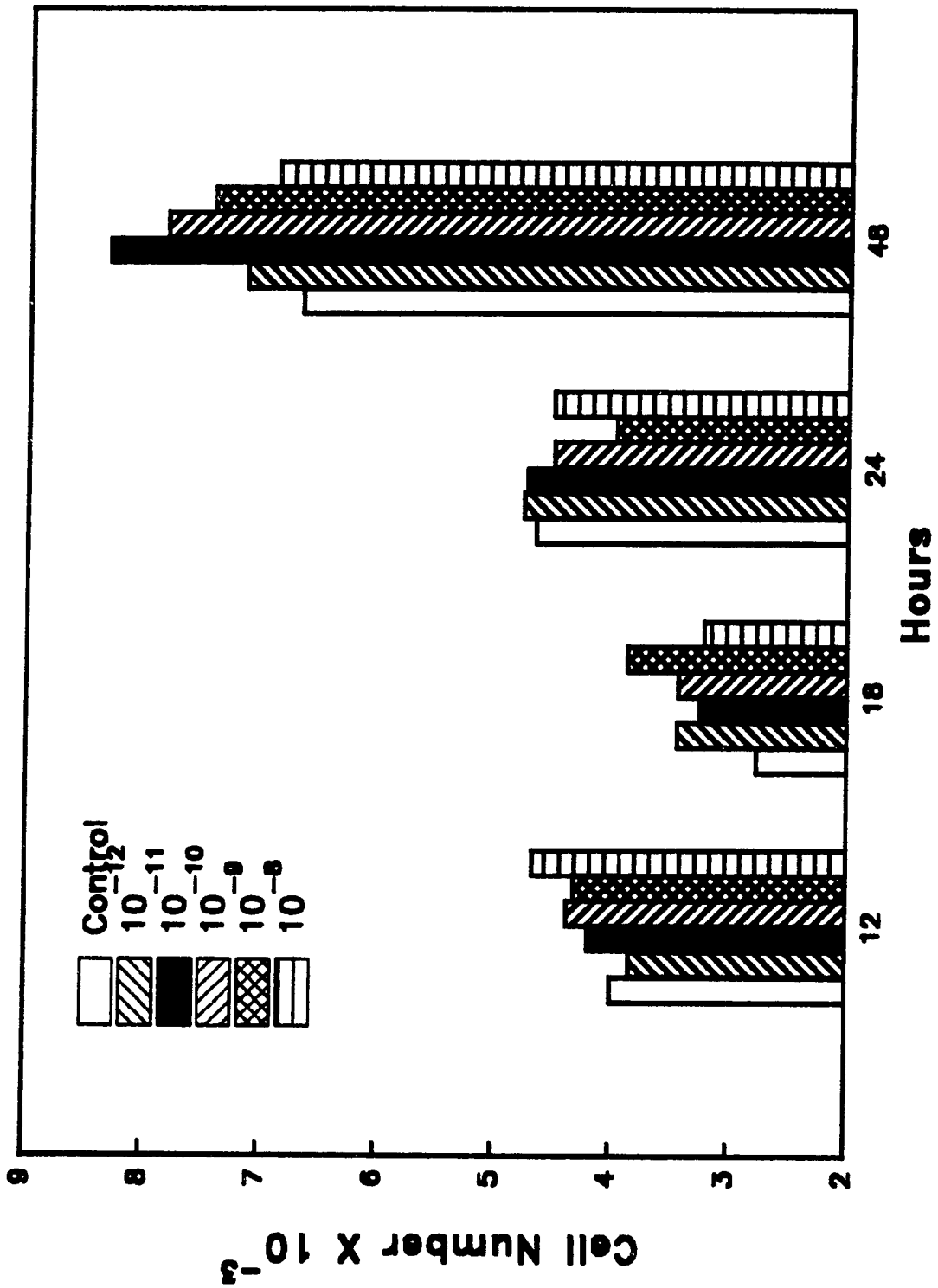

Cell Number X 10^{-3}
9
8
7
6
5
4
3
2
Hours
12
18
24
48
Control
10^{-12}
10^{-11}
10^{-10}
10^{-9}
10^{-8}

Table 1. Dose-response of ROS 17/2.8 cells treated with calcitriol and labelled with [^{35}S]-sulfate for the final 6 h of a 24, 48, or 72 h treatment period.

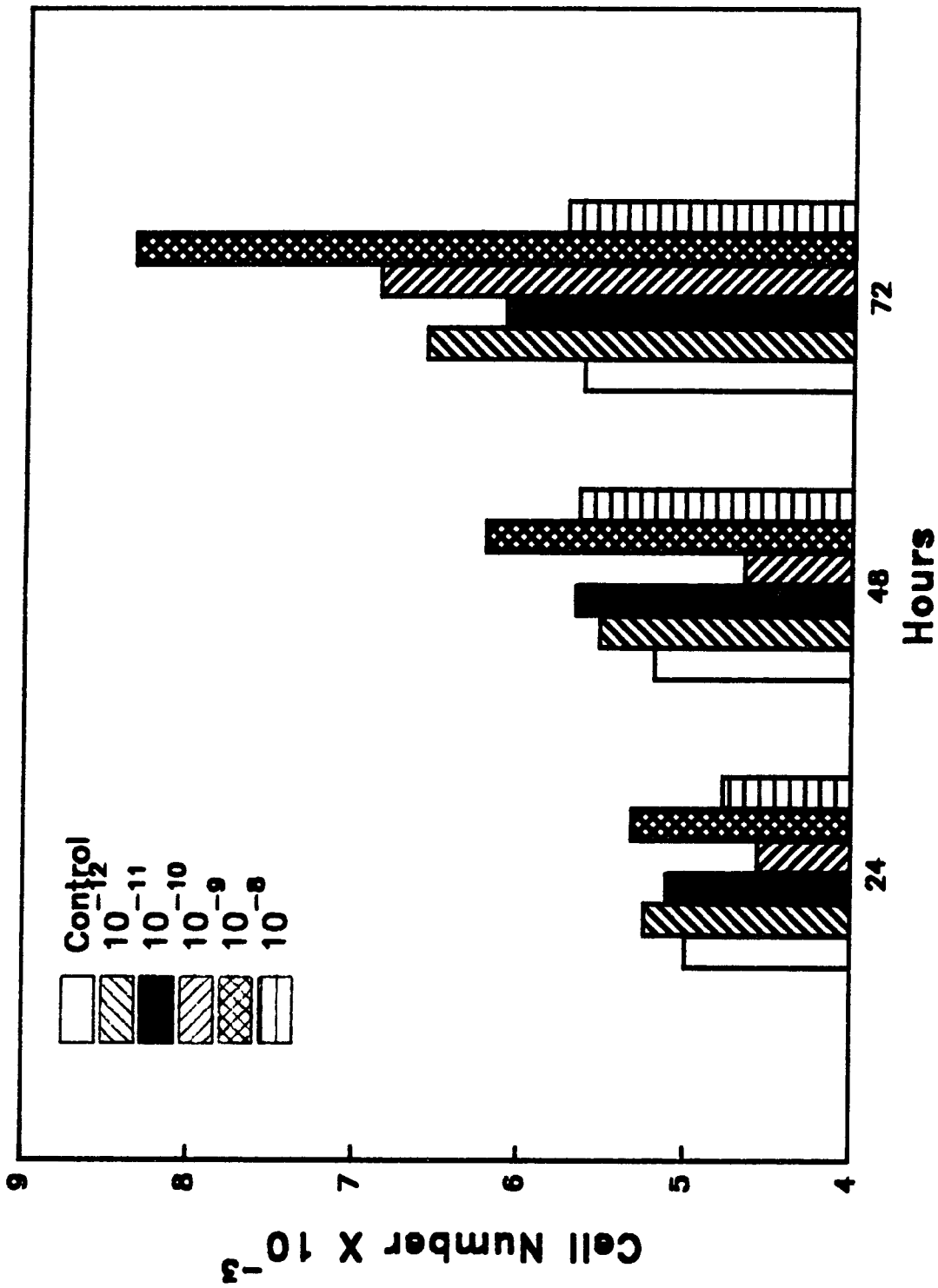
Concentration of $1,25(OH)_2D_3$	[^{35}S]-Sulfate Incorporation % of Controls		
	24 h	48 h	72 h
10^{-8}	71%	56%	47%
10^{-9}	76%	59%	47%
10^{-10}	83%	68%	57%
10^{-11}	86%	84%	70%
10^{-12}	87%	79%	70%

Besides showing a more clear dose-response in this experiment we also demonstrated for the third time the reproducibility of this response and we verified the fact that it took at least 24 h for the response to be observable.

We obtained data on numbers of cells at all three time points in this experiment (see figure 5). At 24 h there were significant differences between some of the means but again these differences did not occur in a dose-dependent manner. Numbers of cells at 48 h were not significantly different from each other while at 72 h there were significant differences between means. For the 72 h time point the means varied in a random fashion as before with the highest numbers of cells being observed at the doses of 10^{-9} M and 10^{-10} M and the number of cells at 10^{-8} M being no different from that of controls. Again we felt there may be random or systematic error involved which is why the means generated were not more similar and, as before, the differences were not dose dependent indicating that calcitriol was probably not the responsible agent.

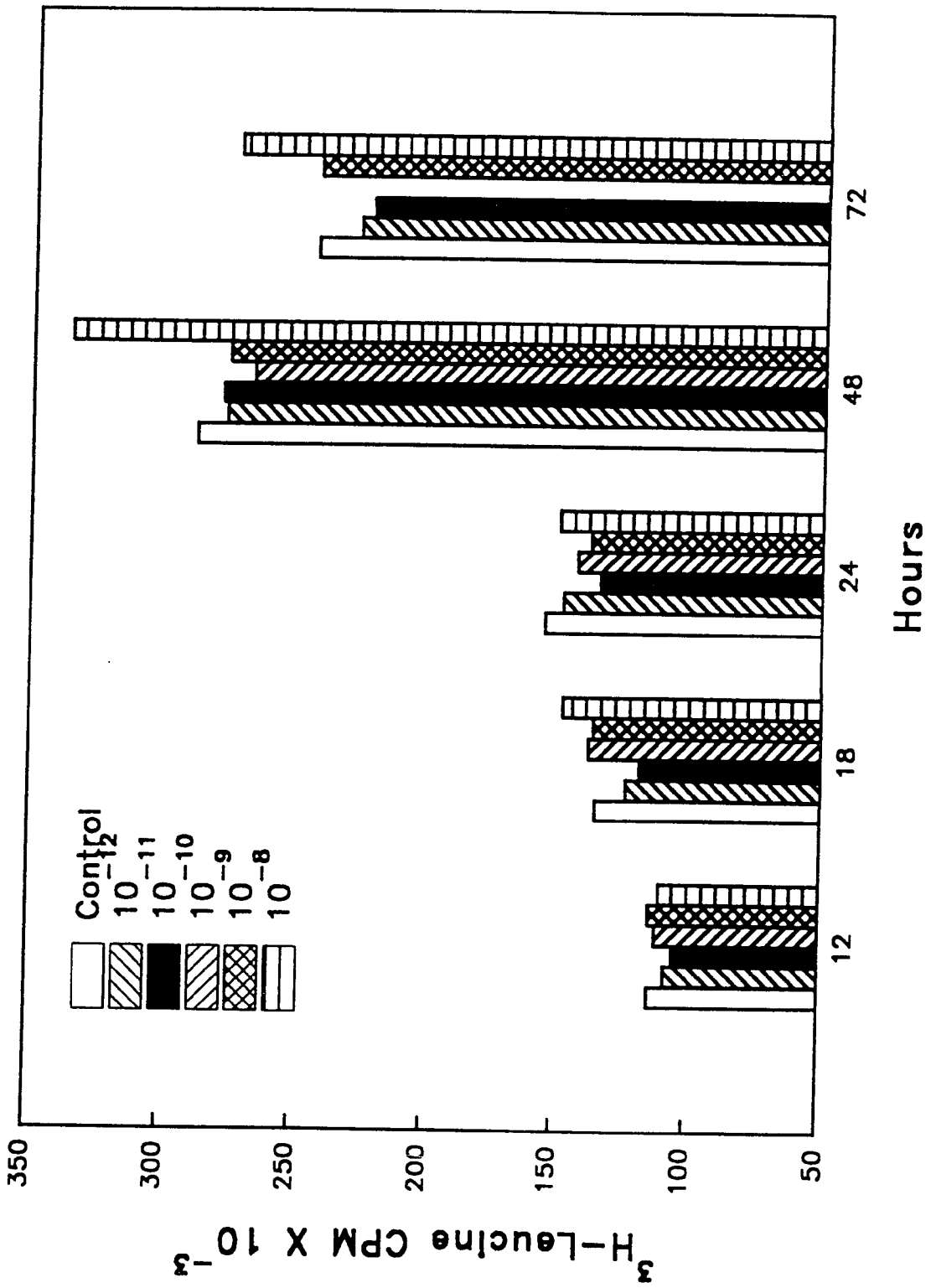
To test the effect of calcitriol on total protein synthesis, we designed a dose-response experiment with five doses of calcitriol given for five different lengths of time and labeled with 10 μCi/mL [^{3}H]-leucine. This experiment did differ from previous dose-response experiments in that the media and cell layer were combined at harvest prior to the initial desalting step. For each length of treatment there

Figure 5. Effect of calcitriol on ROS 17/2.8 cell proliferation as determined by the number of cells counted at various time points after treatment with calcitriol at different doses. Y axis - number of cells X 10^{-3}. X axis - hours of treatment with calcitriol. Insert - Various doses of calcitriol. Four replicates at each dose per time point were averaged. Means are represented by the bars.


Cell Number X 10⁻³
9
8
7
6
5
4
24
48
72
Hours
Control
10⁻¹²
10⁻¹¹
10⁻¹⁰
10⁻⁹
10⁻⁸

were four replicates per dose. The mean incorporated [^{3}H]-leucine radioactivity at each dose was analyzed by One-Way Analysis of Variance. At 12, 18 and 24 h of exposure to calcitriol there were no differences between controls and calcitriol-treated samples in terms of macromolecular [^{3}H]-leucine (see figure 6). At 48 h there was a significant difference between means but it was interesting to note that the highest amount of incorporated [^{3}H]-leucine was seen at the dose of 10^{-8} M calcitriol with the next highest amount seen in the control group. The mean values for the other doses of calcitriol were not different from each other. Since the greatest amount of incorporated [^{3}H]-leucine was seen at 10^{-8} M calcitriol in the cells exposed for 48 h, calcitriol was not diminishing total protein synthesis. Similar, although not as strikingly different, values were observed for cells exposed to calcitriol for 72 h. At 10^{-8} M calcitriol the incorporated [^{3}H]-leucine was highest, followed by the values at 10^{-9} M and those in the control group. The other doses of calcitriol yielded values that were slightly less than those at 10^{-9} M and control. We concluded that calcitriol was not inhibiting protein synthesis. If anything, the hormone was probably inducing the synthesis of some proteins as has been described in the literature. The results of this experiment actually strengthen the earlier data showing a reduction in [^{35}S]-sulfate incorporation: The ratio of incorporated [^{35}S]-sulfate to [^{3}H]-leucine in the 72 h samples, obtained

Figure 6. Effect of calcitriol on total protein synthesis. ROS 17/2.8 cells were labeled with [^{3}H]-leucine and treated with various doses of calcitriol for the lengths of time shown on the X axis (time is in hours). Four replicates of each dose at each time point were harvested and the media and cell layer combined. Average [^{3}H]-leucine CPM of the four replicates were calculated and are depicted by the bars. Y axis - [^{3}H]-leucine CPM X 10^{-3}. Doses of calcitriol are shown in the insert.

