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ANALYSIS OF CRANIOFACIAL EXTRACELLULAR MATRIX PROTEINS DERIVED FROM THE RAT NASAL SEPTUM, MANDIBULAR CONDYLAR, AND SPHENOSYNCHONDROSAL CARTILAGES

by

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Submitted in partial fulfillment of the requirements for the

Degree of Master of Science in Dentistry

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January, 1996

ANALYSIS OF CRANIOFACIAL EXTRACELLULAR MATRIX PROTEINS DERIVED FROM THE RAT NASAL SEPTUM, MDIBULAR CONDYLAR, AND SPHENOSYNCHONDROSAL CARTILAGES

Abstract by

BASSAM A. SHAHEEN

The importance of recent facial growth biology is distinguished by concentrating on the role of major craniofacial cartilages in the final outcome of growth Previous studies have compared craniofacial cartilages, using transplantation studies and concluded dissimilarity exists among them However, the molecular basis for the suggested dissimilarity has never been subjected to experimental confirmation

The present study used one and two dimension polyacrylamide gels electrophoresis (SDS-PAGE) to determine the protein distribution among nasal septum, mandibular condylar, and sphenosynchondrosal cartilages. The craniofacial cartilages were obtained by careful dissection from nine to eleven day old rats and protein extraction with 4M Gu HCl, 0.1 sodium acetate buffer, pH 5.8 containing proteinase inhibitors. After lyophilization, the reconstituted fraction was subjected to electrophoresis on 3-16 % one and two dimensional gels containing 0 1 % SDS The gels were stained with modification of silver nitrate technique

and Coomassie blue. Differences in protein distribution and patterns among nasal septal, condylar and sphenosynchondrosal cartilages were recorded with some proteins of interest isolated for future study.

In summary, the overall goal of this study was geared toward attempts to understand the distribution of the proteins obtained from the rat nasal septum, condylar, and sphenosynchondrosal cartilages and to identify whether these proteins reacted with human proteoglycans from hip and knee using immunological techniques.

DEDICATED

TO

My parents to whom I owe everything.

My brothers and sisters.

Two special people Grace and Nergil.

My country.

ACKNOWLEDGEMENTS

The author wishes to extend his sincere appreciation to the following individuals: Dr Charles Malemud, Professor of Medicine and Anatomy, for his expert guidance in this study and for accepting the responsibility of acting as thesis advisor, and especially for allowing me to conduct this research in his laboratory.

Dr. Mark Hans, Graduate Program Director and Chairman of the Department of Orthodontics, for his guidance in the set-up and coordination of this project.

Dr. Douglas Amberman, for his dedicated help in bringing elegance and style to the manuscript.

My special appreciation to Robert Papay, for his numerous tips and hands-on help with running and trouble-shooting the SDS gels.

The Orthodontic residents, Clay Damon, Seema Dhingra, J. Kevin Holman, and Sharon Schmahl, with whom I have had the pleasure to spend the last two years.

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INTRODUCTION

Several investigators of craniofacial growth think that we are in the middle of conceptual revolution working at the cellular level that controls morphogenesis . The real question is how the cellular level (local growth) work at the tissue level.

Until recently, the interpretation of growth control theories was considered complete, but this is not true any more. We just started knowing what we did not understand at all, and the nature of the real problem is that we are trying to solve these problems. This itself is an outstanding revolution.

First of all, I would like to mention two concepts called remodelling and displacement process (Enlow 1975). Bone resorption and deposition are the processes of remodelling and the regulation of remodelling speed is affected by the soft tissue matrices housing the bone. In displacement, structural adjustment happened so that a functional fitting of all separate bones to each other and to their soft tissue is achieved.

Cartilage, in general, is the smooth wear-resistant material that forms the articulating surfaces while resisting large amount of stresses for long periods of time . These extraordinary capabilities are afforded by the composition and organization of the tissue. For example, studies have shown that the collagen network provides cartilage with its intrinsic tensile stiffness and strength while its proteoglycan content, gives it both static

and dynamic properties Proteoglycans provide the equilibrium bulk compressive stiffness for the tissue and as a result of the counterions with their associated high negative charge density, significant Donnan osmotic swelling pressure can be produced Schmidt (1985).

Other forms of the structure-function interplay associated with controlling cartilages conduct may not be so clear , or need more explanation , or additional research. However, these forms can give us more understanding into how collagen fibrils, extracellular matrices, and proteoglycans, as well as the interstitial water perform in situ and how the interaction between them affects the production of cartilage.

Brigham (1976) brought into light antigenic differences among nasal septal, condylar, and sphenosynchondosal cartilages. He assumed that these antigenic differences were utilized by the tissue to exclusively regulate facial growth. Two years later, Palmer (1987) showed histologic proof of cytopathic effects of antisera on the chondrocytes from the rat nasal septum. Hans (1981) injected polyclonal antisera against the nasal septum in young rats from birth to thirty days old and observed a shrinkage in the midfacial appearance. This study did not identify what proteins reacted with these polyclonal antisera or whether these antigens controlled midfacial growth.

Slodov (1991) found that extracellular matrix protein appeared to be most likely target for monospecific antibodies. Unique proteins were not found on the membranes of chondrocytes. However, there appeared to be a recognizable distinction in the one-dimension protein profiles among the nasal septal, condylar, and synchondrosal cartilages Nguyen (1992) examined the protein structure of nasal septum and mandibular condyle

using Hans' antisera to determine which proteins reacted with the anticartilage antisera.He also used monoclonal antibodies against known structural proteins of cartilage and found that the protein patterns of the nasal septum and mandibular condyle were grossly similar on one dimension gel electrophoresis. He reported that the antisera were reactive with the large and small proteoglycans, link proteins and dermatan sulfate proteoglycans, as well as yet other uncharacterized matrix proteins.

The goals of this study were to directly compare the protein patterns and the differences among nasal septal, mandibular condyle, and synchondrosal cartilages by using a two dimensional gel technique. Using the rat nasal septal, mandibular condyle, and synchondrosal cartilages, this study examined the matrix proteins of the three cartilages. In addition, the protein components of the extracellular matrix were separated, purified, and incubated with the proteoglycan from the human hip and knee and examined using a modification of Western blot technique to determine if the proteoglycans reacted with any nasal septal, mandibular condylar, and synchondrosal matrix proteins.

LITERATURE REVIEW

Facial growth and the role of cartilages in that process are just a few of the several disputed subjects in orthodontics. The nasal septum, mandibular condyle, and sphenosynchondrosis have been regarded for a long time as an area containing some kind of genetic blueprint that restrains and directs growth. There are several causes to controversies in understanding the exact role of the cartilage in facial growth. The main cause of such disputes stems from the fact that it is nearly impossible to assess quantity, quality, and location of facial growth in humans.

Embryonic Development of the Craniofacial Cartilages

The Nasal Septal Cartilage

Production of the nasal septal cartilage is the outcome of interaction of suitable mesoderm with olfactory epithelium. All nasal cartilages are derived from a single cartilaginous framework (Drumkeller1973) with the lateral nasal cartilages being continuation of the septal cartilage.

In the evolving septal cartilage, the speed of mitosis is unequal, and there are dissimilarities within the same species as well as between different species, the location of the nasal septum does not mean that is the site of primary growth; the information are adequately similar with the localization of loci of secondary compensatory growth. In this respect it was noted that chondrocytic hypertrophy and not mitosis, played the more

important role in elongation of the embryonic chick tibial cartilages.

Briefly, embryological information does not support the clinical theories of the role of the nasal septum . If the nasal septal cartilage does grow subordinarily and is equal to the earlier passive movement of the facial bones within the orofacial capsule, it is necessary to suggest a mechanism for such growth .

The Mandibular Condyle Cartilage

At about the fourth or the fifth week of human intrauterine development, the branchial arches are formed and the stomodeum is surrounded by five mesenchymal swellings which are part of what is called the first branchial arch.

Every branchial arch is formed by mesodermal tissue containing immigrated neural crest cells which have their origin in neural plate and give rise to the skeletal components of the face. The mesoderm of the branchial arches will form the musculature of face and neck. On the outside, the embryo is covered by ectoderm, on the inside, it is covered by epithelium of endodermal origin. Somewhat later, cartilage is formed in the first branchial arch . Underneath the future eye, the maxillary cartilage forms, and mandibular cartilage is called the Meckel's cartilage.

Between the third and the fourth month, further growth of the mandible is assured by the formation of three secondary cartilages structures: the condylar cartilage, the angular cartilage, and the symphyseal cartilage. These structures are called secondary to differentiate them from primary cartilage such as Meckel's cartilage and other cartilage structure appearing earlier in development. They also present a different histological

structure : the cells are larger and are surrounded by iess intercellular matrix.

During the twelfth week, the condylar cartilages develops by forming a carrot-shaped cartilage issued from mesenchymal condensation. During the fourteenth week, an endochondral ossification process starts at the condylar cartilage bone. By the twentieth week, the process is finished, and a relatively narrow zone of secondary cartilages remains to assist in the further growth of the condylar head, the condylar neck, and the whole ramus.

Returning to the twelfth week, we can see that the condylar head is formed by cartilaginous center, surrounded by mesenchymal tissue. The further interstitial and oppositional growth of this cartilage will produce the typical rounded form of the condyle covered by a layer of fibrous connective tissue, which is itself surrounded by undifferentiated mesenchyme.

Sphenocciptal Synchondrosis

The growth of the cranial base may be an important factor in facial development because its configuration directly effecting the disposition of the facial complex. There are four growth sites in the base of the skull, namely: (1) sphenooccipital synchondrosis, (2) intersphenoidal synchondrosis, (3)intra-occipital synchondrosis, and (4) sphenethmoidal synchondrosis.