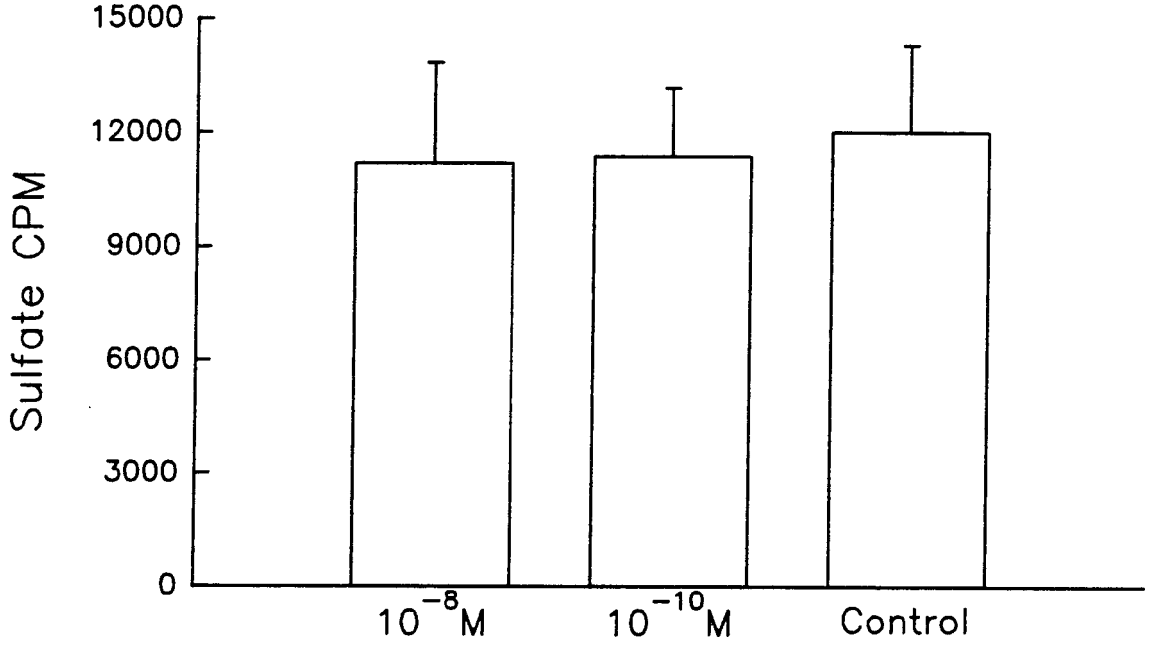

^{3}H-Leucine CPM X 10^{-3}
350
300
250
200
150
100
50
12
18
24
48
72
Hours
Control
10^{-12}
10^{-11}
10^{-10}
10^{-9}
10^{-8}

using data from the previous dose response experiments, is 0.68 to 0.7 for controls, 0.5 for 10^{-9} M calcitriol and 0.45 for 10^{-8} M calcitriol.

We tested the effects of three metabolites of cholecalciferol , 1,25 dihydroxycholecalciferol or 1,25,$(OH)_2$ D_3 (calcitriol), 24,25 dihydroxycholecalciferol or 24,25 $(OH)_2$ D_3 and 25 hydroxycholecalciferol or 25(OH) D_3 on the ROS 17/2.8 cells. Calcitriol or the 1,25 hydroxy metabolite is well known for numerous effects on bone tissue and other tissues as well. The other two metabolites, being similar in structure to calcitriol, may bind to the calcitriol receptor with more or less affinity and may actually cause a measurable response. The 24, 25 metabolite has been found concentrated in fetal bone and injured, healing bone leading to the speculation that it may be necessary for normal bone formation. Our working hypothesis was that the dose-dependent reduction in [^{35}S]-sulfate incorporation was caused specifically by calcitriol. Data from the experiment testing effects of 25 (OH) D_3 are shown in figure 7. This metabolite did not cause a measurable response in the ROS 17/2.8 cell line that differed from what was seen in controls. We concluded that the 25 (OH) D_3 was not an active metabolite in this cell line. Calcitriol, as expected, caused the reduction in sulfate incorporation into macromolecules synthesized by ROS 17/2.8 cells. Unexpectedly, the 24,25 $(OH)_2$ D_3 also caused a reduction in [^{35}S]-sulfate incorporation as shown in figures 8 and 9. All

Figure 7. Effect of 25-hydroxycholecalciferol on [^{35}S] sulfate incorporation into ROS 17/2.8 cell proteoglycans extracted from the media and cell layer. Cells were treated with 25(OH)D_3 for 48 h and labelled with [^{35}S]-sulfate for the final 6 h of the experiment. Four replicates per dose and of the controls were obtained. Average incorporated radioactivity is represented by the bars.

ROS 17/2.8 Media Proteoglycan Response to $25(OH)D_3$



ROS 17/2.8 Cell Layer Proteoglycon Response to $25(OH)D_3$

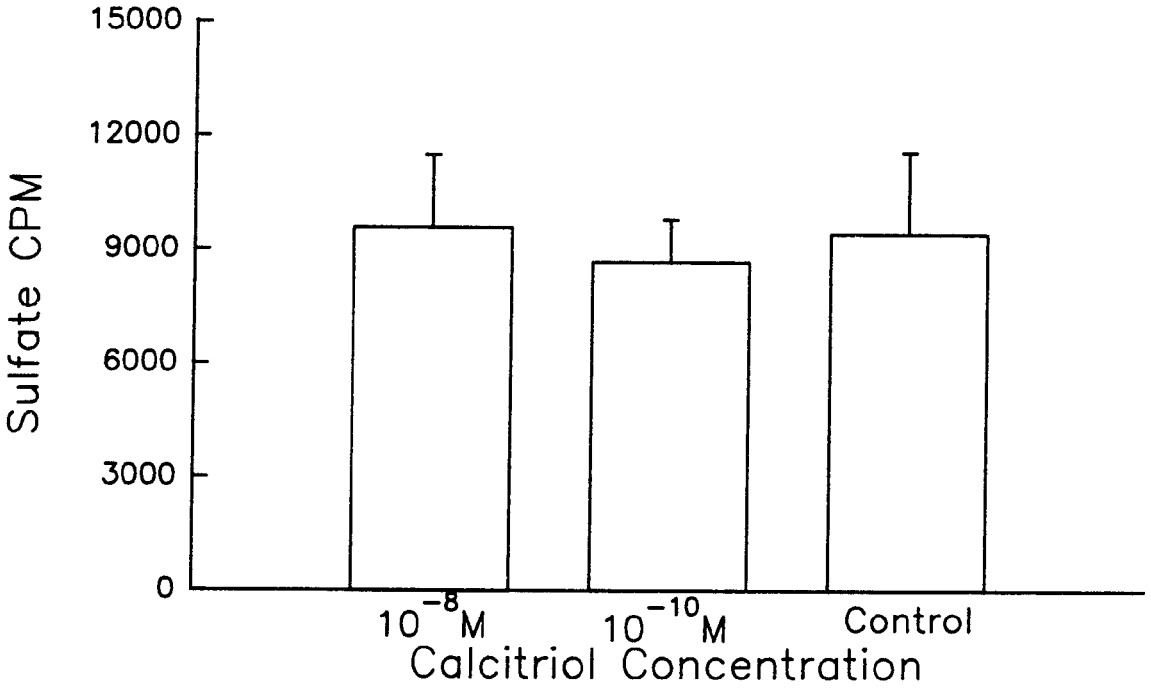
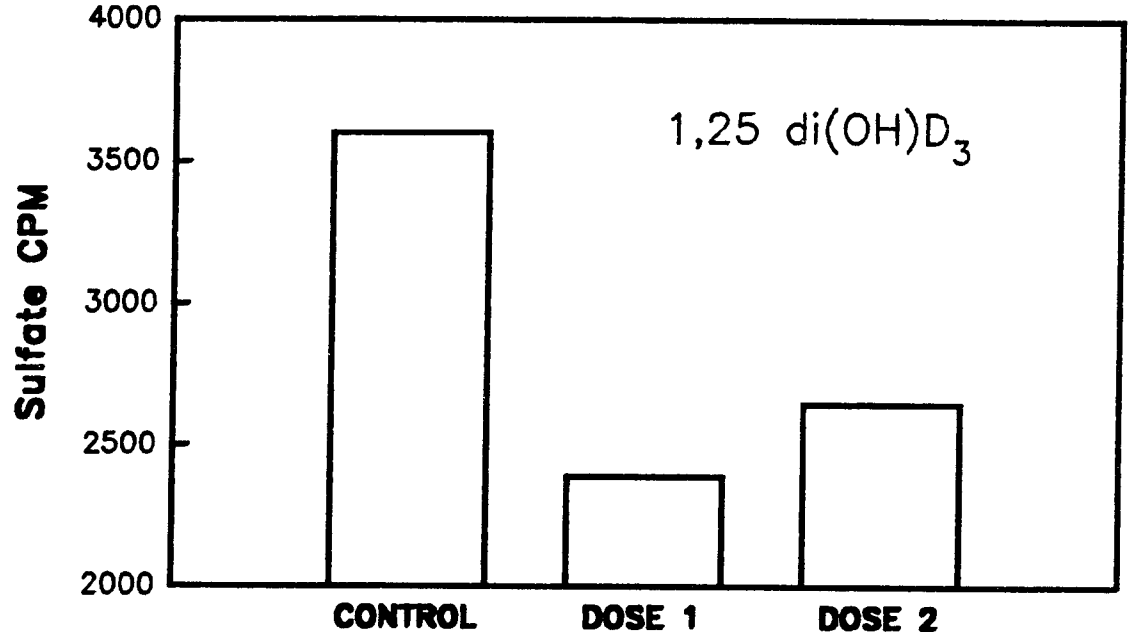
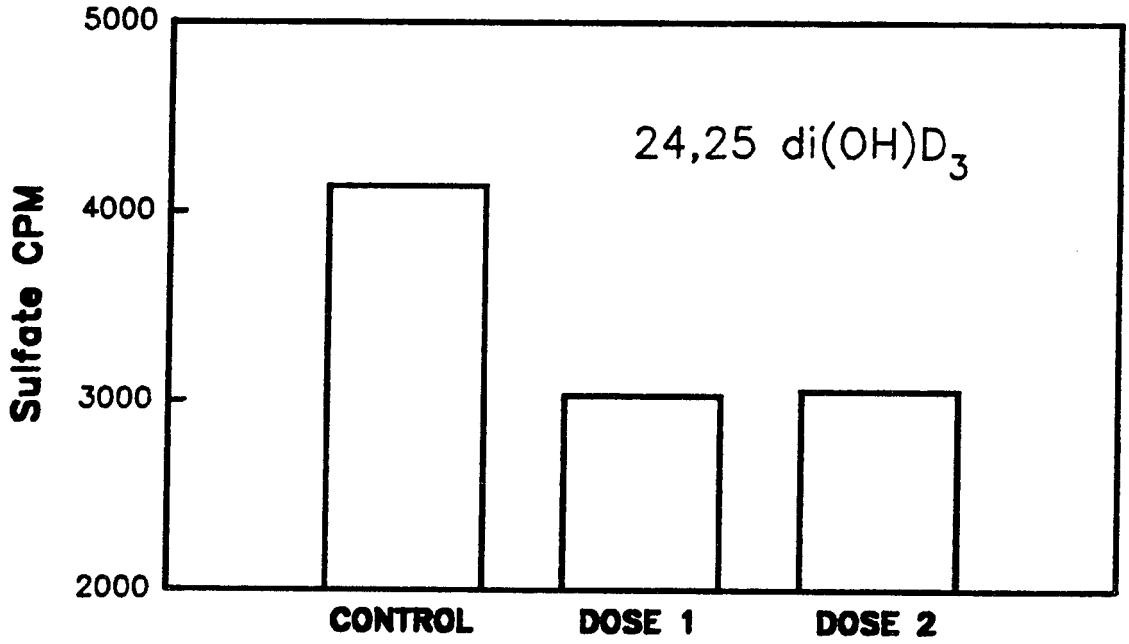


Figure 8. Effect of 1,25-dihydroxycholecalciferol and 24,25-dihydroxycholecalciferol on [^{35}S]-sulfate incorporation into ROS 17/2.8 cell proteoglycans extracted from the media. Two doses of each metabolite were used (10^{-8}M and 10^{-10}M). Cells were treated with a metabolite for 48 h and labeled with [^{35}S]-sulfate for the final 6 h of treatment time. Four replicates of each dose and of the control were harvested. Average CPM are represented by the bars. Y axis - [^{35}S]-sulfate CPM. X axis - different doses and control shown for each metabolite.