These cartilages grow by an endochondral mechanism with the intersphenidal synchondrosis activity stopping at birth and the intra-occipital synchondrosis stopping at the age of three to five. Therefore, the two principal synchondrosis that contribute to the

growth of the cranial base are the spheno-occipital and the spheno-ethmoidal. The spheno-occipital synchondrosis, which stays open until approximately twenty years of age, is clearly the essential impetus to the enlargement of the cranial base. However, it is assumed that this synchondrosis may have behaved to modify the cranial base to the demands of the growing brain and upper respiratory tract. As such, it may represent a more passive mechanism of accommodation. The contribution of the spheno-ethmoidal synchondrosis have been variably assessed with some investigators suggesting closure at the age of seven, while others indicate closure by twenty-five years of age. Its major contribution may have been made by the time the first permanent molar erupts and the anterior cranial base is no longer increasing in size.

Activity at this synchondrosis is accompanied by corresponding remodelling changes which lead to a repositioning of the different parts of the growing bones and generalized cortical drift in an anterior direction . The frontal bone moves outwardly and the frontal sinus is created. As a result of the activity of the spheno-occipital synchondrosis and the continued forward movement of the frontal bone with the maxillary complex being carried upward and forward until the synchondrosis closes . Thus, the direction and rate of the sphenooccipital movement may affect the spatial position of the maxilla and the maxillary teeth with both direction and rate varying throughout the growth span and the general pattern among individuals. The direction and rate of growth can be forward or more vertical, depending on the individual while some midfaces may be deeper or taller than others.

Theories of Craniofacial Growth

Several hypotheses have been suggested to explain facial growth mechanisms. The following is a review of those hypotheses.

Genetic Control Theory

Until 1960, it was claimed that the facial growth was genetically predetermined. Corresponding to this theory, the sutures, the periosteum and the cartilaginous structures, replied to their genetic data to restrain the enlargement of the facial complex, and as a result established the size and shape of their structures.

Cartilage-controlled Growth Theory

This theory states that craniofacial growth is directed by growth of the cartilage: the nasal septum, the mandibular condyle, and the synchondrosis are growth centers and structural growth is adaptive; the growth of the cartilage pushes away the bone structures of the face and the sutures adopt by regional bone formation.

Moss Functional Matrix Theory

The basis of this theory is that the data controlling the size and form of a bone that does not exist in the cells of bone nor in the sutures. Growth of the bones rely completely on the soft tissue and the functioning spaces.

In other words, heredity has no direct effect on the size, shape, or position of the facial bone but rather that they are controlled by outside local influences or functional matrices. These matrices are; (1) perisotal functional matrices (2) capsular functional matrices.

Craniofacial Growth Control Theory

This theory contradicts the functional matrix theory especially when you take into

consideration clinical conditions like hydrocephaly, anencephaly (desmocranium), and craniostenosis (chondrocranium). According to Van Limbourgh (1972), chondrocranial growth is controlled by local epigenetic and environmental factors.

Compromise Theory

This theory combines the findings of Limbourgh (1972) and Moss (1962) in attempting to explain the growth and development of the middle face and the mandible. According to this theory , the maxillary and the mandibular growth are effected by local epigenetic and environmental factors, while at the same time the sphenooccipital synchondrosis exert some indirect effect on the mandible.

Servosystem Theory (Petrovic; 1972)

This theory states that the effect of somatomedin on the growth and development of the primary cartilages of the midface has the cybernetic form of a command, and this complex has a direct effect on the cell proliferation of the secondary cartilages.

In other words, the dispute between the ultimate locations of the dental arches helps according to Petrovic (1972) to serve as peripheral comparator. In the process of growth and development, the maxillary dental arch is in constant change, and the mandibular dental arch is following maxillary dental changes.

The Cells of Cartilages

Articular cartilage is an avascular and aneural containing connective tissue that forms the weight bearing surfaces of synovial joints. The tissue is composed of a highly organized extracellular matrix containing a population of highly specialized cells, the chondrocytes . The cellular composition of articular cartilage is made up of 75% water

and 25% matrix containing 40% collagen and 60% proteoglycans. Proteoglycans in cartilage and bone differ significantly in both size and components. The bone variety possesses a smaller core protein with one or two glycosaminoglycan chains. By contrast, the cartilage proteoglycan is heterogeneous with respect to both number and length of the glycosaminoglycan chains. The most abundant proteoglycan in hyaline cartilage is a high molecular weight aggregate species that contain both chondroitin sulfate and keratan sulfate chains and has an extended "bottle brush" structure in which the protein back-bone is densely substituted with carbohydrates chains.

Collagen Types (Piez, 1987)

The collagens are a heterogenous group of extracellular matrix proteins with a variety of molecular structures and interaction properties. However, they all contain molecules with one or more triple-helical, collagenous domains. In classifying collagens, it is convenient to divide them into two groups: the fibrillar collagens which form characteristic "quarterstaggered" fibrils and the nonfibrillar collagens which do not. Several minor types of collagen are known to exist in the bovine midface which combine with fibrillar collagen and although they constitute only a small percentage of the total content, these minor collagens may have important functions. It is known that collagen is important to many mechanical properties of the bovine cartilage. By forming a fibrous network, which restrains the swelling pressure of the closely packed proteoglycans within the matrix. This creates a resilient material with tensile and comprehensive strength. Collagen is known to exist in at least fourteen molecular types, each type differing in composition and properties. Approximately 80% of the collagen in bovine cartilage is

fibrillar in make up, however, the role of these different types of collagen in the bovine cartilage is unknown. Biochemistry gives little information about their location. By utilizing specific antisera, it is possible to determine where the various types are located within the tissue, and an indication of their functions.

Extracellular Matrix and Development

The molecular basis of development is one of the central problems of modern biology. The major phases of embryonic development include cell differentiation, formation of tissues from differentiated cells and finally, the controlled growth of tissues to generate the characteristic form of the developing embryo. How does the spherical fertilized egg differentiate and develop into the extraordinary human being with perfect bilateral symmetry? The progressive differentiation of specialized cells and their organization into tissues is accompanied by the prominent appearance of extracellular connective tissue matrix. In fact, this matrix functions as the "connective tissue" that binds together the different cell types in a complex tissue or organ.

An analysis of the mechanisms of differential gene expression during development will have important implications for developmental anomalies. While considerable attention has been focused on intracellular molecules involved in the transcription and translation of the genetic information, not much is known about the role of extracellular matrix thus setting stage for investigations of the developmental role of these constituents.

At the anatomical level, the organism can be characterized as a precisely organized array of cells, tissues and organs that varies little among the individual members of a given species. This precise and reproducible pattern of tissues and organs arises during periods

of embryonic and morphogenesis. One of the major problems of developmental biology is to provide a detailed account at cellular and molecular levels of the processes that establish and stabilize the final organization of the adult tissues.

It has long been presumed that adhesive interactions of cell with cell and cell with extracellular matrix play important role in tissue patterning. One of the useful approaches to investigate this presumption developed from the discovery that embryonic tissues could reestablish normal histotypic relationships when tissue fragments or cell reaggregates were maintained in organ culture. These studies have been expanded to a variety of organs, and have demonstrated that embryonic tissues have remarkable powers of self-assembly. Normal relations can be established when homogeneous tissue fragments are cultured in apposition or when random heterotypic aggregates of cells produced by reaggregation of mixed suspensions of dispersed cells are maintained for one or more days in organ culture. In the first situation, cells of one of the tissue fragments spreads over the surface of the partner fragment, modeling the spreading morphogenetic movements occurring in vivo. In the second situation, the different cell types present in the initial aggregate sort out into homogeneous tissue domains that are, themselves, arranged in a fashion reminiscent of that observed in vivo.

The cell types are combined in these two fashions in organ culture need to be from tissues that normally are in association in vivo. Even cells taken from remote sites in the embryo will show regular patterns of tissue spreading if just apposed as contacting tissue fragments with regular patterns of cell sorting if assembled into random reaggregates

Cell to cell and tissue to tissue interactions are known to be critical steps in the

differentiation of all vertebrate tissues. (Wozney 1988) and the other investigators showed that the major advances in the six years between 1977 and 1983 have been in characterizing and documenting the role of matrices in mediating tissue interactions during development. I examined the role played by matrices in the differentiation of that tissue and cartilage.

Hardingham (1983) and Couchman et al., (1984) consider the role of proteoglycans while von der Mark et al., (1984) consider the role played by the collagen. Chondrogenesis occurs in three different regions of the embryo (vertebrae, limb, and cranium) where cartilage differentiates from three different embryonic mesenchymes (sclertotomal, somatopleural, neural crest) under the influence of three different extracellular matrices (perinotochordal and perispinocaudal matrix, limb bud epithelium, pigmented retinal epithelium) (Solursh, 1984).

Matrix-Controlled Osteogenesis

The two systems of cranial and facial osteogenesis represent the most extensively studied examples of matrix-induced bone formation but even there, much more needs to be investigated. There are other systems where a matrix has been shown to induce bone directly without the intervention of a cartilaginous phase, and another where cartilage is induced first because bone is the end result with the matrix induces cartilage not bone. Osteogenesis then follows the pattern typical of endochondral ossification in the fetus, hypertrophy of the chondrocytes, calcification of the cartiginous matrix, invasion of cartilage by blood vessels and deposition of bone by local progenitor cells or by cells transported by the vascular system. The induction of cartilage by a diffusible glycoprotein

localized within demineralized bone or dentin matrix is a good example of such a system. It is very clear that this matrix product controls the differentiation of cartilage. How osteogenesis is controlled is less clear, although there is evidence that indicates that a factor from the cartilage matrix may be involved.

The initiation of osteogenesis in vitro from dissociated mesenchymal cells offers a good system for examining the role of matrix products, although the best bone formation is found in vascular sites rather than in vitro. One such system is osteogenesis in micromass cultures of limb mesenchyme, apparently independent of the cartilage which also forms as well (Osdoby and Caplan, 1979). These osteogenic foci contain bone-specific alkaline phosphatase (Osdoby and Caplan, 1981) and mineral. The microenvironment plays an important role in the differentiation of bone with high cell density (5 X 10^6 cells/ 35 mm culture dish) favors chondrogenesis, while lower cell density (2-2.5 X 10^6 cells) favors osteogenesis. This should prove to be an excellent system for analyzing matrices and osteogenic differentiation.