ROS 17/2.8 Media Proteoglycan Response to Various Metabolites

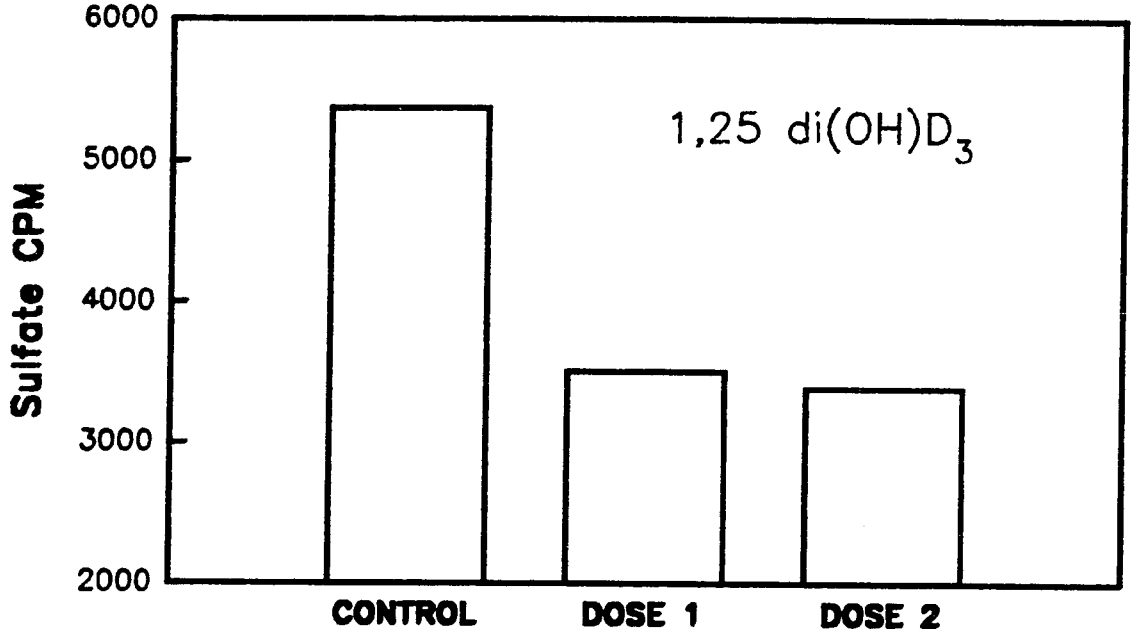


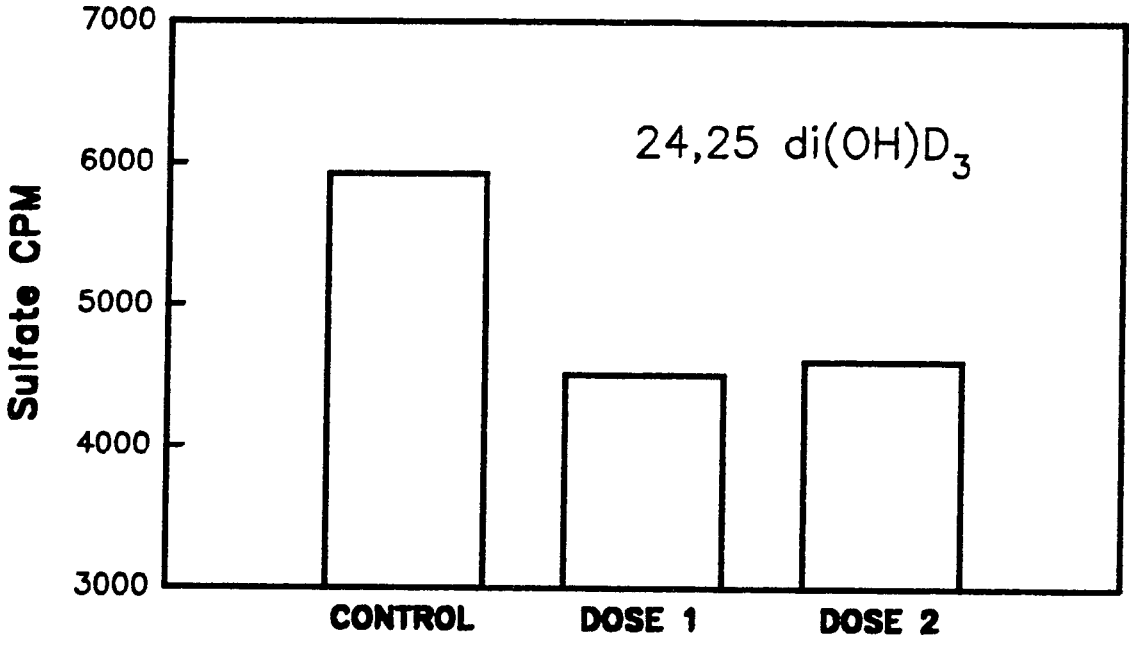


Dose 1 = 10^{-10}M
Dose 2 = 10^{-8}M

Figure 9. Effect of 1,25-dihydroxycholecalciferol and 24,25-dihydroxycholecalciferol on [^{35}S]-Sulfate incorporation into ROS 17/2.8 cell proteoglycans extracted from the cell layer. Two doses of each metabolite were used (10^{-8}M and 10^{-10}M). Cells were treated with a metabolite for 48 h and labeled with [^{35}S]-sulfate for the final six h of treatment time. Four replicates of each dose and of the control were harvested. Average CPM are represented by the bars. Y axis - [^{35}S]-sulfate CPM. X axis - different doses and control shown for each metabolite.

ROS 17/2.8 Cell Layer Proteoglycan Response to Various Metabolites





Dose 1 = 10^{-10}M
Dose 2 = 10^{-8}M

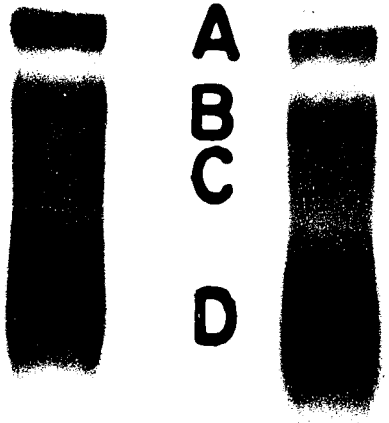
replicates in each group were averaged and the average incorporated [^{35}S]-sulfate analyzed for significance by Analysis of Variance. For media samples treated with calcitriol incorporated [^{35}S]-sulfate was 73% and 66% that of controls at 10^{-8} M and 10^{-10} M calcitriol, respectively. We cannot explain why the lower dose produced a more dramatic response here when a dose-response is usually generated. These means were significantly different from the control mean radioactivity ($p=0.001$). The media samples treated with the 24, 25 metabolite at 10^{-8} M and 10^{-10} M had incorporated radioactivity that was 73% that of controls, a difference that was significant ($p=0.028$). The cell layer samples had 63% the incorporated sulfate of controls when given 10^{-8} M calcitriol for 48 h and 65% that of controls when given 10^{-10} M calcitriol for that amount of time. At both doses the means were significantly different from controls ($p=0.001$). The cell layer samples receiving 24 ,25 $(OH)_2$ D_3 at 10-8 M had 77% the incorporated sulfate of controls and the dose of 10^{-10} M was associated with a drop in sulfate to 76% that of controls. While these differences were not significant, they do show a definite trend toward reduced sulfate. This experiment was repeated once and similar results were obtained. We concluded that 24,25 $(OH)_2$ D_3 was active in this cell line and caused a reduction in [^{35}S]-sulfate incorporation. Due to time restraints we did not pursue work with this metabolite.

As in previous experiments, we examined effects of the cholecalciferol metabolites on cell proliferation. The control cell wells had slightly fewer numbers of cells than did the wells containing media treated with either of the cholecalciferol metabolites but these differences were not significant.

Realizing that the labeled [^{35}S]-sulfate macromolecules eluting at the void volume of the Biogel P-30 columns actually represent several populations of proteoglycans, we were interested in finding out if all these subpopulations of proteoglycans were affected by calcitriol. A fluorogram, prepared as described in the methods section, was analyzed by densitometry in order to answer this question (see figure 10). Densitometry of the 5-15% gradient gel identified four major [^{35}S]-sulfated bands with similar retention times for the control and calcitriol-treated samples. Area percent analysis revealed a slight difference between three of the bands in the calcitriol treated sample as compared to the control sample. Two of the bands in the calcitriol sample (bands A and C , see figure 10) were reduced compared to controls while the fourth band (band D) was increased in terms of area percent. This could reflect a redistribution of radioactivity to the last peak if the first and third peaks were reduced with respect to incorporated sulfate CPM. This would be the outcome since we loaded equal CPM per lane. These results led us to speculate that perhaps certain proteoglycan populations were affected more so by calcitriol

Figure 10. Five to fifteen percent gradient gel processed for fluorography, of the macromolecular sulfated material eluting at the void volume of a P-30 gel size exclusion column. This fluorogram was analyzed by densitometry using the Maxima 820 program. Four major peaks were identified in both control and calcitriol-treated samples as shown. Peaks are identified by retention time and quantified by area percent.

Area Percent	Control Calcitriol	Area Percent
13.53	A	9.96
14.49	B	14.48
15.13	C	10.11
50.48	D	60.52



than others. Based on these results we decided to attempt to separate the various proteoglycan subpopulations and subsequently devised several experiments aimed at doing so. Separation of the different proteoglycans would allow us to better ascertain if calcitriol differentially affected the ROS 17/2.8 cell proteoglycans.

We tried two approaches to separate the ROS 17/2.8 proteoglycans. Initially, immunoprecipitation was attempted using antibodies graciously given to us by Larry W. Fisher and Joachim Sasse. From Larry Fisher's lab we received four polyclonal antibodies, one against a mixture of rat bone PGs, one against human bone PG I and PG II, one against a synthetic peptide of human biglycan and one against a synthetic peptide of human decorin. Joachim Sasse's lab supplied us with three polyclonal antibodies, one against bovine PG I, one against bovine PG II and one against the bovine aggregating cartilage PG. After trying these with several different immunoprecipitation protocols we did not succeed in precipitating any of the labeled material in our samples from the ROS 17/2.8 cells.

Our next attempt at separating these proteoglycans was to try a method reported in the literature by Fedarko et al. (1990) for separating human bone cell proteoglycans. As detailed in the methods section, this approach involved a multistep HPLC scheme. Ion exchange on a Nucleogen-4000-10 DEAE column would be followed by size exclusion on a 1 x 50 cm Omnifit glass column packed with TSK-GEL ToyoPearl HW

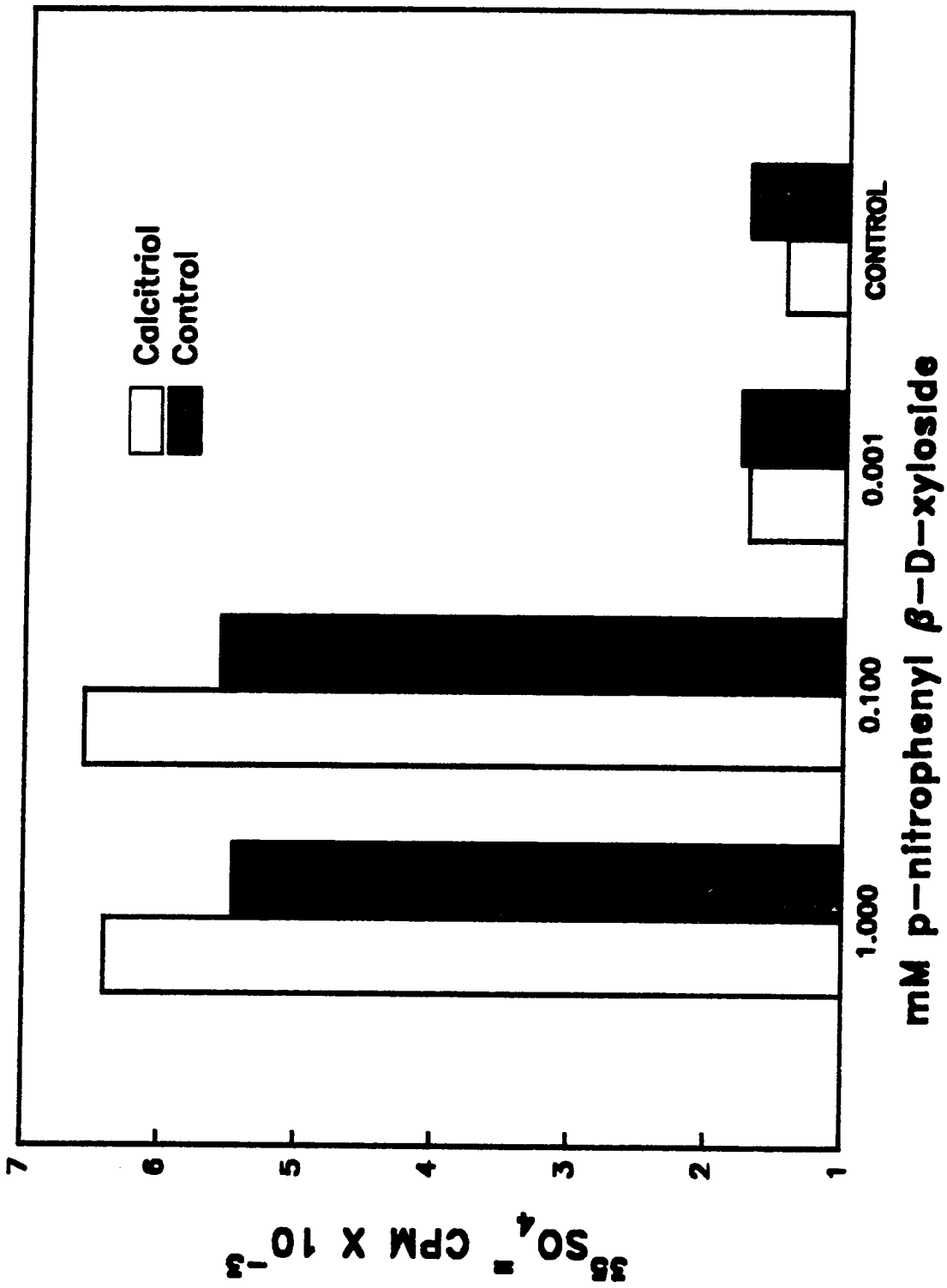
75(F). Labeled peaks were identified by liquid scintillation counting and SDS-PAGE fluorography. Many preliminary runs were required on the Nucleogen 4000 DEAE column to work out satisfactory separation of labeled material. Run times and gradient parameters were key variables. A buffer of 50% formamide with a gradient of 0 to 1.5 M KCl, pH 6.0, over 90 min seemed to work best. We were able to achieve reproducible baseline separation of major labeled peaks and recoveries of better than 90% for the first several runs on this column. For some reason after that number of runs, our recovery dropped to less than 10%. Analysis of the problem involved checking conductivities of all buffers and water, remaking buffers, washing the column, checking the fittings for leaks or blockage and doing a study on the decomposition of formamide. We determined that the column was still binding the sample, but for some reason we could not elute any labeled proteoglycans. Fortunately, a sizeable amount of sample from the ROS 17/2.8 cells had been partially purified prior to the column failing, so we decided to pursue the next step in this scheme, the size exclusion chromatography. Prior to the next chromatographic step we needed to concentrate and desalt the samples so a small volume containing a large quantity of labeled material could be loaded onto the size exclusion column. For both the calcitriol-treated and control samples, peaks of radio-activity eluting from the Nucleogen DEAE column were pooled, dialysed against a large volume of water and lyophilized. We

found that the Triton-X100 present in the buffer interfered with lyophilization. The next attempt involved diluting the pooled samples once with 0.05 M Tris then passing the entire sample volume over a 3 mL column of ToyoPearl DEAE equilibrated in 0.05 M Tris, pH=6.0. The sample was washed onto the column with two to three column volumes of 0.05 M Tris, eluted with five mL of 4 M guanidine/ 0.05 M Tris and washed again with five mL of 0.05 M Tris. One mL fractions were collected. Peaks of radioactivity were identified by scintillation counting, collected and run over PD-10 columns equilibrated in NH_4HCO_3 to remove the guanidine prior to lyophilization. After lyophilization samples were prepared for 5-15% gradient SDS-PAGE to identify the proteoglycans. We found this protocol to work fairly well although by the time we were ready to prepare samples for gel electrophoresis little radioactivity remained, in fact there was not enough to proceed with the size exclusion run. In two separate attempts we harvested large volumes of ROS 17/2.8 cell media labeled with 200 μCi/mL [^{35}S]-sulfate, yet still could not recover enough radioactivity to proceed to the final size exclusion step.

For the experiments designed to test whether or not calcitriol decreased the ability of ROS 17/2.8 cells to synthesize glycosaminoglycan (GAG) chains, we utilized p-nitro phenyl ß D-Xyloside, an artificial initiator of GAG synthesis. This approach worked well in that we could increase GAG production to what appeared to be a maximum

level in ROS 17/2.8 cells with an artificial GAG acceptor and therefore test the cellular capacity for GAG synthesis under various conditions. The 2 X 2 factorial experimental design allowed us to test all combinations of factors at different concentrations. Results are shown in figure 11. When ROS 17/2.8 cells were treated with only the xyloside at 1.0 and 0.1 mM there was an increase in [^{35}S]-sulfate incorporation to 320% the level seen in cells not treated with xyloside. At 0.001 mM xyloside the increase in macromolecular [^{35}S]-sulfate was only 2%. ROS 17/2.8 cells treated with 10^{-9} M calcitriol alone had [^{35}S]-sulfate reduced to 84% of that seen in the control cells. When calcitriol-treated cells were also given 1.0 to 0.1 mM xyloside the decrease in incorporated [^{35}S]-sulfate was abolished and, in fact, the sulfate radioactivity in calcitriol plus xyloside treated cells rose to over 400% of that observed in cells exposed to calcitriol alone. At 0.001 mM xyloside in cells also treated with calcitriol there was an increase in incorporated [^{35}S]-sulfate to 117% over that of the group given only calcitriol. These data suggest that once an acceptor is present, the enzymatic steps required for formation of a GAG chain are not affected by calcitriol. Of course, this experiment did not enable us to ascertain whether or not transfer of xylose to a core protein was the target of calcitriol's action. An attempt was made to perform a xylosyl transferase assay using media from calcitriol-treated and untreated ROS 17/2.8 cultures, but we

Figure 11. Addition of p-nitrophenyl ß D-xyloside to ROS 17/2.8 cell cultures treated with and without calcitriol. Cells were treated with 10^{-9}M calcitriol for 48 h and then labeled with [^{35}S]-sulfate for the final two h of the experiment. Various doses of p-nitrophenyl ß-D-xyloside, shown on the X axis along with the control, were also given for the last two h of the experiment. Four replicates of each combination of factors were obtained and mean [^{35}S]-sulfate CPM calculated. Means are represented by the bars. [^{35}S]-sulfate CPM X 10^{-3} shown on the Y axis.