A number of methods are now available for isolating osteoblasts from the calvaria of new born mice so that they may be maintained in vitro or in diffusion chambers. These systems have the advantage of yielding homogenous cell populations but the disadvantage of isolating the osteoblasts from other cells (osteoclasts) and matrices with which they normally interact. Foetal calvarial cells cultured alone remained fibroblastic, whereas similar cells cultured on devitalized endosteal bone, differentiated into osteoblasts and chondroblasts. Calvarial cells from neonates are able to self-differentiate from calvaria of embryonic chicks have to be folded to bring the osteogenic cells into apposition to create

a microenvironment adequate to maintain osteogenesis in vitro Osteogenic stem cell can be isolated from bone marrow and either maintained in vitro or transplanted in diffusion chambers. As with the calvarial cells, matrix components are important, either for attachment and initiation and proliferation or for induction of stem cells. The primary challenges for the future in these systems are to identify the state of determination of the marrow stem cells (osteogenic, osteogenic and chondrogenic, osteogenic and fibroblastic; determined or inducible) and how matrices direct their differentiation.

Another important area for future research is the role of regulators of osteogenesis produced by osteoblasts themselves and deposited into their normal bony matrix. It has been known for some time that cartilage matrix contains such factors, some of which are normal structural constituents of the matrix. Similar components are now being isolated from bone, e.g., a 60-80,000 M.W. component in chick bone which specifically stimulates DNA synthesis in both osteoblasts and their precursors.

Clearly, we shall have to look at several matrices in the future, all of which play their role in the differentiation of bone. This is the osseous matrix which surrounds the osteoblasts and osteocytes, the microenvironment surrounding each bone and the tissues adjacent to bone or to osteogenic mesenchyme. Documenting their relative roles as controllers and maintainers of the expression of the "osteogenic" portions of the genome is an exciting prospect for future cell and developmental biology.

A major goal of students of morphogenesis is the establishment of explanation for specific morphogenetic events at cellular and molecular levels. The program of study described in this project represent an attempt to realize these goals for a model

morphogenetic system The analysis emphasizes the importance of extracellular matrix molecules, in the behavior of mesenchymal tissues. Whether the analysis can be generalized beyond the hard tissue remains a subject for further investigation.

Epithelial tissues are organized with extensive contact between cell surfaces whereas mesenchymal tissue is organized with the extracellular matrix forming the continuous phase with the cells embedded in it, often with little cell-cell surface contact. It is attractive to propose that the segregation behavior of epithelial is regulated by the integral membrane proteins involved in cell-cell adhesion, whereas that of mesenchymal tissue is not regulated.

STATEMENT OF THESIS

It was very well-known and accepted by many researchers that the craniofacial cartilages play as a "growth center." Recently, however, concentration is put into the clear differences in the field of growth found in these many craniofacial cartilages.

The feedback we get from the results of many experimental outline is to compare the craniofacial cartilages, in general, and the results of transplantation studies. In particular, many researchers reached the conclusion that dissimilarity exists in the fundamental properties of the craniofacial cartilages. The reality of this emulation has not been tested, and whether or not there is a molecular basis for the suggested dissimilarity has never been subjected to experimental confirmation.

The present study has used one and two dimension polyacrylamide gels electrophoresis to establish the protein distribution among the craniofacial cartilages namely, nasal septum, mandibular condyle, and sphenosynchondrosal. The craniofacial cartilages were obtained by careful dissection from 9-11 day old rates and protein extraction with 4M Guanidine HCl 0.1 sodium acetate buffer, pH 5.8 containing proteinase inhibitors. After lyophilization, the reconstituted fraction was electrophoresed on 3-16% one- and two-dimensional gels containing 0.1% SDS. Gels were stained with modification of the silver nitrate technique and Coomassie blue

Differences in protein distribution and patterns among nasal septal, mandibular condyle, and sphenosynchondrosal cartilages were seen, and by mapping these protein, we were able to single out some proteins of interest and have it ready for more study.

In summary, the overall goal of this study was geared toward attempts to understand distribution of the proteins obtained from the rat nasal septal, condylar, and sphenosynchondrosal cartilages and whether we'll be able to isolate these proteins.

MATERIALS AND METHODS

Harvesting the Extracellular Matrix Proteins (ECMP):

Dissection of the sample study

Sample cartilages used in this study were obtained from eight litters of nine to eleven day old Sprague-Dawley rats that were injected with pentobarbital 2 ml syringe. An incisional opening was made along the tip of the nose with a #15 surgical blade. A pair of surgical scissors was used to open the incision further therapy, exposing one of the nares by slightly cutting into the snout. Dissection pliers were then used to separate the nasal septum from its embedded base of insertion.

Removal of the condylar and synchondrosal cartilage was also performed. A bilateral opening was made from the joint of the mouth to the ear to expose the condyle thereby opening the mouth wider allowing entry to the synchondrosal cartilage. The samples were then washed in cold Gey's Balanced Salt Solution (GBSS) to dilute excess blood and surrounding tissue materials that were attached to the sample cartilage. These samples were then restored in Gey's solution at 4^0 C until the end of the dissection session. Keeping the samples in this type of solution which was isotonic, and at a cold temperature was highly important at this stage to prevent samples from degradation by endogenous tissue proteinase. At the end of each dissection, samples were kept at -75^0 C for few weeks before initiating tissue extraction.

Extraction of the ECMP using 4 M Guanidine HCl.

The following methods of extraction were used for this study:

Freezing and milling

The dissected nasal septal, condylar, and synchondrosal cartilage samples were subjected to freezing with liquid nitrogen and ground into powder using a freezer/mill apparatus (Spex Industries Inc. NJ). This method required a great deal of extra caution to place the impactor chip in the center of the sample tube to prevent contact with the wet component of the cartilage. This procedure would prevent adherance to the chip and would cause as an incomplete grinding thereby reducing the surface area of tissue material available for subsequent extraction.

Guanidine Hydrochloride Extraction

Another method that was employed in this study was subjecting the samples to guanidine hydrochloride. Much of the proteoglycan tissue extraction was obtained with high concentration of this protein denaturant . The samples were then subjected to use of 4 M guanidine HCl in 0.1 M sodium acetate buffer, pH 5.8 containing proteinase inhibitors (100 mM 6-amin-hexanoic acid, 5 mM benzamidine hydrochloride, 10 mM Na_2 EDTA, 20 ug/ml ovomucoid) then added to the milling powder. The samples were left for extraction for 24 hours on a magnetic stirrer at 4^0 C . The extracts were then centrifuged for ten minutes at 2400 rpm (Sorvall J-6B) to remove any unextracted remains of the samples.

Dialysis

To remove the guanidinium salt and to allow proteoglycan aggregates to reform, the extracts were then dialysed thoroughly against double distilled deionized water for 24 hours, at 4^0 C, on a magnetic stirrer, using porous membrane tubing with molecular weight cut-off of 6000 to 8000 daltons. Water was changed every 6 hours.

15-K Fractions

A precipitate was formed during dialysis and the dialysates were then centrifuged at 15,000 rpm (SS-34 rotor, Sorvall centrifuged), for ten minutes to produce a 15-k supernatant and the 15-k pellet fractions. These samples were assayed for hexuronic acid and protein.

Lyophilization

The 15-K supernatant which contained most of the proteins was frozen with dry ice in ethanol or acetone and lyophilized overnight. The 15-K pellet was stored at -4^0 C for later comparison with other samples.

Cesium chloride Density Gradient Separation

The 15-K lyophilized supernatant was then dissolved in 12 ml of 4M guanidium HCl in 0.1 M sodium acetate buffer, at pH 5.8, and centrifuged at 2400 rpm for ten minutes producing gradient pellets and gradients supernatants. The gradient pellets were stored at -20^0 C. while cesium chloride was added to the gradient supernatant (2.6 g of cesium chloride to every 4 ml of solution). The density of both was then adjusted to 1.5 0.0049 g/ml, using micropipets calibrated for 100 ul and weighted on a precision balance (Mettler analytical balance). The samples were

then centrifuged at 35,000 rpm (gravity=105,000 g) for 45 hours at 4^0 C, yielding four density fraction D4 -D1, bottom to top

Dialysis

To remove CsCl and guanidinum HCl, the extracts were then dialysed thoroughly against double distilled deionized water overnight, at 4^0 C, on a magnetic stirrer, using porous membrane tubing with molecular weight cut-off of 6,000 to 8,000 daltons (Medical Spectrum Industries, LA,CA). Water was changed twice resulting in the final four gradient fractions D1-D4.

Uronic Acid Analysis and protein analysis

Quantification assays were performed on different fractions: The 15-K supernatants and pellets, the gradient supernatants and pellets and the final extracts. Uronic acid analysis was performed to determine the concentration of proteoglycans, (see Table 1). Bradford micro-assay was performed to quantify the amount of protein extracted in each fraction (Bradford, 1976):Bradford micro-assay for protein, (see Tables 2,3 and 4):

1) Total volume of each tube is 1 ml.

2) Add to each sample tube 200 ul of dye (Bio-Rad Protein assay, cat. 500-0006) and for a blank tube , add 800 ul dd H_2O, Vortex and for sample tube, add 50 ul of sample and 750 ul of double distilled deionized (dd) H_2O,

3) Vortex and for standard tubes, Bovine serum albumine (BSA) was prepared at 100 ug/ml kept at 4 C. The standard tubes will be loaded in the following manner:

Read optical density (O D) after five minutes, on spectrophotometer at 595 nm, visible light and compute standard regression for fraction and from standard regression fraction, compute sample protein for 50 L and deduct sample concentration.