Calcitriol
Control
7
6
5
4
3
2
1
$^{35}SO_4^{=}$ CPM X 10^{-3}
1.000
0.100
0.001
CONTROL
mM p-nitrophenyl β-D-xyloside

were not able to detect activity in the media retrieved from cell cultures. While xylosyl transferase probably was present, these levels were below the sensitivity of the assay. Xyloside treatment, alone or with concurrent calcitriol treatment, did not affect ROS 17/2.8 cell proliferation.

The decrease in incorporated macromolecular [^{35}S]-sulfate seen in the media and cell layer fractions isolated from ROS 17/2.8 cultures could be due to an enhanced rate of degradation of proteoglycans. We tested this hypothesis by adding partially purified, labeled ROS 17/2.8 proteoglycans back to cultures of the cells, then assessing degradation after specific amounts of time had elapsed. Further details are found in the methods section. The results are displayed in figure 12. For each time point shown, two separate size exclusion runs on Sepharose CL6B are shown, one representing elution of control material and one representing elution of calcitriol-treated material. When exogenously added proteoglycans were incubated with ROS 17/2.8 osteoblastic cells for 2 h, very little degradation was observed. This was evident because there was little [^{35}S]-sulfate labeled material eluting at or near the V_t, which would represent the degraded proteoglycan-derived material. The large peak eluting at or near the V_o corresponds to the intact proteoglycans. Note that at all time points there is not a difference between controls and calcitriol-treated samples with respect to the amount of labeled material

Figure 12. Sepharose CL6B chromatography of [^{35}S]-sulfate labeled ROS 17/2.8 proteoglycans added to ROS 17/2.8 cell cultures treated with (-----) and without (———) 10^{-8}M calcitriol. Degradation of the exogenously added proteoglycans was quantified at 2 h, 6 h, 12 h, 24 h and 48 h and is seen as material eluting toward the total volume of the column. At each time point the percent CPM recovered from the later-eluting peaks did not vary with the treatment mode.

Buffer – 4M Guanidine, 0.05M Tris, 1% Triton

Packing Material – Sepharose CL6B

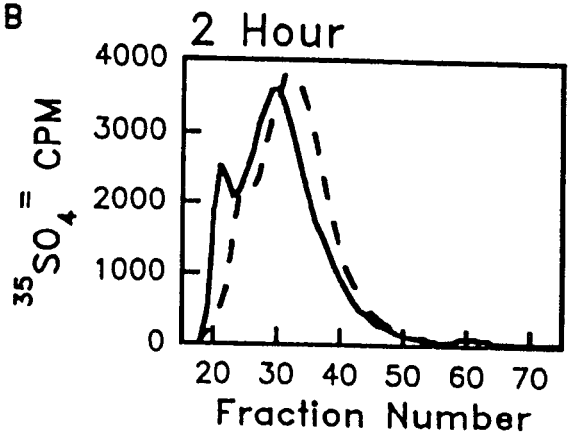
Flow Rate – 1 ml/5 min.

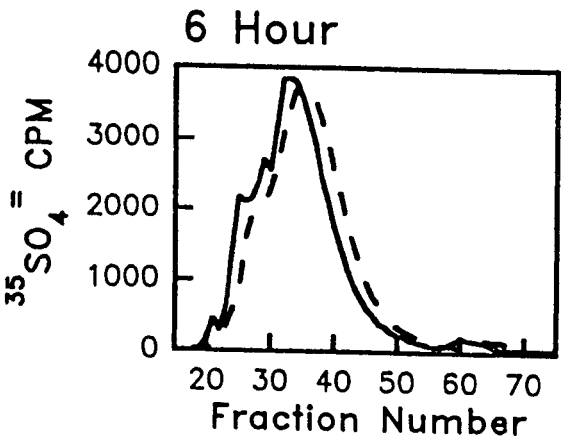
V_0 = 24 ml

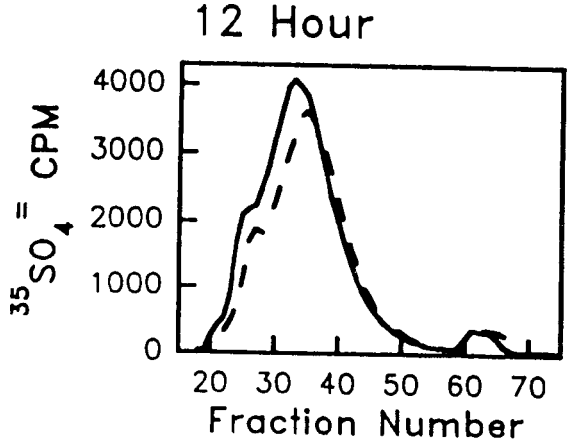
V_t = 68 ml

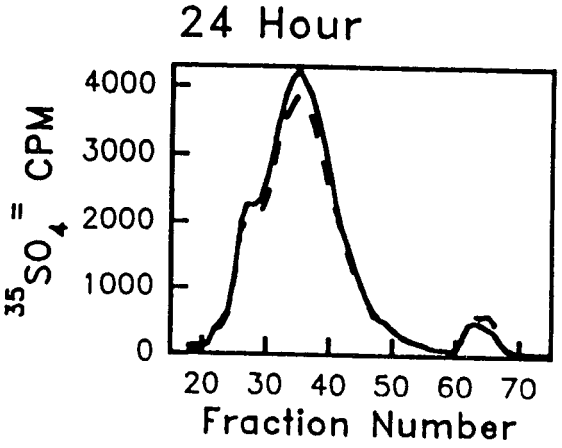
—— Control

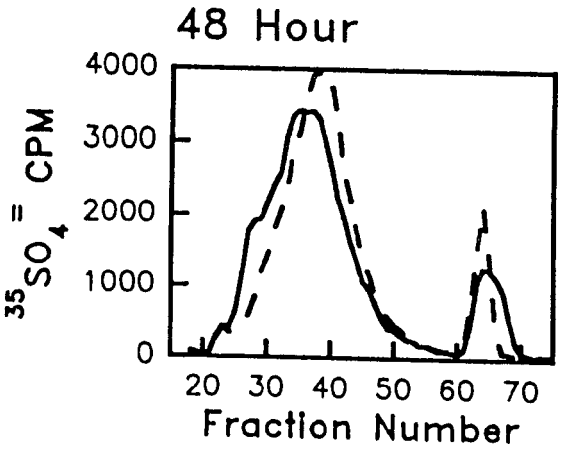
- - - Calcitriol











eluting at V_o. This is shown in table 2. Similarly, while the labeled material eluting at V_t did increase with time, indicating that degradation of proteoglycans was occurring, there was no difference in rate or degree of degradation between the control and calcitriol-treated samples. By 48 h the [^{35}S]-labeled degradation products eluting at the total volume accounted for 11.4% of the total loaded CPM in controls and 10.4% of total loaded radioactivity in the calcitriol treated sample. No autodegradation of proteoglycans occurred.

In order to find out if calcitriol caused shorter glycosaminoglycan chains to be synthesized, we liberated GAG chains from partially purified proteoglycans by papain digestion, then assessed relative size of GAG chains on a Sepharose CL6B column. Results of this experiment are shown in figure 13. Glycosaminoglycan chains were labeled in this case with [^{3}H]-glucosamine. From the chromatographic results the calculated Kav of both the calcitriol-treated and control GAG chains was approximately 0.30, indicating that GAG size was not affected by calcitriol.

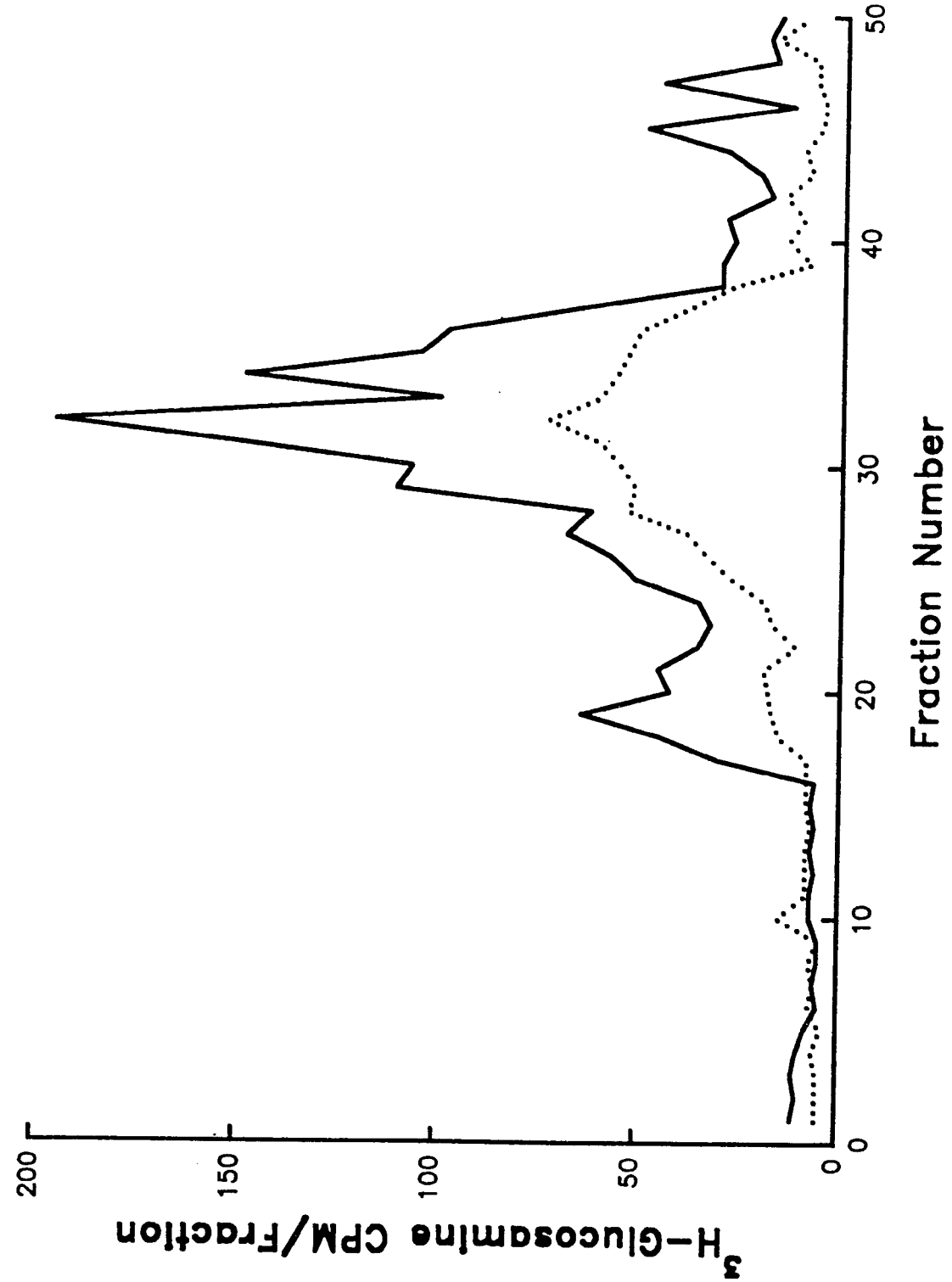
The effect of calcitriol on GAG synthesis was further examined by quantification of [^{3}H]-glucosamine incorporation into ROS 17/2.8 cells treated with and without calcitriol. This allowed us to determine whether fewer GAG molecules were being synthesized or if they were undersulfated due to calcitriol treatment. In the same experiment the effect of

Table 2. Degradation of exogenously added proteoglycans by calcitriol-treated and control ROS 17/2.8 cells. Percent of total added radioactivity eluting at the V_0 and V_t are shown for the various experiment termination times.

Percent of $^{35}SO_4$ = CPM Recovered from Total Recovered

Time, hours	Control		Calcitriol	
	V_0	V_t	V_0	V_t
48	88.6	11.4	89.6	10.4
24	90.0	4.0	94.5	5.5
12	96.0	4.0	95.6	4.3
6	98.5	1.6	98.5	1.5
2	99.4	0.6	99.4	0.5
48, no cells	99.7	0.6		

Figure 13. Effect of calcitriol on the relative size of glycosaminoglycan chains liberated by papain digestion from ROS 17/2.8 cell proteoglycans. Partially purified papain digested material was chromatographed on Sepharose CL6B in a 1cm X 120cm column eluted with 4M guanidine HCl/50mM Tris, pH 7.06. Axis - [^{3}H]-glucosamine CPM per fraction number. Estimated K_{AV} shown in insert.


^{3}H–Glucosamine CPM/Fraction
200
150
100
50
0
0
10
20
30
40
50
Fraction Number

cell density on the action of calcitriol was tested because we observed varying results in terms of [^{3}H]-glucosamine incorporation during the partial purification of proteoglycans. Separate batches of material harvested from the ROS 17/2.8 cells had different amounts of [^{3}H]-glucosamine in peaks resolving from the DEAE Sepharose column and one variation we could attribute this to was cell density at the time of calcitriol treatment. The questions we asked were whether or not calcitriol reduced [^{3}H]-glucosamine incorporation into macromolecules synthesized by ROS 17/2.8 cells and if this effect was cell-density dependent. Details of the experiment are outlined in the methods section and the results are shown in figures 14, 15 and 16. Figure 14 shows DEAE Sepharose chromatography of multiple replicates of control and calcitriol-treated ROS 17/2.8 cell media. Note that three peaks resolved from this column as it was eluted with 0 to 1.0 M NaCl and that the second and third peaks were particularly affected by treatment. The second peak may correspond to the elution of hyaluronic acid while the third peak marks the elution of proteoglycans, distinguished by their characteristic displacement from a DEAE column at approximately 0.6 M NaCl. Plate A displays four control replicates at the highest cell density while plate B shows the four calcitriol-treated replicates at that density. There was a notable difference between the control samples

Figure 14. The effect of 10^{-8}M calcitriol and cell density on the incorporation of [^{3}H]-glucosamine into marcomolecules synthesized by ROS 17/2.8 cells. Cells were cultured to three different densities prior to starting the 48h treatment with calcitriol. [^{3}H]-glucosamine label was applied for the final 24h of the treatment time. Four replicates at each density were obtained for both calcitriol-treated and control cells. Following desalting, each replicate was chromatographed on DEAE Sephrose and eluted with a step-wise NaCl gradient.
Graph A. - Chromatographs of four control replicates at the highest density. Graph B. - Chromatographs of four calcitriol-treated replicates at the highest density. Graph C. - Chromatographs of four control replicates at intermediate density. Graph D. - Chromatographs of four calcitriol-treated replicated at intermediate density. Graph E - Chromatographs of four control replicates at the lowest density. Graph F. - Chromatographs of four calcitriol-treated replicates at the lowest density. In all instances, fractions 1-2 were eluted with 4ml of 0.15M NaCl, fractions 3-7 were eluted with 10ml of 0.37M NaCl and fractions 8-12 were eluted with 10ml of 0.68M NaCl.

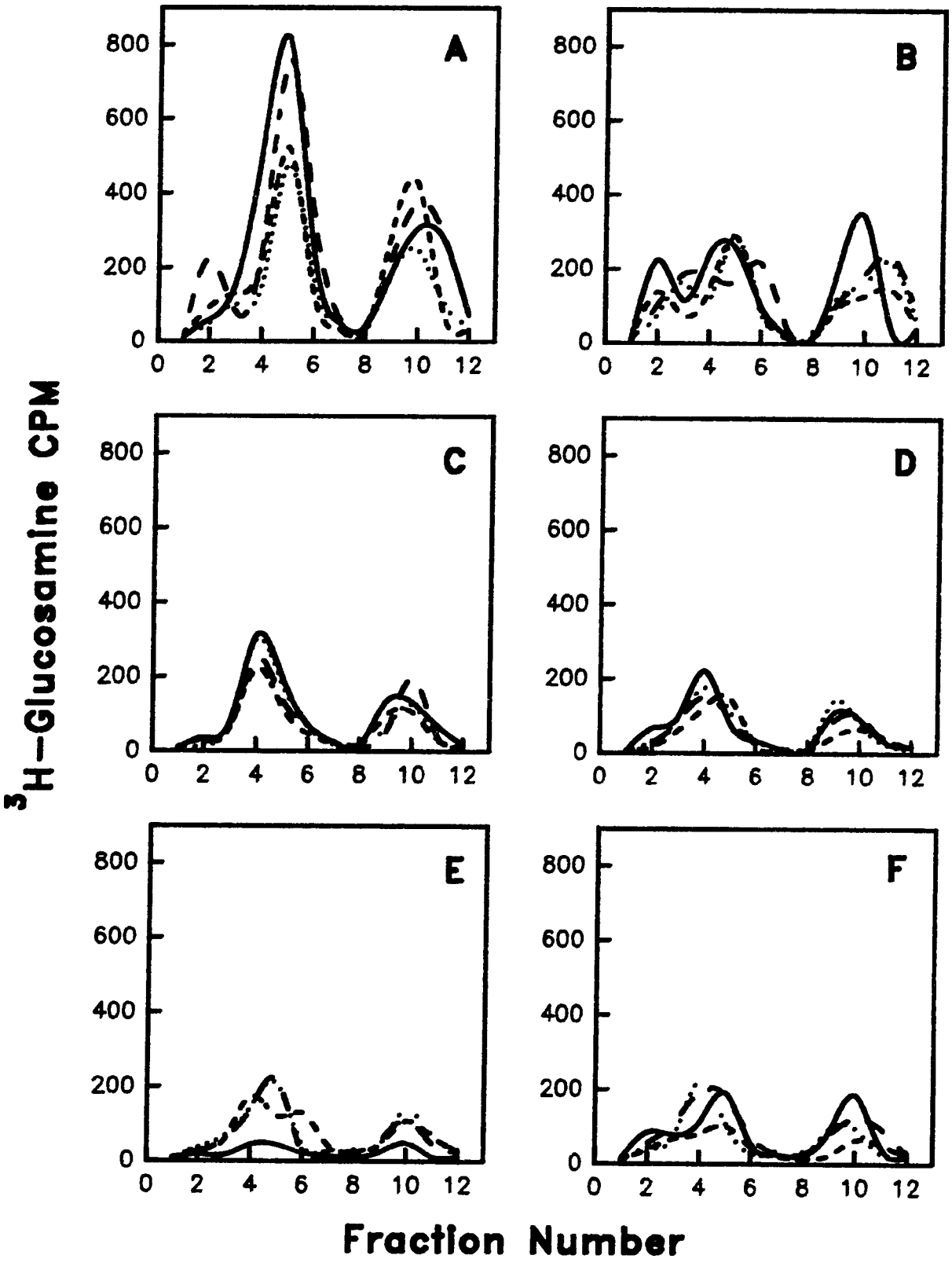

A
B
C
D
E
F
800
600
400
200
0
0 2 4 6 8 10 12
^{3}H–Glucosamine CPM
Fraction Number

Figure 15. The effect of cell density on the action of calcitriol in ROS 17/2.8 osteoblastlike cells. Three different cell densities are shown on the X axis for both calcitriol-treated and control cell cultures. Average [^{3}H]-glucosamine CPM are shown on the Y axis. The averages are taken from four replicates at each density for the peak eluting from the DEAE Sepharose columns at 0.38M NaCl (peak 2).

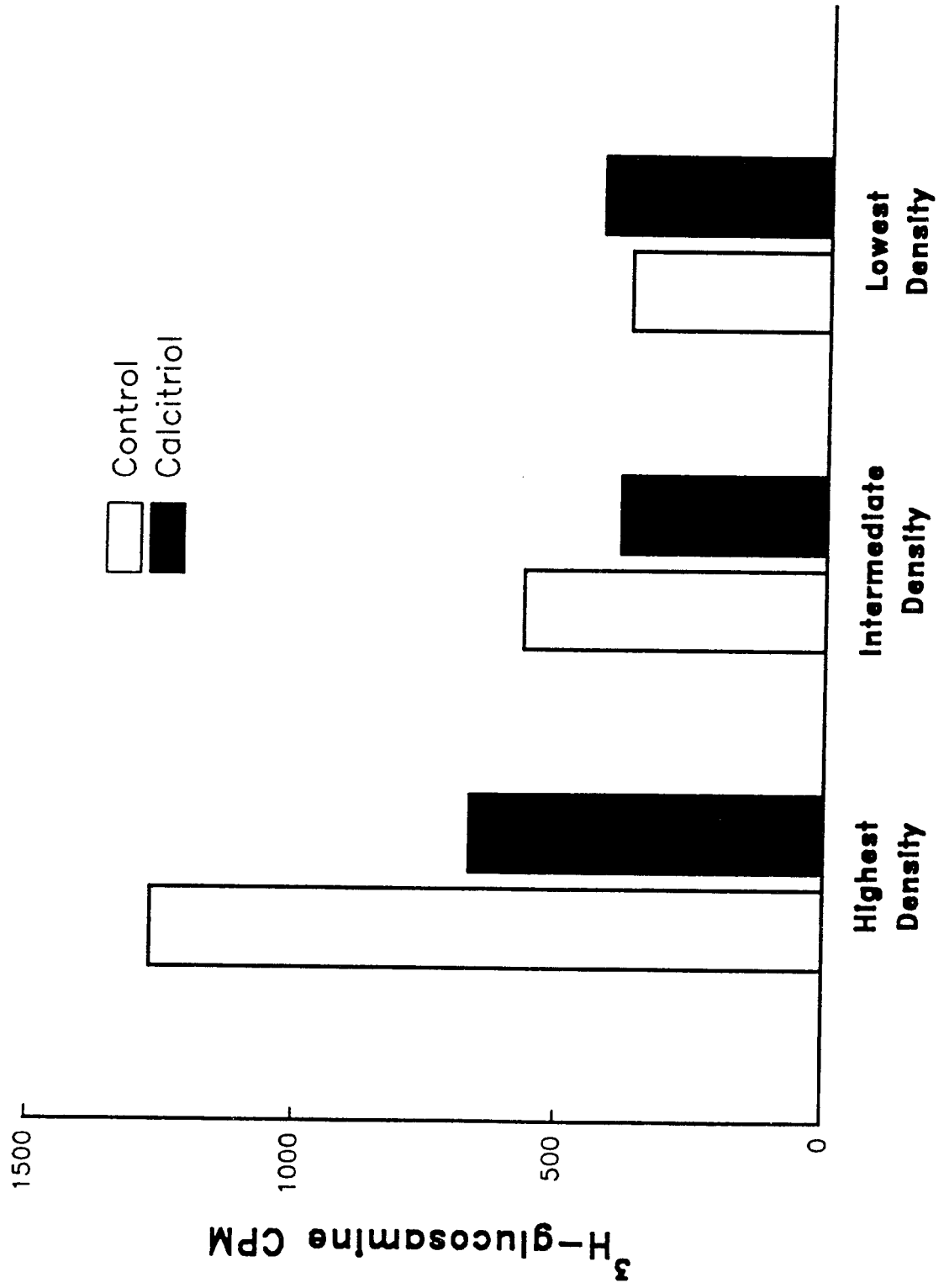
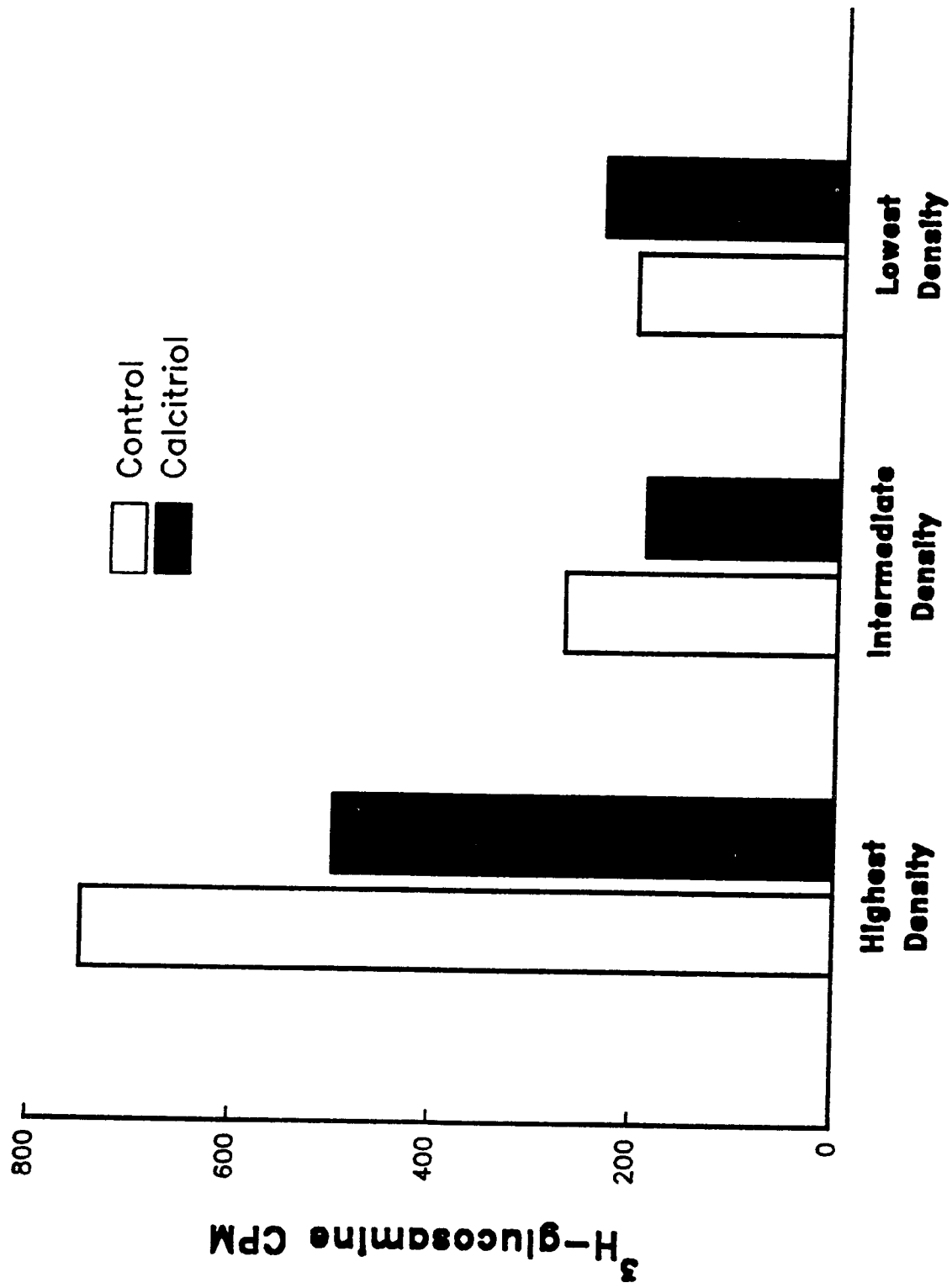

^{3}H–glucosamine CPM
1500
1000
500
0
Control
Calcitriol
Highest Density
Intermediate Density
Lowest Density

Figure 16. The effect of cell density on the action of calcitriol in ROS 17/2.8 osteoblastlike cells. Three different cell densities are shown on the X axis for both calcitriol-treated and control cultures. [^{3}H]-glucosamine CPM are on the Y axis. Average [3H]-glucosamine CPM are represented by the bars and are calculated from four replicates at each density for the peak eluting from the DEAE Sepharose columns at 0.67M NaCl.


Control
Calcitriol
800
600
400
200
0
^{3}H–glucosamine CPM
Highest Density
Intermediate Density
Lowest Density

and the calcitriol-treated samples in this high cell density group. Plates C and D represent the control and calcitriol-treated chromatograms from the intermediate density groups respectively. Plates E and F show replicate chromatograms from control and calcitriol-treated groups, respectively, that were treated when cells were at the lowest density. At the lowest density there was not a noticeable difference between treated and control groups in terms of [^{3}H]-glucosamine incorporation into the second and third peaks. Since multiple replicates were generated at each cell density we were able to analyze data by independent T tests on average incorporated radioactivity for each peak at each density, comparing controls versus calcitriol-treated samples. At the highest density, the difference in [^{3}H]-glucosamine incorporated into the third peak of control and calcitriol-treated samples was significant at the $p=0.05$ level. The amount of incorporated [^{3}H]-glucosamine eluting in the third peak when cells were treated at an intermediate density was significantly different between controls and treated cells. In both cases calcitriol caused a reduction in the incorporated [^{3}H]-glucosamine and therefore a decrease in GAG synthesis since the third peak corresponds to the proteoglycan fraction. At the lowest density there was no difference between controls and calcitriol treated samples when analyzing the CPMs in the last peak. The incorporated [^{3}H]-glucosamine showing up in the second peak eluting from this column was significantly different between

samples at the highest density ($p=0.05$) and at the intermediate density. Again, there was a reduction seen in the calcitriol-treated samples. There was not a difference at the lowest density. Figures 15 and 16 are graphic displays of these results showing the differences between control and calcitriol-treated samples at each density for a given peak. Regardless of treatment mode or cell density, the amount of radioactivity eluting in the third peak was between 30% and 38%. There was between 59% and 64% of the total recovered radioactivity eluting in the second peak regardless of cell density or treatment except for the calcitriol group at the highest density which had 51% of total radioactivity eluting at the second peak. It appears that the action of calcitriol may be dependent on cell density although we cannot say from this experiment if the effect of calcitriol on sulfate incorporation specifically would be cell density dependent. Calcitriol may be independently regulating both sulfate metabolism and glucosamine metabolism.

We wanted to find out if there was less total sulfate being transferred to the disaccharide units of the glycosaminoglycans on proteoglycans synthesized by ROS 17/2.8 cells treated with calcitriol. [^{3}H] glucosamine-labeled proteoglycans were Chondroitinase ABC digested and the constituent disaccharide units analyzed for total sulfate and sulfate placement using an Alltech Lichrosorb

amino column (250 mm x 4.6 mm). Further details can be found in the methods section.