Table 1 -- Tubes used in the uronic acid analysis

STDS (BSA* in ug)	ul (of100 ug/ml BSA)	dd** H2O in ul
1	10	790
2	20	780
4	40	760
6	60	740
8	80	720
10	100	700
12	120	680
14	140	660

*Bovine serum albouman
**double distilled water

A Modified Uronic Acid Carbazole Reaction

The reaction of uronic acids with carbazole is the most satisfactory method of estimating uronic acids in chromatographic fractions but requires two hours for the full development of color with certain compounds, the color is partially suppressed by salts, is unstable and sensitive to overheating or dilution by water, and impurities in the reagents or the sample interference.

Table 2.-- Bradford Assay· Protein content in the SPNT.&PELLET (RAT) 15Ks of the Nasal Septum, Mandibular condyle and Synchondrosal cartilages.

Samples	B.S.A. ml	d H2O ml	A Coomassie	A 595
Blank	0	800	200	0.000
1	10	790	200	0.088
2	20	780	200	0 112
5	50	750	200	0.237
7.5	75	725	200	0.339
10	100	700	200	0.485
12.5	125	675	200	0.565
15	150	650	200	0.632
17.5	175	625	200	0.642
20	200	600	200	1.605
N.S. SPNT.	50	750	200	0.134
N.S. SPNT.	200	600	200	0.411
N.S. PELLET	50	750	200	0.260
N.S. PELLET	200	600	200	0.565
M.C. SPNT.	50	750	200	0.010
M.C. SPNT.	200	600	200	0.096
M.C. SPNT.	50	750	200	0.295
M.C. SPNT.	200	600	200	0.665
SYNC. SPNT.	50	750	200	0.015
SYNC. SPNT.	200	600	200	0.030
SYNC. PELL	50	750	200	0.160
SYNC. PELL	200	600	200	0.275

Table 3.-- Bradford Assay protein content in the D4 fraction of the SPNT. & PELLET (RAT) 15 Ks of Nasal Septum, Mandibular Condyle & Synchondrosal

Samples	B.S.A. ml	d H2O ml	Coomassie	A 595
Blank	0	800	200	0.000
1	10	790	200	0 085
2	20	780	200	0.118
5	50	750	200	0 266
7.5	75	725	200	0 397
10	100	700	200	0.493
12.5	125	675	200	0.587
15	150	650	200	0.698
17.5	175	625	200	0.850
20	200	600	200	0 879
N.S.SPNTD4	50	750	200	0.231
D3	50	750	200	0.103
D2	200	600	200	0.096
D1	200	600	200	0.120
N.S.PELLD4	50	750	200	0.088
D3	50	750	200	0.062
D2	200	600	200	0.044
D1	200	600	200	0.058
M.C.SPNT4	50	750	200	0.075
D3	200	600	200	0.102
D2	200	600	200	0.030
D1	200	600	200	0.019
M.CPELLD4	50	750	200	0.085

Samples	B.S A ml	d H2O ml	Coomassie	A 595
D3	200	600	200	0 130
D2	200	600	200	0 038
D1	200	600	200	0.011
SYNCSP D4	200	600	200	0.092
D3	200	600	200	0 056
D2	200	600	200	0.025
D1	200	600	200	0.010
SYNCPE D4	50	750	200	0.052
D3	50	750	200	0.315
D2	200	600	200	0.259
D1	200	600	200	0.057

Table 4 -- Uronic Acid Assay on the SPNT &PELLET (Rat) 15s Nasal Septum, Mandibular condyle & Synchondrosal Cartillages

Sample	ML	VL/ml A	530
Blank			0
5			0.040
10			0.116
20			0.207
30			0.360
40			0.481
50			0.642
N.S. SPNT.	20	44	0.042
N.S. PELLET	20	20	0.047
M.C. SPNT.	20	62	0.002
M.C. PELLET	20	20	0.015
SYNC. SPNT.	20	48	0.009
SYNC. PELLET	20	20	0.005
N.S. SPNT.	50	44	0.111
N.S. PELLET	50	20	0.310
M.C. SPNT.	50	62	0.107
M.C. PELLET	50	20	0.190
SYNC. SPNT.	50	48	0.062
SYNC. PELLET	50	20	0.079
N.S. SPNT.	100	44	0 240
N.S. PELLET	100	20	0.262
M.C. SPNT.	100	62	0.017
M.C. PELLET	100	20	0 066
SYNC. SPNT.	100	48	0.000
SYNC. PELLET	100	20	0.035

IEF First Dimension Casting IEF Tube Gels

For reproducible results with tube gels, it is essential that the gels be the same length and that the polymerization be identical from day to day. Care must be taken in pouring the gels to the same height, and in overlying, so that the polymerization height will be the same from tube to tube.

Mark the tubes to the desired gel length with a marking pen.

Set the tubes in a level casting stand. If the tubes are not perfectly vertical, the gels will polymerize at an angle to the pull of gravity and the proteins may band at the same angle. If the tube gels are the first dimension gels of a 2-D run, the slanted bands will cause distortion, overlap of polypeptide spots and irroproducibility in spot location in the second dimension.

Prepare the monomer solution and degas for at least 15 minutes. Removal of molecular oxygen by degassing is essential for reproducible polymerization. Add the APS and TEMED, swirl gently 8 to 10 times, and cast the tube gels. For gels 3 mm I.D. and wider, use a long pasteur pipet or a syringe with a long needle.

Capillary tube gels 2.5 mm I.D., must be poured with a syringe and fine gauge needle long enough to reach the bottom of the tubes. Slowly, inject the acrylamide solution into the tube, applying a slight extra force to initially dislodge any air bubble at the bottom of the tube. Withdraw the needle as the acrylamide enters the tube and fill to the determined mark. Repeat this process until all the tube gels are cast.

Overlay the gels carefully with equal volumes of overlay buffer.

Polymerization should occur within one hour.

After polymerization, rinse tops of the gels thoroughly with distilled water. Then, place tubes back in the casting stand and reapply a volume of overlay buffer to height of 1 cm above the gel surface.

Molecular Weight Standards For Electrophoresis

1) Myosin	200,000 KD
2) Phosphorylase (b)	97,400 KD
3) Bovine Serum Albumin	69,000 KD
4) Ovalbumin	46,000 KD
5) Carbonic Anhydrase	30,000 KD
6) Trypsin Inhibitor	21,000 KD
7) Lysozyme	14,300 KD

Procedure for silver staining of gels

A simplified ultrasensitive silver stain was used for detecting protein on the polycrylamide gels. and the materials needed are: Gel fix: 50% methanol,10% acetic acid, in glass distilled water. Gel rinse: 5% methanol, 7.5% acetic acid, 3.7% formaldehyde (1:10 dilution of commercial) 10% glutaraldehyde, 1% citric acid, freshly made. Concentrated ammonum hydroxide.

Take a pint bottle and prepare an aliquot of 5 ml in 5-10 ml screw cap vials, with

two layers of parafilm between cap and vial, and many layers of parafilm wrapped around the cap. Mark the 5 ml level in the vial and check for evaporation before use.

Three immacutalely clean 8" by 8"silver nitrate staining pans/gels, are used with tightly fitting lids and flat bottoms.

Reducer solution

NaCl (37 g) and CuSo4 (37 g) are dissolved in 850 ml of distilled water in a glass container. Concentrated NHO4OH is added until the precipitate which first forms is completely dissolved with the volume adjusted to 1 liter.

Immediately before use,equal volumes of A and B are mixed and diluted to appropriate strength using 75 ml each diluted to 500 ml.

Stop solution

24 g of Kodak Hypo clearing agent/liter and Gels: 140 x 140 x 0.8 mm (10%) and after gels are run, the following steps are carried out:

1) 500 ml gel fix, 12 hours or more

2) 500 ml gel rinse, 1 hour or more

3) 150 ml 10% glutaraldehyde

4) 500 ml glass-distilled water, 15', repeated four times

5) 500 ml 0.02 Tris-HCl, pH 9, 30', repeated twice. We have found this step necessary for staining 2D gels of which the first dimension is the O'Farrel non-equilibrium PH gradient method.

6) Repeat step 4.

All the above steps should be carried out with mild agitation (50 rpm on a New Brunswick Shaker). For the following steps (7-10),

agitation should be increased to prevent the gel sticking to the bottom of the pan.

7) Drain off water and add freshly made ammoniacal silver solution (prepared within 5 minutes of use) For 150 ml, add 2.1 ml fresh NH_4OH to 31.5 ml 0.36% NaOH With vigorous stirring, add 6 ml 19 4% $AgNo_3$ (1.2 g $AgNo_3$ + 6ml glass-distilled water). Add glass-distilled water to a final volume of 150 ml. Agitate at 75 rpm for 15 minutes and keep the lid shut tightly.

8) Transfer gel to pan containing at least 500 ml water and agitate for two minutes.

9) Transfer gel to pan containing 5 ml 1% citric acid and 5 ml 3.7% formaldehyde/liter. Stained spots may appear in this solution anytime from 5-30 minutes or more. Transfer gel to distilled water when spot intensity is judged sufficient because the staining reaction does not stop immediately.

10) We have found that the gels need to be destained somewhat, because a brown background develops and/or mirror-like deposits of silver form on the surface of the gel. This is done with the reducer solution which should be monitored closely with a light box and is stopped with the stop solution. Gels may be stored in distilled water but should be photographed within 24 hours (kept in stop solution) as they tend to fade.

General precautions

Glass-distilled water must be used throughout and precautions should be taken to prevent fingerprints, halide ions, etc, from contaminating the solution and gels. Used diammine solution should be thrown out or acidified with HCl right after use. Glass plates are cleaned with chromic acid and rinsed with 100% methanol before use. Plexiglass gel chambers are washed thoroughly, swabbed with 7% acetic acid and then with 100% methanol. If staining is unsuccessful for some reasons, gels may bleached to transparency with full-strength reducer for 1-15 minutes, rinsed exhaustively with distilled water.