Disaccharide units were separated on this column based on the amount of total sulfate present and also on whether the sulfate is present in the four or six position on the N-acetyl galactosamine. Labeling with [^{3}H]-glucosamine allowed us to observe the nonsulfated disaccharides. Nonsulfated, four-sulfated and six-sulfated disaccharide standards were first eluted from this column for calibration purposes. Reproducible baseline separation was achieved with the buffer system of 20% to 80% 0.8M phosphate in 20 minutes (see figure 19). Figure 17 shows the results of the first experiment with ROS 17/2.8 media proteoglycans treated with or without calcitriol. The calcitriol-treated sample had a greatly reduced amount of four-sulfated disaccharide as compared to the control sample. In addition, a later eluting peak which we suspect as being sulfated at both the four and six positions was also reduced in the calcitriol treated sample. We repeated this experiment and essentially found identical results (see figure 18). Tables 3 and 4 display these data differently, as percent total radioactivity recovered per peak. There was a shift of labeled material in the calcitriol treated samples from the four-sulfated disaccharide to the unsulfated disaccharide. The four-sulfated peak was reduced by about 10% in calcitriol samples while the unsulfated peak was increased by that amount.

Figure 17. Analysis of [^{3}H]-glucosamine labeled disaccharides obtained by chondroitinase ABC digestion of ROS 17/2.8 proteoglycans treated with and without calcitriol. Unsulfated and sulfated disaccharides were eluted from a Licrosorb Amino column with a continuous gradient of 0.16 to 0.64M phosphate. Fraction number vs CPM per fraction are shown on the axes.

Di-0S - peak corresponding to unsulfated material
Di-6S - peak corresponding to six-sulfated disaccharide
Di-4S - peak corresponding to four-sulfated disaccharide
Di-diS$_D$ - peak corresponding to disaccharide sulfated at the four and six positions
——— Elution profile of material from control cultures
- - - Elution profile of material from calcitriol-treated cultures

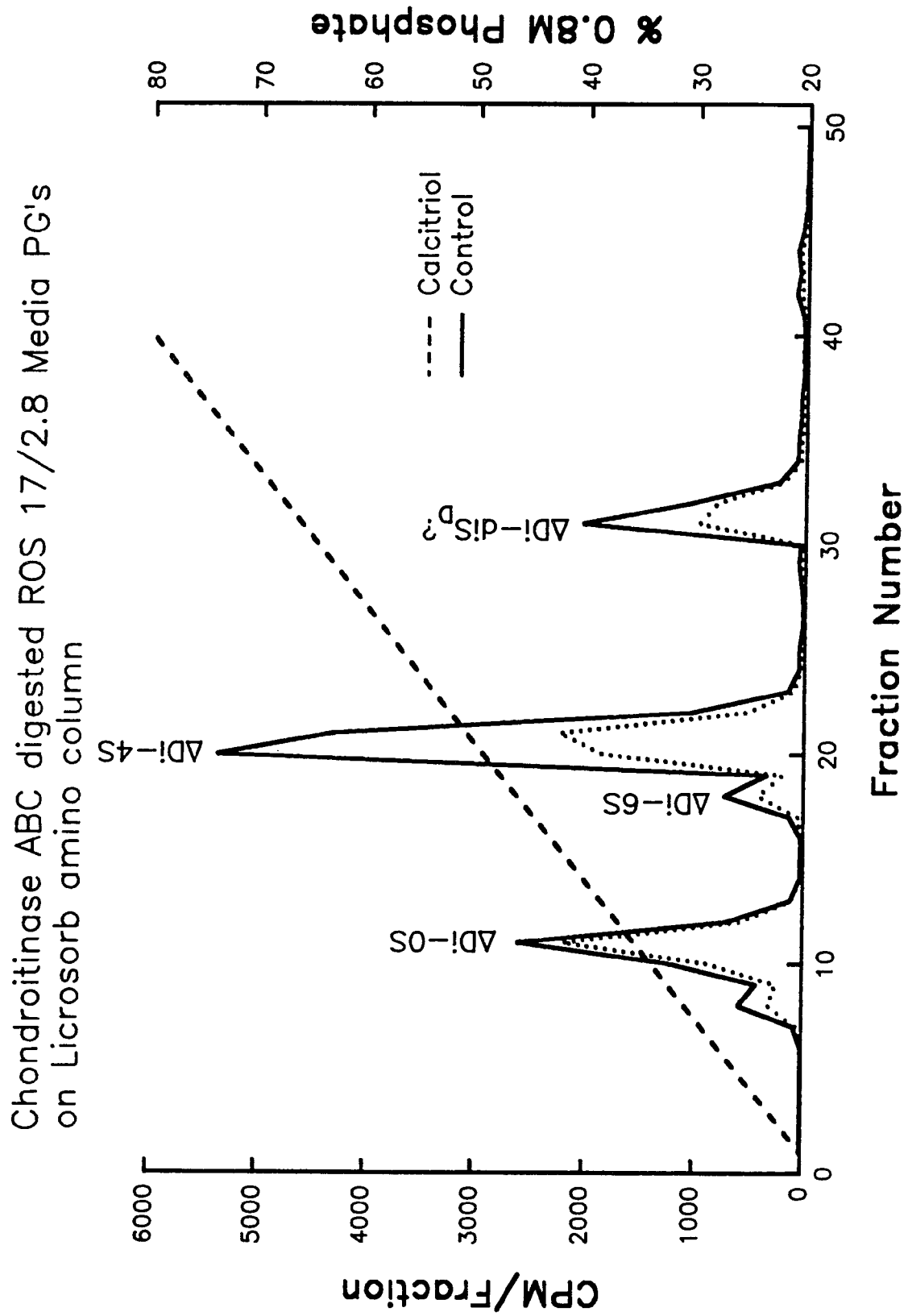

Chondroitinase ABC digested ROS 17/2.8 Media PG's
on Licrosorb amino column
CPM/Fraction
% 0.8M Phosphate
Fraction Number
Calcitriol
Control
ΔDi-0S
ΔDi-6S
ΔDi-4S
ΔDi-diS$_D$?

Figure 18. Analysis of [^{3}H]-glucosamine labeled disaccharides obtained by chondroitinase ABC digestion of ROS 17/2.8 proteoglycans treated with and without calcitriol. Unsulfated and sulfated disaccharides were eluted from a Licrosorb Amino column with a continuous gradient of 0.16 to 0.64M phosphate. Fraction number vs CPM per fraction are shown on the axes.

Di-0S - peak corresponding to unsulfated material
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Di-diS_D - peak corresponding to disaccharide sulfated at the four and six positions
——— Elution profile of material from control cultures
- - - Elution profile of material from calcitriol-treated cultures

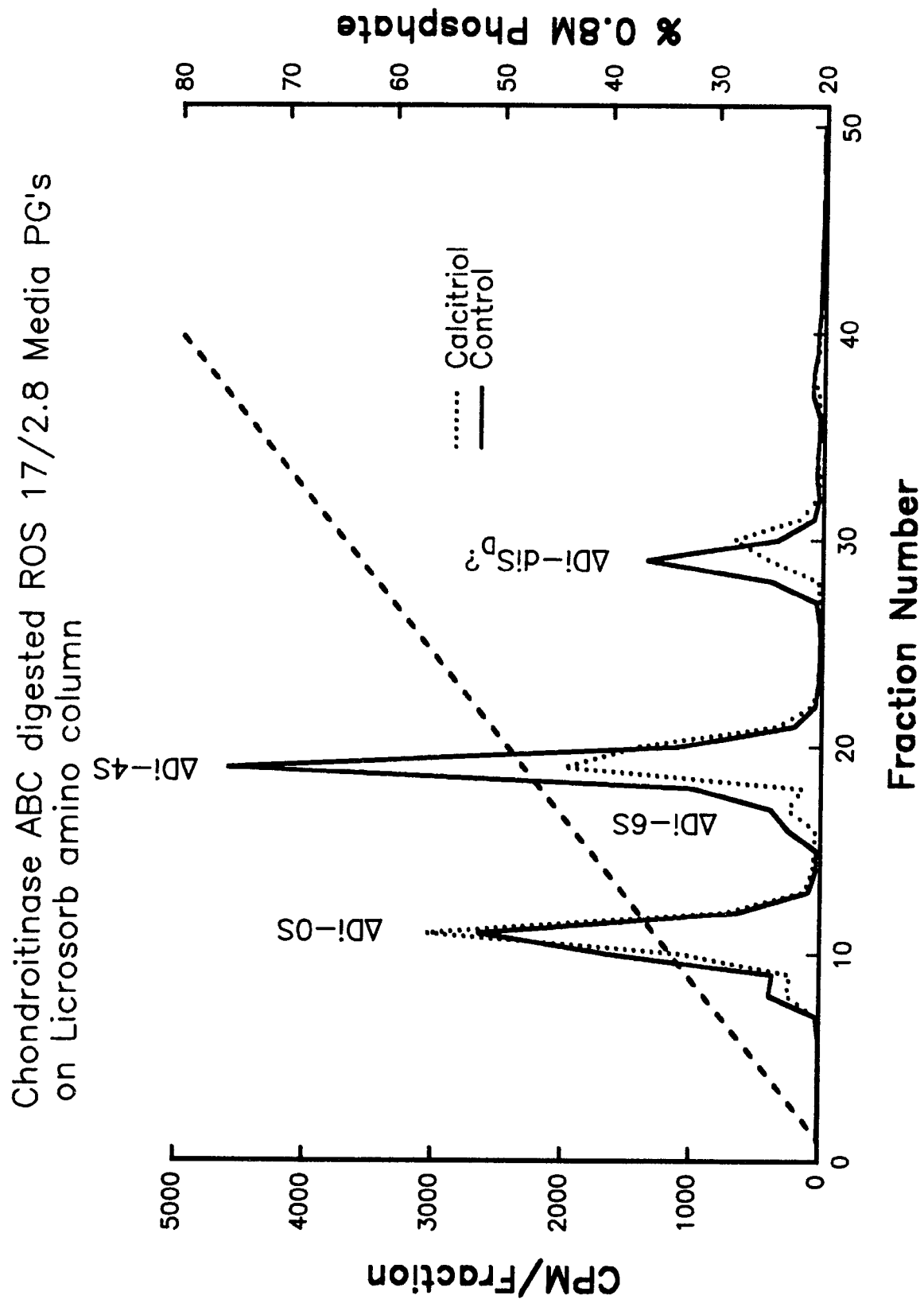
Chondroitinase ABC digested ROS 17/2.8 Media PG's
on Licrosorb amino column
CPM/Fraction
% 0.8M Phosphate
Fraction Number
Calcitriol
Control
ΔDi-0S
ΔDi-4S
ΔDi-6S
ΔDi-diS$_D$?
0
1000
2000
3000
4000
5000
20
30
40
50
60
70
80
10
20
30
40
50

Figure 19. Licrosorb Amino column analysis of unsulfated and sulfated disaccharide standards. Eluted with a continuous phosphate gradient of 0.16M to 0.64M. X axis - real time in minutes. Retention times are shown by each peak. Peaks are identified as follows:

Di0S - Unsulfated disaccharide standard
Di6S - six-sulfated disaccharide standard
Di4S - four-sulfated disaccharide standard
$DidiS_D$ - four and six-sulfated disaccharide standard

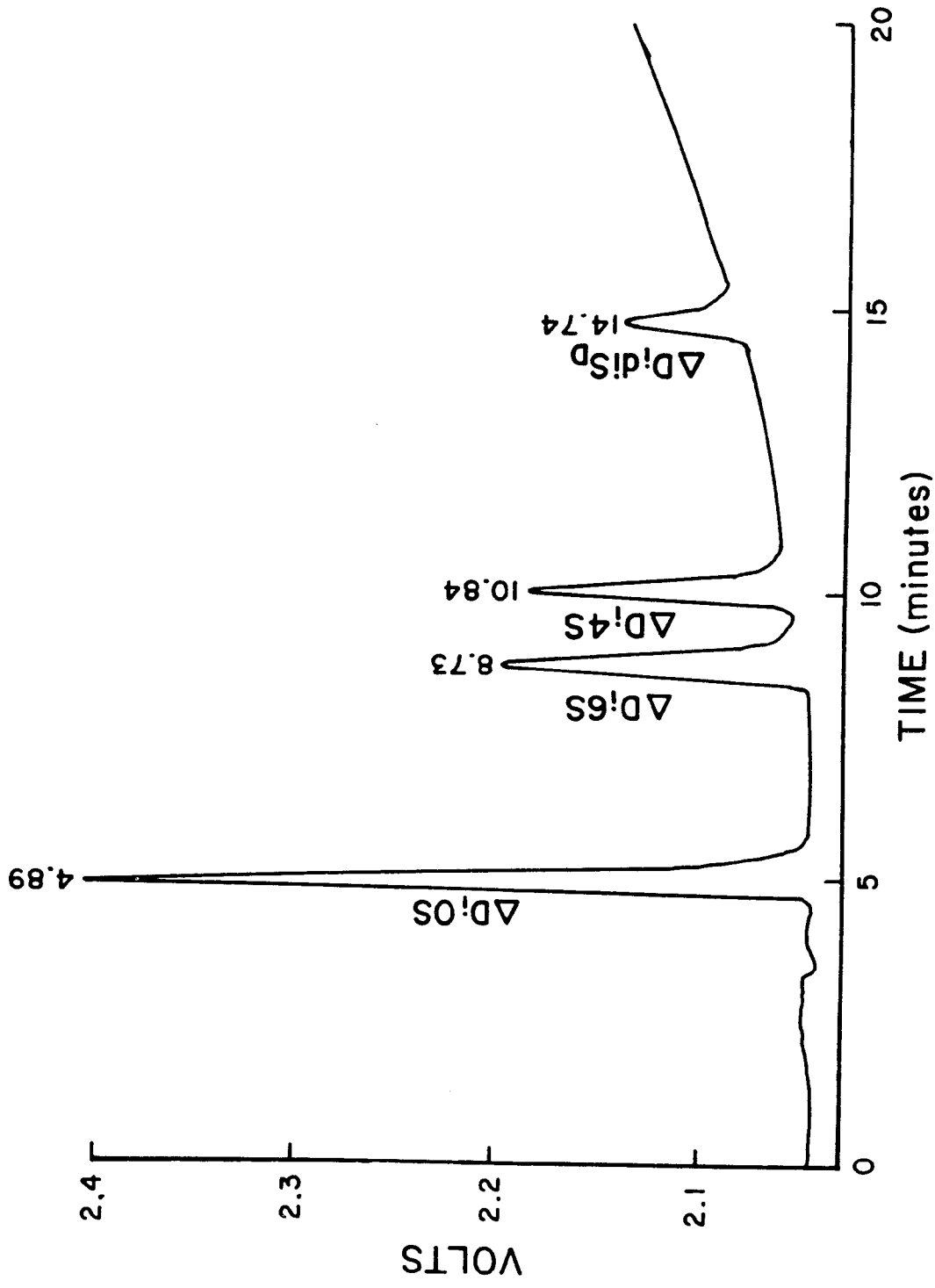
VOLTS
2.4
2.3
2.2
2.1
0
5
10
15
20
TIME (minutes)
ΔD;0S
4.89
ΔD;6S
8.73
ΔD;4S
10.84
ΔD;diS$_D$
14.74

Table 3. Table of data from figure 17. The five major peaks eluting from the Licrosorb Amino column are identified by peak number and by corresponding fraction numbers as shown in columns one and two. The combined [^{3}H]-glucosamine CPM for these fractions are shown in the third column. The fourth column shows the percent of total radioactivity recovered per peak.

Control cells

Peak #	Fractions	CPM	% of total recovered
1	7-9	1166	5.2
2	10-13	4820	21.6
3	17-19	1278	5.7
4	20-23	11207	50.3
5	29-35	3802	17.1
Total		22273	99.9

Calcitriol treated cells

Peak #	Fractions	CPM	% of total recovered
1	7-9	658	5.4
2	10-13	3883	31.9
3	17-19	705	5.9
4	20-23	4858	39.9
5	31-33	2068	17.0
Total		11118	100.0

Table 4. Table of data from figure 18. The five major peaks eluting from the Licrosorb Amino column are identified by peak number and by corresponding fraction numbers as shown in columns one and two. The combined [^{3}H]-glucosamine CPM for these fractions are shown in the third column. The fourth column shows the percent of total radioactivity recovered per peak.

Control cells

Peak #	Fractions	CPM	% of total recovered
1	7-9	794	5.0
2	10-13	5039	32.1
3	16-17	651	4.1
4	18-21	6908	44.1
5	27-32	2262	14.4
Total		15654	99.6

Calcitriol treated cells

Peak #	Fractions	CPM	% of total recovered
1	7-9	513	4.6
2	10-13	4991	44.9
3	16-18	403	3.9
4	19-22	3833	34.4
5	29-32	1338	12.0
Total		11118	99.8

Thus, calcitriol causes a specific reduction in the amount of four-sulfated glycosaminoglycan in ROS 17/2.8 cell proteoglycans. Whether there is direct regulation of GAG synthesis or an indirect effect because of core protein synthesis remains to be determined.

DISCUSSION

The goal of specific aim 1 was to verify that the [^{35}S]-sulfate labeled macromolecules were proteoglycans. Since the majority of the media-derived macromolecules were digested with Chondroitinase ABC, we were able to conclude that they were proteoglycan. The cell layer derived proteoglycans proved to be an exception in that they were not easily digested with chondroitinase ABC and the recovery from the P-30 desalting columns was less than 20%. Because of this we decided to observe the cell layer labeled macromolecules by SDS-PAGE fluorography allowing us to judge whether or not these molecules were proteoglycans, depending on the appearance. We did not expect to see a large proportion of [^{35}S]-sulfate label in any other macromolecules since there are no other macromolecules sulfated to the extent as are proteoglycans.

It is difficult to say why we had such poor recovery following the digestion with chondroitinase ABC for the cell layer fraction. It has been reported that small amounts of sample, such as those used from radiolabeling experiments, often give poor recovery from size exclusion columns and that carrier, unlabeled proteoglycan sufficient to yield 1-2 mg/mL sample concentration will result in better recovery (Hascall and Kimura 1982)

We did not add carrier proteoglycan so perhaps this was a problem. Also, for these size exclusion runs, both before and following digestion, the running buffer was NH_4HCO_3 instead of 4 M guanidine which may make a difference. Guanidine, a chaotropic agent which disrupts ionic and hydrophilic bonds, is used to dissociate aggregating proteoglycans. The bone proteoglycans are not of the aggregating variety so chromatography with a nondissociating running buffer should work. During the second step we were chromatographing disaccharide units and not intact proteoglycans so again the use of a nondissociating buffer should not be a problem. In subsequent experiments we found NH4HCO3 to be a suitable buffer for eluting proteoglycans from P-30 gel columns. The inclusion of a detergent also minimizes loss of material on a column but including detergent prior to lyophilization was not practical. These samples were lyophilized before the digestion step to minimize volumes and so samples could be redissolved in the appropriate buffer for chondroitinase digestion. In addition, the lyophilized samples were easier to process for fluorography. Some type of agent capable of disrupting hydrophobic bonding may have been needed in the running buffer because the cell layer contains the lipid-rich cell membranes within which some proteoglycans may be bound. While these proteoglycans would have been released during the harvest extraction with 4 M guanidine, maybe they later

formed aggregates with cell membrane debris and were not adequately digested or were rendered incapable of entering the size-exclusion gel matrix. This would explain the apparent loss of radioactivity and poor recovery following chondroitinase ABC digestion. We could have sampled the top cm of gel to find out if radiolabel was located there and concluded that radiolabeled molecules did not enter the gel.

The next set of experiments dealt with dose-response, length of treatment and cellular viability. We observed a clear dose-response in our initial experiment with five different concentrations of calcitriol which spanned the physiological range. These results also indicated that cells were exposed to the hormone for a sufficient period of time for a response to be generated. We refined the dose-response by reducing the labeling period to the last 6 h of calcitriol treatment. In all experiments, treatment with calcitriol was for at least 24 h, and was usually 48 h. While some hormonal effects can be observed in a matter of minutes, genomic effects often are not seen for hours or days. Calcitriol's effect on [^{35}S]-sulfate incorporation into macromolecules synthesized by osteoblastlike MC3T3-E1 cells was reported by another group during the course of this study (Takeuchi 1989). This group also found that 24 to 48 h of treatment were required for the effect of calcitriol to be seen.

Evaluation of cell viability included monitoring the effects of calcitriol treatment on cell proliferation and assessing the incorporation of [^{3}H]-leucine into media and cell layer-derived macromolecules. Results from the first dose-response experiment suggested that calcitriol had a proliferative effect on ROS 17/2.8 cells, but later data proved that this was not the case. Because of the random fashion in which differences in mean cell numbers occurred, these differences were most likely due to systematic or random error, both being errors of measurement. This was quite possible considering the large number of replicates per experiment and the fact that the techniques used for cell counting involved repeated experimental manipulation.

Overall, the cell number data suggest that calcitriol does not alter cell proliferation in a detrimental manner. Calcitriol was reported to inhibit human osteoblast proliferation and human and articular cartilage cell proliferation, yet increase cell proliferation in perosteal cells and mesenchyme-derived chondrocytes (Schwartz 1989). The investigators found that the response to calcitriol was dependent on cell type and degree of cell differentiation or maturation. Another group reported that calcitriol did not alter the total protein or DNA content of MC3T3 osteoblastic cells, if the hormone was applied during the growth phase, but that it might stimulate cell proliferation when applied during the early phase of culture (Kurihara 1986). We

applied calcitriol during the late growth phase, so in concurrence with the results of Kurihara, we would not expect to see alterations in cell number or protein synthesis due to calcitriol treatment. In labeling ROS 17/2.8 osteoblastic cells with [^{3}H]-leucine we found no differences in total protein synthesized between calcitriol treated cells and controls. Differences that did occur between mean incorporated [^{3}H]-leucine did not follow the dose response pattern expected if these differences were due to calcitriol. Instead they occurred randomly leading us to conclude that calcitriol did not diminish overall protein synthesis. There have been numerous reports of calcitriol stimulating the synthesis of bone extracellular matrix proteins such as osteocalcin, osteopontin, and matrix Gla protein but, since these matrix proteins comprise a relatively minor proportion of total proteins, it is doubtful we would see an increase in total protein synthesis based on synthesis of these alone. Others have found essentially similar results as ours when labeling calcitriol treated ROS 17/2.8 cells with [^{35}S]-methionine and observing total protein synthesis by two dimensional SDS-PAGE fluorography. Murray et al. (1990) saw no alteration of total protein synthesis in the ROS cell lines exposed to 10nM calcitriol for 72 h. This group did find however that specific proteins, comprising a minor proportion of the total, were induced by calcitriol. Our results and the

results of others demonstrate that calcitriol does not cause a deleterious effect on the ROS 17/2.8 cells or their metabolism. Thus, the reduction in [^{35}S]-sulfate incorporation was specific and not part of a generalized response.

By doing several dose-response experiments, we showed that the effect of calcitriol on sulfate reduction is reproducible. Another dose-response experiment (data not shown) was done 2 yr after the initial experiments and verified that the reduction in [^{35}S]-sulfate incorporation still occurred.

Once we established that the reduction in [^{35}S]-sulfate incorporation was dose dependent and was not the result of a widespread toxic cellular event, we outlined several specific aims that would enable us to understand more about the nature of calcitriol's action. In consideration of specific aim 2b we developed the hypothesis that the effect of calcitriol was metabolite specific. The results, as detailed in the previous section, proved this hypothesis incorrect. We found that both 1, 25 dihydroxycholecalciferol and 24, 25 dihydroxycholecalciferol caused less [^{35}S]-sulfate incorporation into macromolecules synthesized by ROS 17/2.8 cells. Although 24,25 $(OH)_2$ D_3 has been reported to cause a variety of changes in cartilage cell metabolism, it was somewhat surprising to find an effect in osteoblast-like cells. In chondrocytes, this metabolite stimulated [^{3}H]-

uridine incorporation at 10^{-8} M and 10^{-9} M (Schwartz et al. 1989) and [^{35}S]-sulfate incorporation into proteoglycans (Corvall et al.1978). More recently, Schwartz et al. found that resting zone chondrocytes respond preferentially to 24,25 $(OH)_2$ D_3 by increasing alkaline phosphatase activity and the production of noncollagenous proteins and by decreasing phospholipase A2 activity (Schwartz et al.1991). Growth zone chondrocytes responded to 1,25 $(OH)_2$ D_3 primarily by increasing alkaline phosphatase and phospholipase-A2 activities and increasing collagen production. 1,25,$(OH)_2$ D_3 activity is receptor-mediated so one would expect 24,25 $(OH)_2$ D_3 to function via a receptor also and that target tissues would contain these receptors. The receptor for the 1,25 dihydroxylated metabolite does bind the 24,25 metabolite although only 4% as well. Separate receptors for the 24,25 metabolite were found in developing endochondral bone, growth plate chondrocytes and chick embryo limb bud mesenchymal cells (Norman et al.1982). Since a separate receptor exists for this metabolite, it may have a separate function. Takeuchi et al. (1989) did study the effect of 24,25 $(OH)_2$ D_3 on MC3T3-E1 cells and found it caused little difference in the incorporation of [^{35}S]-sulfate and [^{3}H]-glucosamine into macromolecules synthesized by these cells. However, when examining the data from this group, I noticed there was a significant reduction in the incorporation of both precursors when treating with the

24,25 metabolite at 10^{-7} M. In addition, there was a dose-dependent, yet, statistically insignificant trend toward decreased glucosamine incorporation at doses ranging from 10^{-12} M to 10^{-8} M. In a review, Norman mentioned a possible cooperative action between calcitriol and the 24,25 metabolite in bone formation. A finding supportive of this idea was that 1,25 alone elevated serum levels of osteocalcin but bone concentrations of this matrix protein were increased only with coadministration of 1,25 and 24,25 (Weintroub et al. 1987b). The 24,25 dihydroxylated compound supposedly has been localized in developing bone and, while evidence for its having exclusive effects on bone are scarce, there may be enough to suggest that it has an independent role in the elaboration of new osseous tissue. We found 24,25 $(OH)_2$ D_3 to independently reduce the incorporation of [^{35}S]-sulfate into ROS 17/2.8 macromolecules. These cells go through several developmental stages in culture. Perhaps during one of these stages 24,25 $(OH)_2$ D_3 exerts an influence on these osteoblastlike cells as it seems to do during certain stages of bone development.

To accomplish specific aims 5 and 8 we employed several methods to see if calcitriol was altering the manner in which glycosaminoglycan chains were being synthesized. For specific aim 5, which was to determine if calcitriol altered the capacity of the ROS 17/2.8 cells to synthesize GAG chains, we utilized p-nitro phenyl ß-D xyloside, a molecule

that is a substrate for galactosyl transferase-1 and thus act as an exogenous acceptor of glycosaminoglycan synthesis. Our results indicated that calcitriol was not inhibiting the capacity of the ROS 17/2.8 cells to synthesize GAGs, at least past the addition of the xylose residue to the protein core. The ability of cells treated with calcitriol to synthesize GAGs was no different from that of controls; both treated and untreated cells greatly increased the production of GAG molecules when xyloside was added to cultures, as was expected. Takeuchi et al. (1989) exposed MC3T3-E1 cells to calcitriol, labeled with [^{35}S]- sulfate, and added both cyclohexamide and xyloside to these cultures. A significant reduction in incorporated [^{35}S]- sulfate was seen in calcitriol treated cells as compared to controls even in those cultures with added xyloside. This cell line did not recover from the calcitriol-induced sulfate reduction when xyloside was added to cultures suggesting that calcitriol caused a lower capacity to synthesize GAGs. These results were not in agreement with ours. One explanation for this was the use of a different cell line although both are osteoblastlike. Neither cell line has had the proteoglycan population completely characterized, so it is possible that they secrete somewhat different proteoglycans that respond differently to calcitriol. Another explanation comes from the manner in which the experiments to test cellular GAG synthesis under the influence of calcitriol were performed.

We administered hormone when cells were approximately 70% confluent as opposed to Takeuchi's procedure of applying calcitriol when cells were already confluent. There can be altered responses dependent on cell density at treatment time as was found by Lars Uhlin-Hansen (1988). In this study on cell density dependent expression of chondroitin sulfate proteoglycans in cultured human monocytes, different responses to benzyl-ß-D-xyloside were noted depending on cell density and stage of differentiation. A tenfold increase in GAG synthesis with xyloside addition was seen in monocyte derived macrophages at high density while high density monocytes increased GAG synthesis only 1.6 times after xyloside treatment. Monocyte derived macrophages showed a dramatic drop in the response to xyloside as the cell density decreased. These results illustrate how cell density and cell maturation can affect the response to xylosides (Uhlin-Hansen and Kolset 1988).

Another important variation was that we administered the hormone for 48 h and then used only p-nitro phenyl ß-D xyloside on the control and treatment groups. Takeuchi et al. (1989) treated with hormone for a similar amount of time, but then applied both cycloheximide (100 ug/mL) and xyloside to control and treated cells. We noticed a dramatic increase in [^{35}S]-sulfate incorporation (three to four times the levels normally found in control and calcitriol treated cells) when xyloside was added to cultures. Takeuchi et al.

noted a reduction in [^{35}S]-sulfate to 4-5% of background levels when giving only cycloheximide to calcitriol-treated and control cells. When they added both cyclohexamide and xyloside to cultures, there was an increase in [^{35}S]-sulfate incorporation above that seen with cycloheximide alone but, in both calcitriol-treated and control cultures, the increase was only 30% that of the levels observed without xyloside and cycloheximide. In other words, neither control or calcitriol-treated cells fully recovered the ability to synthesize GAGs when cycloheximide and xyloside were added. The cycloheximide alone caused a reduction in [^{35}S]-sulfate that was not overcome by the addition of ß-D-xyloside. Perhaps cycloheximide affected the enzymes responsible for GAG synthesis. Nonetheless, the data of Takeuchi et al. do suggest that calcitriol may be limiting the ability of the MC3T3-E1 cells to produce GAGs, whereas our data do not. We evaluated the effect of the various concentrations of ß-D-xyloside on the rate of cell proliferation. We did not observe a decrease in cell number in this experiment probably because we exposed cells to xyloside for only 2 hours. Inhibition of cell proliferation was seen in cultured aortic smooth muscle cells given p-nitrophenyl ß-D-xyloside for 2 to 14 days at doses of 1.0 to 5.0 mM (Wight et al.1989).