Western Blot Analysis

0.5 ml aliquots of 35S-methionine A4 human cartilage extracts were reduced and subjected to formaldehyde and then treated with Chondroitinase ABC (ABC) for 3 hours and after ABC, 25 ml of 5 X gel sample buffer were added to each sample (0.312 M Tris, 10% SDS, 100 mm DTT, 25% glycerol, bromophenol blue) which was then boiled for 5 minutes and samples were diluted to 500 ul with IX gel sample buffer. 50 ml were used per gel and samples were run on a 3-16% gradient gel overnight at 40 V and gels were incubated with transfer buffer 15-20 minutes prior to actual transfer and (0.125 M Trizma base, 0.96 M glycine, 12.5% methanol) and immobilon transfer sheets (0.45 Millipore) were employed in the transfer after the following treatment and wet membrane 1-3 seconds in methanol and immerse membrane 1-2 minutes in water and equilibrate membrane in transfer buffer for a few minutes.

The gel was then placed onto two layers of Whatman N= 3MM chr, filter paper soaked with transfer buffer. The Immobilon membrane was placed on the top of the gel followed by two more soaked pieces of filter paper. Two others sandwiches were made for two other gels with Scotch-Bride pads separating each sandwich and the filter unit was closed and placed into the E-C electro transfer apparatus filled with cold transfer buffer. The transfer was carried out for 4 hours at 1.5 at 4^0 C and the membrane were blocked with 3% BSA in the TBS Tween (20 mM Tris, 500 mM Ancl, pH 8 plus 0.05% Tween-20 for 1 hour on a shaker) and the membrane were then incubated overnight with the respective human proteoglycans on a shaker and membranes were washed 3X10 minutes using 40

ml TBS-Tween and membranes were then incubated for 2 hours with the second antibody at a 1.7.500 dilution in TBS-Tween: alkaline phosphatase-conjugated goat antimouse and the membranes were again washed 3X10 minutes with TBS-Tween and the alkaline phosphatase substrate was then added to each dish for up to 30 minutes in the dark and the membranes then washed with d H_2O and air dried and the membranes were exposed to Betamax Hyperfilm in an X-ray cassete for ten days at -70^0 C prior to developing the A R G with D-19 and rapid fixer.

RESULTS

Comparison of Protein Distribution

In the Nasal Septal, Mandibular Condyle, & Synchondrosal Cartilages

The samples used in the study namely nasal septal, mandibular condylar, and synchondrosal cartilages were dissolved to high protein concentration (20 ug of protein per 100 ml in distilled H_2O) and then further diluted. 300 ul of samples were utilized from this dilution to form different gradient levels producing D1, D2, D3, and D4 samples.

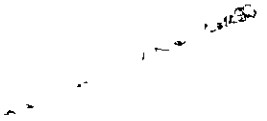
Samples derived from nasal septal, condylar and synchondrosal cartilages were subjected to high speed (15,000 rpm) centrifugation. The high molecular weight proteins and other material precipitate to the bottom and are called 15K pellet the low molecular weights proteins will be on top and are called 15K supernatant. Both samples were electrophoresed using two-dimensional gel technique 3-16% gradient (SDS-PAGE and IEF with silver-stained).

In the 15K S fraction, the protein distribution and isoelectric points (PK) among nasal septal, condylar and synchondrosal were completely different from one another. The proteins in the nasal supernatant fraction (Figure 1) (Table 1) ranged in the apparent size from 69- 46 K_D whereas the proteins of the mandibular condylar 15KS fraction. (Figure 2) and synchondrosal 15KS fraction (Figure 3) fractions were spread widely between 69-14.3 K_D.

The mandibular condylar 15K S (Figure 2) and synchondrosal supernatants (Figure 3) also showed microheterogenity which was not obvious in the nasal septal

supernatant fraction (Figure 1).

Comparing the synchondrosal 15K pellet fraction with nasal septal pellet (Figure 4) and mandibular condylar 15K pellet fractions (Figure 5), we see that most of the proteins in the synchondrosal 15K pellet fraction were found in a range between 69 to 46 KD whereas the proteins noted in the nasal septal (Figure 4) and condylar (Figure 5) pellets were found widely spread between 97.4 to 14.3 KD. The synchondrosal 15K pellet fraction (Figure 6) showed increased microheterogenity which was not obvious in the nasal septal 15K pellet (Figure 5) fractions. The quantity and quality of protein distribution and location observed in the mandibular condylar pellet fraction (Figure 5) were more prominent than those noted in the nasal septal (Figure 4) and synchondrosal pellet (Figure 6), (Table 6).



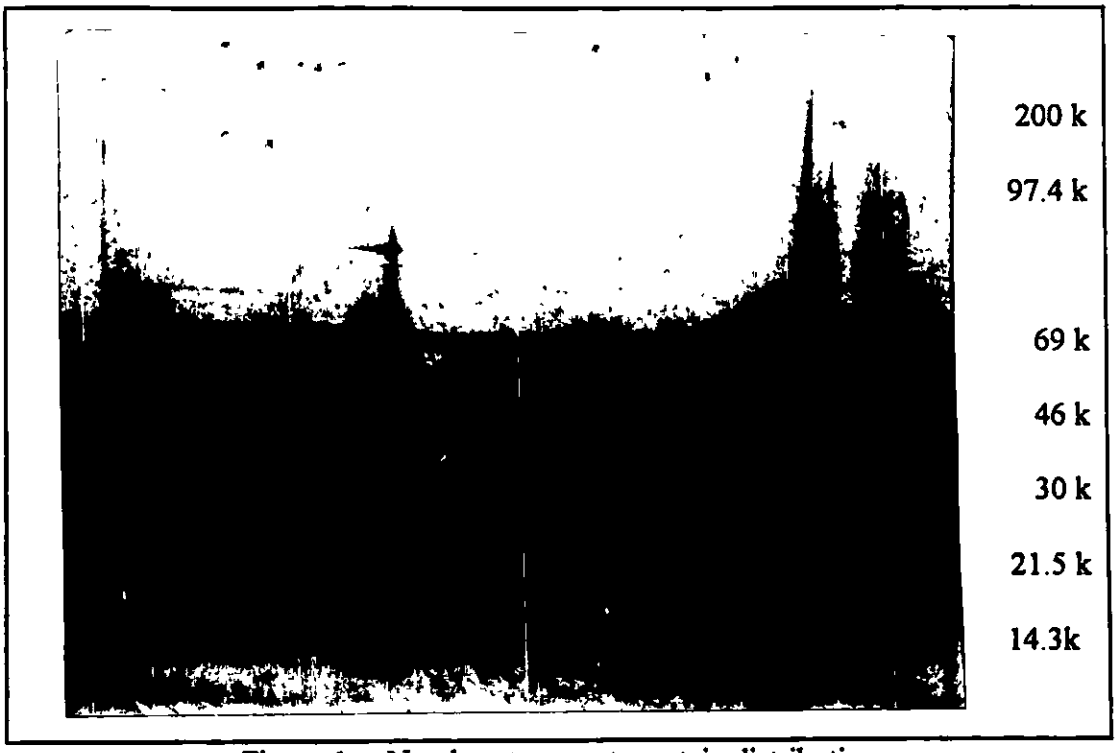


Figure 1.-- Nasal septum spnt. protein distribution
15 Ks rat nasal septal-mandibular condyle-syncondrosal
3-16% acrylamide gel/stain: silver stain
electrophoresis was for 4 hours at 200 ma.

Table 5.-- Nasal Septal Supernatant Fraction 2D Gel
Protein P I. and M.W.

Protein Fraction	Prot No.	Prot P I	Prot. M.W
N.S. SPNT.	1	6.9	69
	2	6.9	50
	3	6.9	46
	4	6.3	97.4
	5	6.3	50
	6	6.3	46
	7	6.3	30
	8	4.95	69
	9	4.95	50

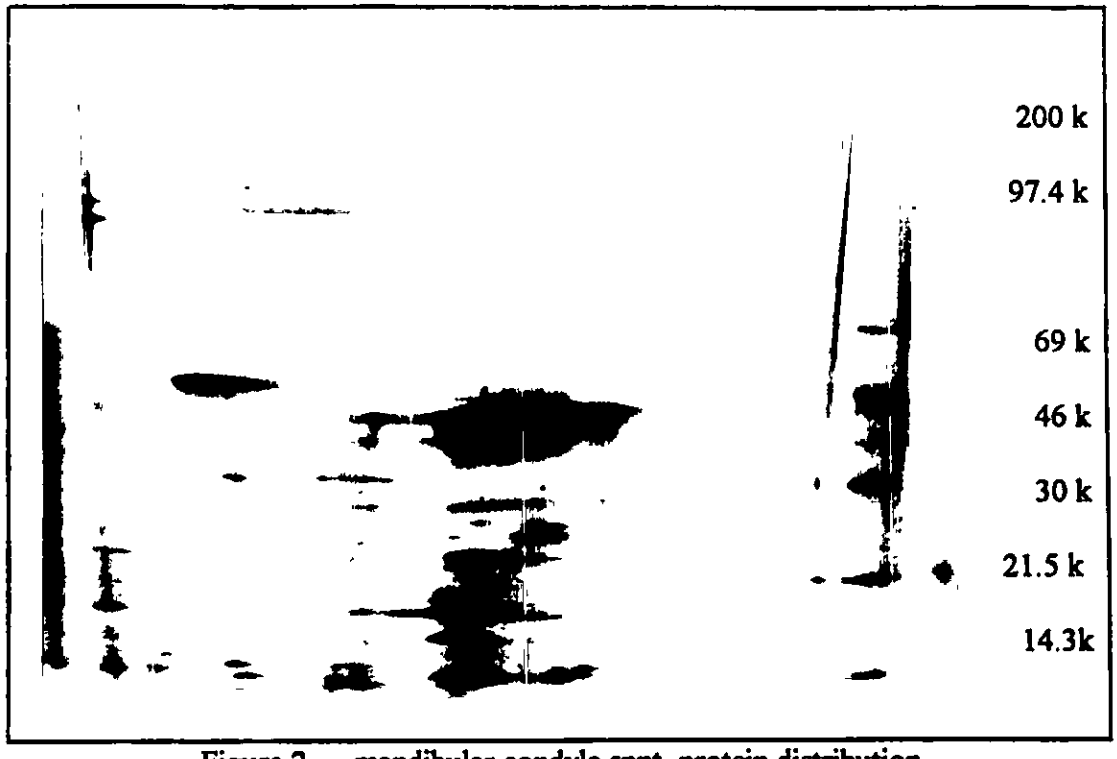


Figure 2.-- mandibular condyle spnt. protein distribution
15 Ks rat nasal septal-mandibular condyle-syncondrosal
3-16% acrylamide gel/stain: silver stain
electrophoresis was for 4 hours at 200 ma

Table 6 -- Mandibular Condyle Supernatant Fraction 2D Gel
Protein P I. and M.W.

Protein Fraction	Prot. No.	Prot. P.I.	Prot. M.W.
M.C SPNT.	1	6.9	25
	2	6.9	21.5
	3	6 9	14.3
	4	6.5 - 6.9	69
	5	6.8	14.3
	6	6.7	14.3
	7	6.3	69
	8	6.3	30
	9	5.08 - 6.1	69
	10	5.8	30
	11	5.5 - 6.1	30
	12	5.5 - 6.3	21.5
	13	6.1	21.5
	14	6	14.3
	15	5.4	14.3

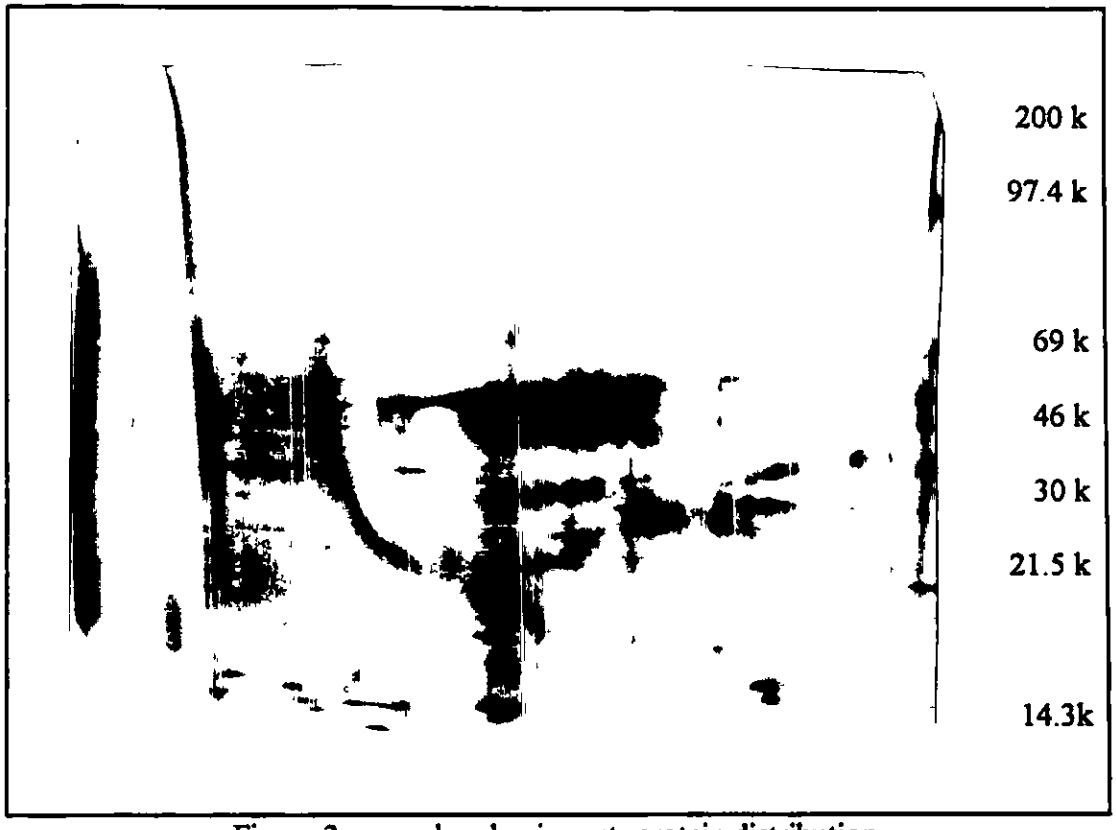


Figure 3.-- synchondrosis spnt. protein distribution
15 Ks rat nasal septal-mandibular condyle-syncondrosal
3-16% acrylamide gel/stain: silver stain
electrophoresis was for 4 hours at 200 ma

Table 7 -- Sphenosynchodrosal Supernatant Fraction 2D
Gel Protein P.I. and M.W

Protein Fraction	Prot No	Prot. P I.	Prot. M.W.
SYNC. SPNT.	1	7	21.5
	2	6.9	21.5
	3	6 9	14 3
	4	6 8	14 3
	5	6 7	14.3
	6	6.6	69
	7	6.6	50
	8	6.3	30
	9	6.3	25
	10	6.4	21.5
	11	6 3	14 3
	12	5.8 - 6.5	69
	13	6.2	46
	14	6.1	46
	15	6.1	50
	16	6.2	30
	17	5.08 - 5.8	46
	18	5.08 - 5.8	30
	19	5.4	21.5
	20	5.15	14.3

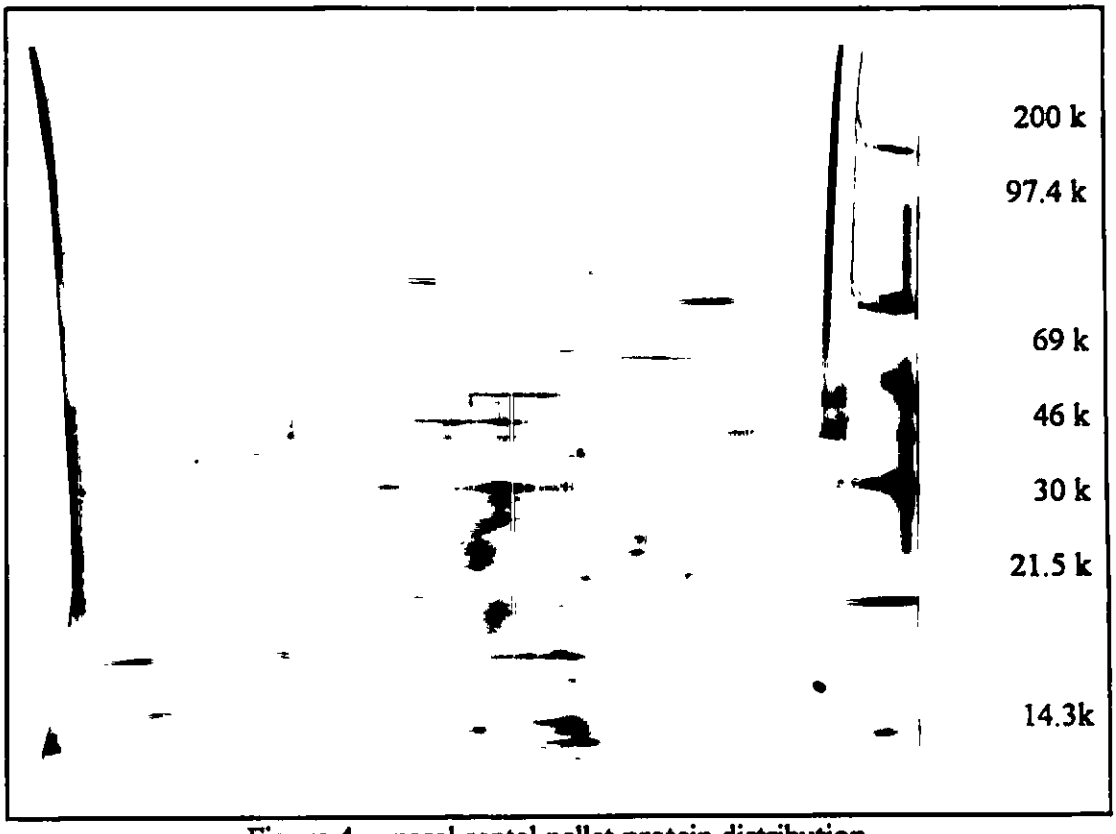


Figure 4.-- nasal septal pellet protein distribution
15 Ks rat nasal septal-mandibular condyle-syncondrosal
3-16% acrylamide gel/stain: silver stain
electrophoresis was for 4 hours at 200 ma

Table 8 -- Nasal Septal Pellet Fraction 2D Gel Protein P.I. and M W.