To accomplish specific aim 4, we prepared partially purified [^{35}S]-labeled proteoglycans and resolved them on a

5-15% SDS-PAGE gradient gel which was processed for fluorography and then analyzed by densitometry. This method allowed us to obtain semiquantitative information on the relative abundance of the proteoglycan species. This analysis would tell us whether calcitriol affected all of the proteoglycan species or one in particular. Our hypothesis was that calcitriol would affect all of the proteoglycans synthesized by the osteoblast like cell line, ROS 17/2.8. If the proportions of PG species were affected by the hormone we could observe this by the differences in distribution between controls and calcitriol treated proteoglycans. There seemed to be slight differences in the distribution between the two samples as shown in figure 10. One can compare the four major peaks which resolved on the gel in terms of area percent. Bands A and C in the calcitriol-treated samples are about 5% less than in the control samples while band D was about 10% higher in the calcitriol-treated sample. These results may indicate that calcitriol affects particular proteoglycans and not others. In a review of bone biology Robey (1989) stated that calcitriol did not affect decorin but did decrease the synthesis of biglycan. Biglycan and decorin most likely exist in the population of proteoglycans synthesized by ROS 17/2.8 cells along with other proteoglycans secreted by this cell line. Since we have only partially characterized the ROS 17/2.8 PG population, we cannot say with certainty which

PGs make up the population. Our evidence here merely suggests that differential regulation may exist for the proteoglycans synthesized by ROS 17/2.8 cells.

Since it was possible that the calcitriol-induced reduction in [^{35}S]-sulfate incorporation could also be caused by an increase in degradation of labeled proteoglycans, rather than an decrease in synthesis, we assessed degradation rates of calcitriol-treated cells and control cells, as stated in specific aim 6. We partially purified proteoglycans from untreated, [^{35}S]-sulfate labeled ROS 17/2.8 cell cultures. These proteoglycans were then added to control and calcitriol-treated ROS 17/2.8 cell cultures and then degradation was monitored by size exclusion chromatography on columns of Sepharose CL6B eluted with 4 M GdnHCl/ 50mM Tris/HCl, pH 7.3. As explained by Roden (1980), if degradation occurs outside the cell, fragments consisting of oligo and polysaccharides with small peptides attached will result from the action of proteases and carbohydrate cleaving enzymes. These products are normally small enough to be passed in the urine so one would expect them to separate well from the intact proteoglycans. Heinegard stated that protein core cleavage occurred first during PG degradation then the GAGs with peptides attached were degraded in the lysosomes to monosaccharides, amino acids and sulfate (Heinegard and Paulsson 1984). We observed no difference in degradation rate between control and

calcitriol treated ROS 17/2.8 cells. There was no appreciable degradation of proteoglycans before 12 h of exposure to cells, and we found no autodegradation of proteoglycans. Following 12 h of exposure to cell cultures, [^{35}S]-sulfate labeled proteoglycan degradation products appeared at the V_t. The amount of degradation steadily increased with time, as expected. Takeuchi et al. (1989) reported increased degradative activity upon treatment of MC3T3-E1 cells with 10^{-8} M calcitriol. In their experiments they measured the rate of disappearance of [^{35}S]-sulfate labeled PGs from medium of treated and untreated cells. Additionally, they labeled treated and untreated cultures with [^{35}S]-sulfate for 16 h then studied the degradation rate of the labeled PGs during a 48 h chase. This group found that calcitriol stimulated the rate of cellular uptake of labeled PGs from medium and consequently increased the intracellular degradative process, as indicated by the increased rate of small molecule secretion back into the medium. This group stated that during the 48 h chase, they observed a difference in degradative rate with cell layer associated proteoglycans and not proteoglycans remaining in the media. It was not clear from their discussion, however, whether the large amount of cell associated proteoglycans included PGs taken up from the media, although one expects that they were. Takeuchi et al.(1989) also demonstrated a remarkable relocation of 70% of the exogenous PGs from the

media to the cell layer in calcitriol-treated cultures. In our study we measured degradation of exogenously added media-derived proteoglycans. This [^{35}S]-labeled material was later retrieved from both the media and cell layer fractions of ROS 17/2.8 cultures. We found very little radioactivity in the cell layer, most remaining in the media. This may have several explanations. The ROS 17/2.8 cells may have a low capacity for endocytosis of exogenous PGs. Since we added only media derived PGs to cell cultures, we were not observing degradation of cell layer molecules and if those PGs specifically were degraded faster due to calcitriol treatment we would not have seen this. Also we found almost no uptake of exogenous radiolabeled molecules from media. At 48 h the majority of label was still in the media, there was none in the cell layer, and the radioactivity in degradation products never exceeded 11% of the total. In contrast, results from the pulse chase experiment by Takeuchi et al. showed 70% of total exogenously added radioactivity eluting at the V_t after 48 h, while cell associated macromolecular radioactivity was approximately 20% of total and medium-derived macromolecular radioactivity was about 12% of total. In adding exogenous proteoglycans to MC3T3 cultures, Takeuchi et al.(1989) did not see quite as dramatic a response. The added PGs in the medium decreased by over 30% in calcitriol treated cultures and by over 20% in controls while small molecules in the

medium rose to 15% in control cultures and to 25% in the calcitriol treated cultures. These differences indicate that the ROS 17/2.8 cells do not take up exogenous proteoglycans from the medium to the extent as do the MC3T3 cells which may, in part, account for some of these discrepancies. Roden (1980) stated that an important factor in degradation of proteoglycans was the recognition and cellular uptake of the molecules. He referred to Truppe et al. (1977) who demonstrated the selectivity of various cell types in this regard. Another possible variation between these two experiments includes the degree of confluency at the time degradation was being measured. Takeuchi et al.(1989) did not indicate confluency during this time and in our experiments we treated cells with calcitriol at 70-80% confluence for 48 h after which we started monitoring degradation. Our cells were confluent during the time degradation was being assessed.

Calcitriol has been shown to stimulate degradation of growth cartilage proteoglycans (Uchida and Shimomura 1989). This was thought to occur via activation of macrophage derived degradative enzymes. The speculation was that calcitriol did not act directly but rather through an activating factor. The investigators felt this supported a role for calcitriol in promoting calcification if indeed proteoglycans suppressed mineral acquisition. If proteoglycans synthesized by osteoblasts were also degraded in

part from macrophage or osteoclast enzymes then we would need to use macrophage conditioned media in order to see increased degradation. This does not seem likely as Takeuchi did not use conditioned media. While our results indicate no increase in degradation of proteoglycans due to calcitriol treatment, we cannot ignore the fact that they do not agree with the findings of others most notably Takeuchi.

In the results section we discussed our attempts to separate the core proteins from the various PG species, a goal set forth in specific aim 7. We were not successful in separating these core proteins in spite of trying several methods including immunoprecipitation and HPLC. A major problem with the HPLC technique seemed to be the poor recovery from columns which we could not explain. Also we did not have good results with the antibodies we used for immunoprecipitation. The antibodies we received from several outside labs were against rat, human and bovine PGs and had not been previously used with ROS 17/2.8 cell PGs. Using two different protocols we tried screening these antibodies to determine if there was activity against the ROS 17/2.8 cell PGs and we found no evidence of precipitation using these antibodies. One can conclude that either the antibodies did not recognize the ROS 17/2.8 proteoglycans or that we needed to develop another protocol.

Specific aim 8a was to determine if the GAG size of calcitriol-treated proteoglycans was less than that of

controls. We did not find any difference in the size of GAG chains liberated by papain digestion from proteoglycans synthesized by calcitriol treated or control ROS 17/2.8 cell cultures. Furthermore, the fluorogram shown in figure 10 provides evidence that the proteoglycans in calcitriol-treated and control cultures are approximately the same size. Takeuchi et al.(1989) also found that calcitriol did not alter the molecular size of GAG chains elaborated by MC3T3 cells. Calcitriol-deficient hatchling chicks synthesize proteoglycans with smaller chondroitin sulfate chains in the growth cartilage indicating that in the deficient state reduced GAG size may be present (Carrino et al.1989). We have not found evidence of the calcitriol-replete state being associated with diminished GAG size.

We tested whether or not calcitriol reduced the incorporation of [^{3}H]-glucosamine into both proteoglycans and hyaluronic acid. The hypotheses we developed were that calcitriol reduced the incorporation of [^{3}H]-glucosamine into macromolecules synthesized by ROS 17/2.8 cells and that the action of calcitriol was cell density dependent.

Calcitriol caused a reduction in [^{3}H]-glucosamine to 66% the level of controls in proteoglycans synthesized by ROS 17/2.8 cells. This was seen when the ROS 17/2.8 cells were treated with calcitriol when they were approximately 80% confluent. When hormone treatment commenced when cells were at the intermediate density (approximately 60%

confluent) the decrease in incorporated precursor was 71% that of controls. Low density cell cultures treated with calcitriol did not show a difference in the amount of [^{3}H]-glucosamine incorporated into proteoglycans compared to controls. The incorporation of [^{3}H]-glucosamine into hyaluronic acid was reduced by 52% in the high density cultures treated with calcitriol and by 68% in intermediate density, calcitriol-treated cultures. Low density ROS 17/2.8 cultures treated with calcitriol were not different from controls in terms of [^{3}H]-glucosamine incorporated into hyaluronic acid. There was appreciable synthesis of hyaluronic acid and proteoglycans at the low cell density but it was less than that seen at the higher cell densities. One group has shown chondroitin sulfate proteoglycan expression to be cell density dependent although they found more [^{35}S]-sulfate incorporation at the low cell density in cultures of monocytes and monocyte-derived macrophages. This group thought there was a process of down regulation operative in the high cell density cultures (Uhlin-Hansen and Kolset 1988). Others have found calcitriol to decrease the incorporation of [^{3}H]-glucosamine into macromolecules. Takeuchi et al. (1989) reported that 10^{-8} M calcitriol dropped the incorporation of [^{3}H]-glucosamine in hyaluronic acid synthesized by MC3T3 cells to 70% that seen in controls. Additionally they observed decreased [^{3}H] label in the proteoglycans synthesized by these cells. This group did

not test whether the effects of calcitriol were cell density dependent. We routinely administered calcitriol when cells were 70% confluent which corresponds to the intermediate cell density in this experiment. This allowed us to obtain consistent data since our evidence suggests the action of calcitriol to be cell density dependent. Our finding that calcitriol reduces the incorporation of [^{3}H]-glucosamine into proteoglycans indicates that there may be fewer proteoglycans and/or GAGs synthesized in ROS 17/2.8 cells treated with calcitriol.

The final set of experiments were designed to fulfill specific aim 8b. Our hypothesis was that calcitriol reduced the degree of sulfation of glycosaminoglycan chains on proteoglycans synthesized by ROS 17/2.8 cells.

In two separate experiments we found that calcitriol specifically reduced the amount of four-sulfated disaccharides obtained from chondroitinase ABC digestion of ROS 17/2.8 proteoglycans. The six-sulfated and bi-sulfated disaccharides were similar quantitatively between control and calcitriol- treated cell cultures. There can be several explanations for these results. One is that the 4-sulfotransferase may be specifically regulated by calcitriol, either by a direct effect on enzyme activity or indirectly perhaps by regulating the intracellular concentration of divalent cations. Delfert and Conrad (1985) have shown that the ratio of four sulfation to six sulfation

in PGs synthesized by chick embryo chondrocytes changed depending on stage of differentiation or growth conditions. They postulated that this suggests physiological significance for alteration of sulfation ratios. This group also found that 4-and 6-sulfotransferase activities were differentially altered by the concentration of divalent metal ions, most notably Mg++ which was stimulatory. Fe++, Cu++ and Zn++ each had different effects on each of the sulfotransferases. In addition, reducing conditions of the sulfotransferase assay affected the enzymes. Dithiothreitol increased the activity of the 4- but not the 6-sulfotransferase. These results, along with ours, show that the 4-and 6-sulfotransferases can be regulated separately. If calcitriol altered any of these conditions in cell culture, it could indirectly affect the sulfotransferase activities. Another possible mechanism involves the fact that calcitriol has been shown to regulate polyamine metabolism in intestinal cells (Suda et al.1990). Polyamines are small polycationic molecules that are important in cell proliferation and differentiation. Reportedly calcitriol increases the activity of ornithine decarboxylase and spermidine/spermine N-acetyltransferase, which catalyze reactions leading to an increase in the polyamine, putricine. Interestingly, polyamines have been shown to alter the activities of chondroitin 4-and 6-sulfotransferase from chick embryo cartilage (Habuchi and Miyashita 1982).It

is noteworthy that these investigators found the 6-sulfotransferase activity stimulated to a greater degree than the 4-sulfotransferase activity by polyamines. Calcitriol could thus be regulating the activities of the ROS 17/2.8 chondroitin sulfotransferases through polyamine metabolism.

Our results suggest down-regulation of the 4-sulfotransferase activity occurs concurrent with reduced GAG synthesis, evidenced by lower [^{3}H]-glucosamine incorporation, in ROS 17/2.8 cells treated with calcitriol. However, the results shown in figure 11, from the experiment where cells were treated with ß-D Xyloside, provide evidence that this is not the case. We labeled with ^{35}S-sulfate and found sulfate incorporation in calcitriol-treated cultures to equal that of controls, when xyloside was provided as an exogenous acceptor of glycosaminoglycan synthesis. It is possible that in calcitriol-treated cells the 4-sulfotransferase activity is decreased and when ß-D-xyloside is added to cultures only the activity of the 6 sulfotransferase increases with added substrate while in control cells both enzymes increase activity when additional substrate is present. A future experiment would be to add ß-D-Xyloside to calcitriol-treated and control cell cultures, harvest and digest the proteoglycans with chondroitinase ABC and analyze the constituent disaccharides with respect to sulfate position. This would reveal whether activities of both

sulfotransferases were equally increased by the addition of xyloside. It is interesting to speculate as to whether the PG core protein itself might have a regulatory effect on the sulfotransferase activities and the aforementioned experiment would not test this possibility. PG core protein conformation or net charge could alter enzyme activity if, for example, the protein core were folded in such a manner that a GAG were sandwiched between two regions of protein thus altering the microenvironment around the GAG or causing limited access between enzyme and substrate. If absence of certain PG core proteins altered the microenvironment this could conceivably have a regulatory effect on the sulfotransferases. These ideas have yet to be tested. Sugahara et al. (1989) has shown that the polylysine segment of the c-Ki-ras 2 protein can activate serum glycosaminoglycan sulfotransferases, which demonstrates that these enzymes are affected by protein charge and pH.

Another explanation of our results is that calcitriol may be causing down-regulation of a specific proteoglycan core protein that attains highly 4-sulfated GAGs. 4-sulfated GAGs are the predominant species synthesized by the ROS 17/2.8 cells, as shown by analysis of unsaturated disaccharides from PGs harvested from control cells, so down regulation of one or several proteoglycans from these cells would result in fewer 4-sulfated disaccharides upon chondroitinase ABC digestion of a sample. We have shown that

the GAG size, relative PG mass and cellular capacity to produce and sulfate GAGs are not reduced by calcitriol, and we have shown that calcitriol does not increase proteoglycan degradation in these cells. Since [^{3}H]-glucosamine incorporation into the PG fraction is diminished by calcitriol, yet GAG size is not reduced by the hormone, there are fewer GAGs probably because there are fewer total proteoglycans synthesized. Consistent with its effects on other bone matrix proteins, calcitriol may be altering synthesis of proteoglycans by acting at the transcriptional level to regulate PG core protein synthesis. Another future area of research would involve using specific cDNAs to probe for PG core protein message as a means of knowing whether PG core protein synthesis was affected by calcitriol. There are cDNAs available for biglycan and decorin, both of which are synthesized by osteoblasts.

In conclusion, we have shown that calcitriol regulates proteoglycan metabolism in the osteoblastlike cells, ROS 17/2.8. Our results indicate that the dose-dependent reduction in [^{35}S]-sulfate incorporation could be due to diminished activity of chondroitin 4-sulfotransferase, or reduced synthesis of specific PG core proteins for PG assembly.

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