Protein Fraction	Prot No.	Prot.P I.	Prot.M.W.
N S. Pellet	1	6 9	21.5
	2	7	14.3
	3	6.7	21.5
	4	6.5	25
	5	6 3	25
	6	6.1	25
	7	5.8	50
	8	5.8	30
	9	5.4	40
	10	5.4	30
	11	6.1	14.3
	12	5.8	21.5
	13	5.5	14.3

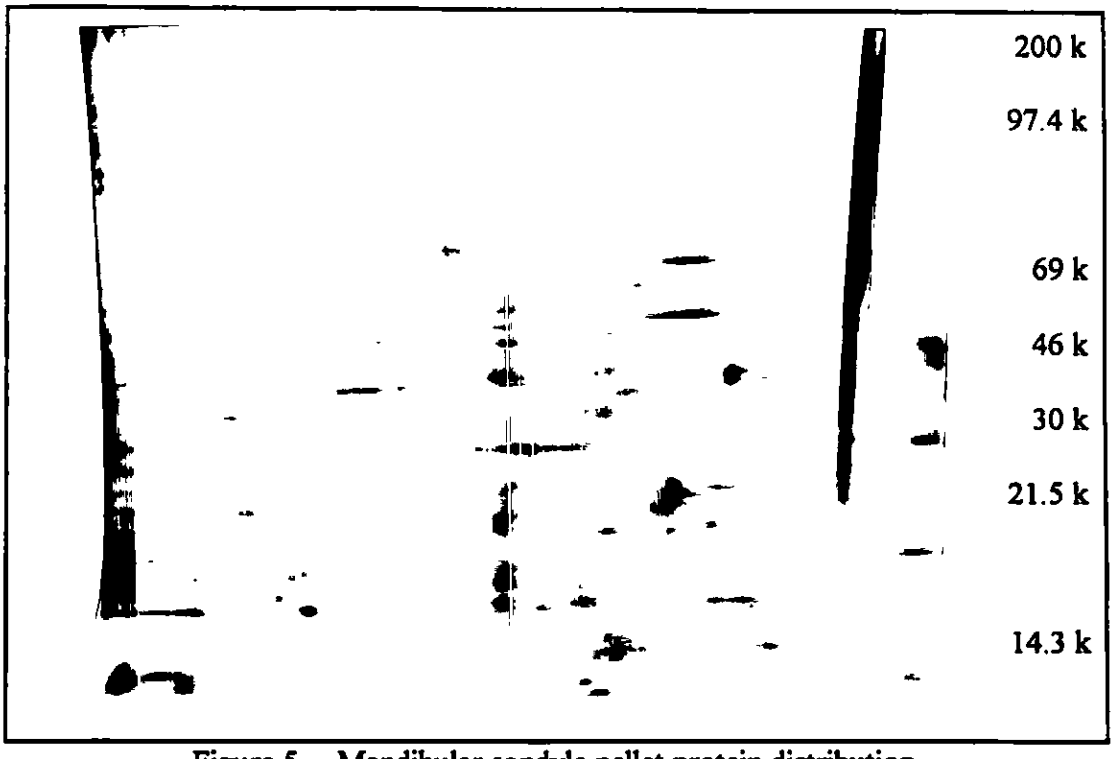


Figure 5.-- Mandibular sondyle pellet protein distribution
15 Ks rat nasal septal-mandibular condyle-syncondrosal
6-16% acrylamide gel/stain: silver stain
electrophoresis was for 4 hours at 200 ma

Table 9 -- Mandibular Condyle Pellet Fraction 2D Gel Protein P.I and M.W.

Protein Fraction	Prot No.	Prot P I	Prot.M.W
M.C PELLET	1	7	46
	2	7	21.5
	3	6.9	30
	4	6.9	21.5
	5	6.9	14.3
	6	6.8	50
	7	6 8	40
	8	6.8	30
	9	6.7	30
	10	6.7	25
	11	6.7	21.5
	12	6.5	46
	13	6 5	25
	14	6.3	46
	15	6.2	46
	16	6	25
	17	5.8	14.3
	18	5.4	46
	19	5 3	30
	20	5.08	69
	21	4.95	97.4
	22	4.95	21.5

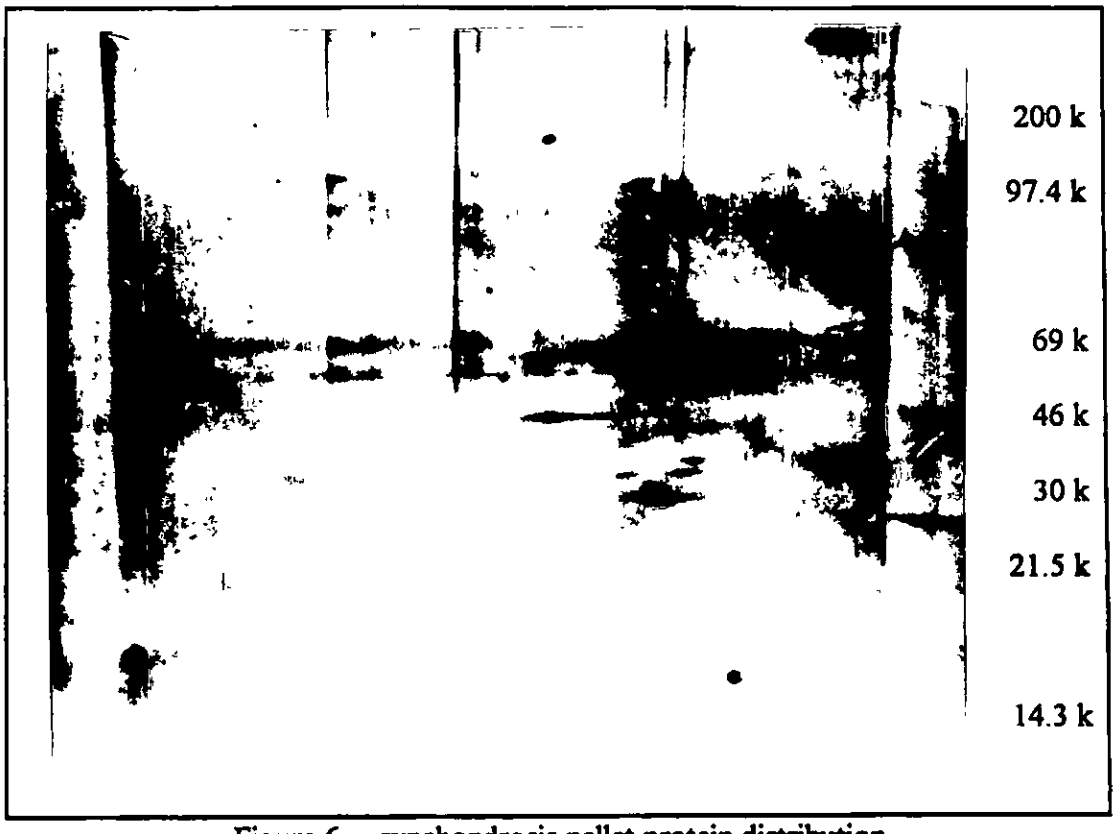


Figure 6.-- synchondrosis pellet protein distribution
15 Ks rat nasal septal-mandibular condyle-syncondrosal
3-16% acrylamide gel/stain: silver stain
electrophoresis was for 4 hours at 200 ma

Table 10.-- Sphenosynchondrosal Pellet Fraction 2D
Gel Protein P I. and M.W.

Protein Fraction	Prot.NO	Prot P I	Prot M.W
SYNC. Pellet	1	6.7	50
	2	6.3	50
	3	6.1	49
	4	4.95 - 6.1	50
	5	6	46
	6	5 8	30
	7	5.5	25
	8	5.4	30
	9	5.4	25

Comparison of D4 Fractions among

Nasal Septal, Mandibular Condylar, and

Synchondrosal Supernatants and Pellets

The rationale behind this experiment was drawn from the fact that non-proteoglycan proteins were concentrated in the D4 fraction. The decision was made to divide the whole sample of nasal septa, mandibular condyle, and synchondrosal into four fractions called: D4, D3, D2, and D1 from top to bottom by ultracentrifugation.The high molecular weight proteins will be found in D1 fraction while the low molecular weight proteins will be found in D4.

D4 gradient nasal septal, mandibular condylar, and synchondrosal supernatants were then electrophoresed using the 2-dimensional 3-16% gradient SDS-PAGE and silver staining. The results showed that the protein distribution and location for all three cartilaginous supernatants were completely different but all seemed to fall in the range between 97.4 to 14.3 KD. One noticeable difference was the presence of microheterogeneity in the mandibular condylar (Figure and Table 8) and synchondrosal supernatant D4 (Figure and Table 9) fractions which was not seen in the nasal septal supernatant D4 (Figure and Table 7) fraction.

Comparing nasal septal pellet D4 (Figure and Table 10) fraction with mandibular condylar (Figure and Table 11) and synchondrosal D4 (Figure and Table 12) fraction pellet, the results showed no similarities among these cartilaginous

elements. Protein distribution and location were completely different among the cartilaginous pellets as the nasal septal (Figure 10) and mandibular condylar D4 (Figure 11) fractions ranged between 97.4 to 14.3 KD, while the proteins found in the synchondrosal D4 fractions pellets (Figure 12) ranged between 69 to 20 KD.

Microheterogeneity was present in the mandibular condylar D4 (figure 11) and synchondrosal D4 fraction pellets (Figure 12) whereas this feature was not found in the nasal septal D4 fraction pellet (Figure 10).

The quality and quantity of the proteins were very prominent in the nasal septal and mandibular condylar D4 fraction pellets while they were less prominent in the synchondrosal D4 fraction pellets. (Figures 10-12)There were some similarities in the protein distribution and location between the synchondrosal supernatant and synchondrosal pellet D4 fractions. (Figures 9-12) Such similarities that existed between synchondrosal supernatant and pellets D4 fraction would support the need to further study in identifying these unique proteins. Therefore a decision was made to map all proteins on the 2-dimensional gel 15 K SPNT and 15 K Pellet D4 fraction:

Table 11.-- Nasal Septal Supernatant D4 Fraction
Comparing the protein weights in the nasal septal supernatant and pellet

Protein Fraction	Protein No.	Prot. P.I	Prot. R.M.	Prot. M.W.
NS SPNT D4	1	5.5	0.51	58
NS SPNT D4	2	6.9	0.44	65
NS SPNT D4	3	6.9	0.63	36.5

Table 12.-- Nasal Septal Pellet D4 Fraction

Protein Fraction	Protein No	Prot. P.I.	Prot. R.M.	Prot. M W.
NS PELLET D4	1	6.8	0 47	36
NS PELLET D4	2	5.3	0.46	36
NS PELLET D4	3	5.8	0 55	40

Comparing the protein weights in the nasal septal supernatant and nasal septal pellet D4 fractions, the proteins in the nasal septal pellet D4 have lower molecular weights (Figure 7-10) than the proteins found in the nasal septal supernatant D4 fraction which have high molecular weights. (Table 11,12).

Table 13.-- Mandibular Condylar Supernatant D4 Fraction

Protein Fraction	Protein No.	Prot. P.I	Prot. R.M.	Prot. M.W.
MC SPNT D4	1	6.7	0.43	61
MC SPNT D4	2	5.3	0.40	69
MC SPNT D4	3	6.3	0.40	69

All proteins have close molecular weights but differ in their protein P.I. and need to be studied more.

Table 14.-- Mandibular Condylar Pellet D4 Fraction

Protein Fraction	Protein No.	Prot.P.I.	Prot. R.M.	Prot. M.W.
MC PELLET D4	1	6.5	0.69	14.1
MC PELLET D4	2	6.7	0.57	23
MC PELLET D4	3	6.5	0.61	20

The mandibular condylar pellet have low molecular weights and are very similar in protein P I (Figure 11, Table 13,14)

Comparing mandibular condylar supernatant and pellet D4 fractions, the proteins found in the mandibular condylar pellets have lower molecular weights than the supernatants. (Figures 8-11, Table 13,14).

Table 15.-- Synchondrosal Supernatant D4 Fraction

Protein Fraction	Protein No.	Prot. P.I.	Prot. R.M.	Prot. M.W.
SYNCH SPNT D4	1	5.8	0.63	31
SYNCH SPNT D4	2	6.9	0.86	14.2

The proteins in the Synchondrosal supernatant D4 fraction have low molecular weights. (Figure 9, Table 15,16)

Table 16.-- Synchondrosal Pellet D4 Fraction

Protein Fraction	Protein No.	Prot. P.I.	Prot. R.M.	Prot. M.W.
SYNCH PELLD4	1	6.1	0.63	28.5
SYNCH PELLD4	2	5.8	0.72	20.5

The proteins in the synchondrosal 15 KD D4 fraction have low molecular weights. (Figure 12, Table 15,16).

When comparing the nasal septal supernatant and pellet D4 fractions, the

supernatants have higher molecular weights than the pellets (Figure 7, 10).

Between the mandibular condylar supernatant and pellet D4 fractions, the supernatant have lower molecular weights than the pellets (Figure 8, 11, Table 15,16).

Lastly, comparing the synchondrosal supernatant and pellet D4 fractions, the supernatants and pellets have both similar molecular weights (Figure 9,12, Table 15,16.

2-D Gel Electrophoresis and Western Blot Analysis

The rationale behind this experiment was drawn from the favorable results of D4 fraction two dimensional gel electrophoresis. Using the Western Blot technique the number of proteins in D4 fraction on the transfer paper did not show many proteins. So the decision was made to combine D4 and D3 fractions hoping this will enable us to see more proteins (dots) on the Western blot transfer paper.

Samples of D3 and D4 gradient obtained from nasal septal, mandibular condylar, and synchondrosal supernatants and pellets were subjected to 2-D gel electrophoresis and Western blot analysis. Transfer papers were incubated with human cartilage proteoglycan labelled by in vitro with 35SO4 which was then subjected to autoradiography.

The binding between the rat proteins and the human proteoglycans was nonspecific and only took place with the nasal septal D4 supernatant, mandibular condylar D4 pellet, and synchondrosal D4 pellet. There was no binding with the nasal septal D4 pellet, mandibular condylar D4 supernatant, and synchondrosal D4

supernatant.

A1 A1 D1 Gradient

One species of proteoglycan with two associative and one dissociative gradient was used (Prepared by Dr. Malemud's Laboratory). The first associative gradient produced the A1 fraction which was used to run the second associative gradient. The fraction A1 A1 was produced and used to create the dissociative gradient which resulted in fraction called A1 A1 D1 (Figure 13).

The gradient A1 A1 D1 was labelled using 35SO4

and the formula for that labelling is:

35SO4 (50uc/ml)m DME

35SO4 + 10% FBS, P,F,M.

20 hours.

2-D Gel Electrophoresis (D3 and D4) Fraction

Samples of D_3 and D_4 gradient obtained from nasal septal, condylar, and synchondrosal supernatants and pellets were subjected to 2-D gel 3-16% gradient SDS-PAGE electrophoresis and Western blot analysis. The transfer papers were stained with Coomassie Blue showing proteins clearer in the mandibular condylar D_3 and D_4 supernatants but not showing on the other cartilaginous gradient samples. A possible reason for this could be that the sensitivity of the transfer paper with the Coomassie Blue might not be high enough to show these proteins.

One and Two Dimensional Gel Electrophoresis

Samples of D_3 and D_4 nasal septal and mandibular condylar supernatants were subjected to one and two dimensional gel electrophoresis. One of the gels was stained with Coomassie Blue and the other gel was subjected to Western blot transfer stained with Coomassie Blue.

The results revealed that on the one dimensional gel mandibular condylar D_4 fraction supernatants, bands of proteins were spotted in the range between 79 to 14.3 K_D, and, on the two dimensional gel mandibular condylar D4 fraction supernatants, the same dots of proteins were seen spreadly between the range of 69 to 14.3 K_D. The transfer gel of the same fraction revealed some bands of proteins on the one dimensional gel and while there were no noticeable dots on the two dimensional gel. The same results also were revealed for the D_3 fraction mandibular condylar supernatants when they were subjected to the same process.

Human Proteoglycans (A_1 A_1 D_1) and Rat Proteins

Samples of D_3 and D_4 nasal septal and mandibular condylar supernatant were subjected to the two dimensional gel electrophoresis and Western blot analysis using $35SO_4$ to label A1 A1 D1 human cartilage proteoglycan. The transfer paper were incubated overnight with A1 A1 D1 and were exposed to X-ray films. The end results revealed no binding between the human proteoglycans and the rat proteins.

A1 A1 D1 2-D Gel Electrophoresis

Chondroitinase ABC-pretreated gradient samples of A1 A1 D1 human proteoglycans were subjected to two dimensional gel electrophoresis. The gel was

dried and stored in a cold environment with the X-ray film. After several days, the X-ray film showed heavy molecular weights in the first well.

One Dimensional Gel Electrophoresis and Western Blot Transfer

Samples of D4 fraction of nasal septal, mandibular condylar, and synchondrosal supernatants and pellets were subjected to one dimensional gel electrophoresis. The samples used were in full strength of protein concentration. One of the gels was stained with Coomassie blue and the second gel was used to do the western blot transfer. The transfer which was successful showed light bands of proteins with Coomasie blue staining. On the X-ray film, the transfer and binding of proteins with human proteoglycans gave negative results. The experiment was repeated showing better transfer but continued to show no binding of rat proteins with human cartilage proteoglycan.

[illegible]

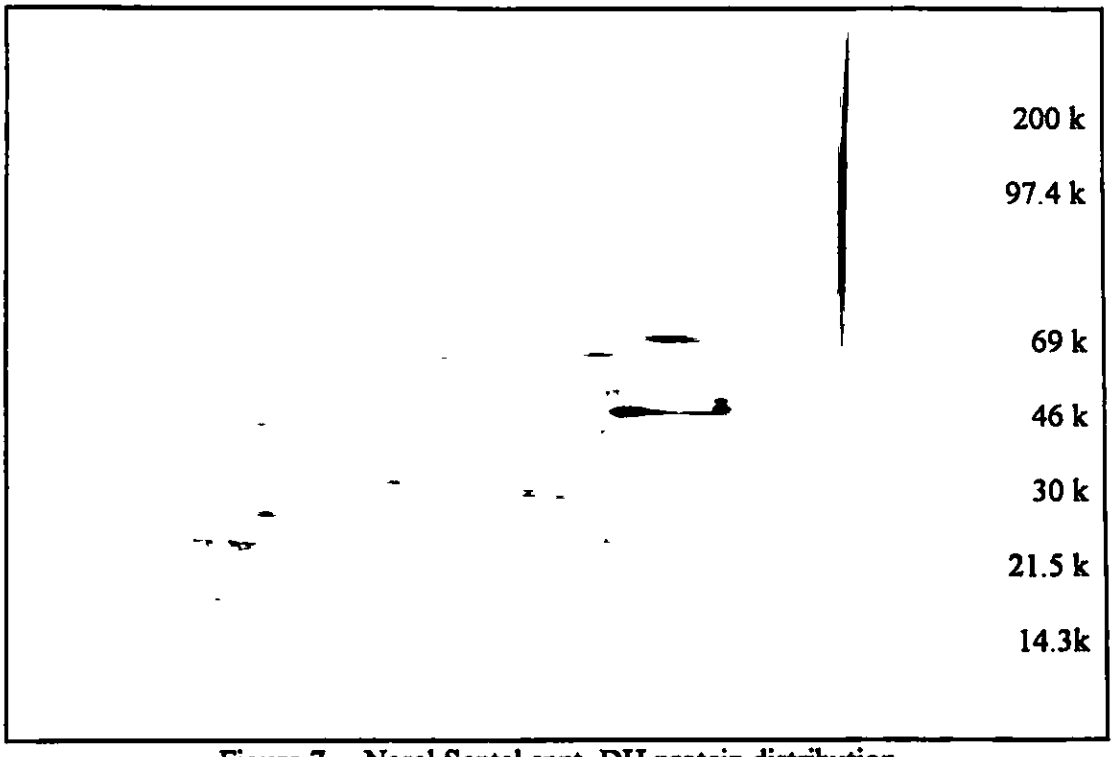


Figure 7.-- Nasal Septal spnt. DH protein distribution
15 Ks rat nasal septal-mandibular condyle-syncondrosal
3-16% acrylamide gel/stain: silver stain
electrophoresis was for 4 hours at 200 ma

Table 17 -- Nasal Septal Spernatant D4 Fraction 2-D Gel Protein P I. and M.W

Protein Fraction	Prot. No.	Prot. P.I.	Prot. M W
N S. SPNT. D4	1	6.8	69
	2	6 8	30
	3	6.7	21.5
	4	6.6	30
	5	6.4	30
	6	6.2	97.4
	7	6.1	69
	8	5.9	40
	9	5.8	40
	10	5.5	69
	11	5.5	46
	12	5.5	25
	13	5.5	14.3
	14	5.3	69
	15	5.4	30
	16	5.15	21 5
	17	4.95	69

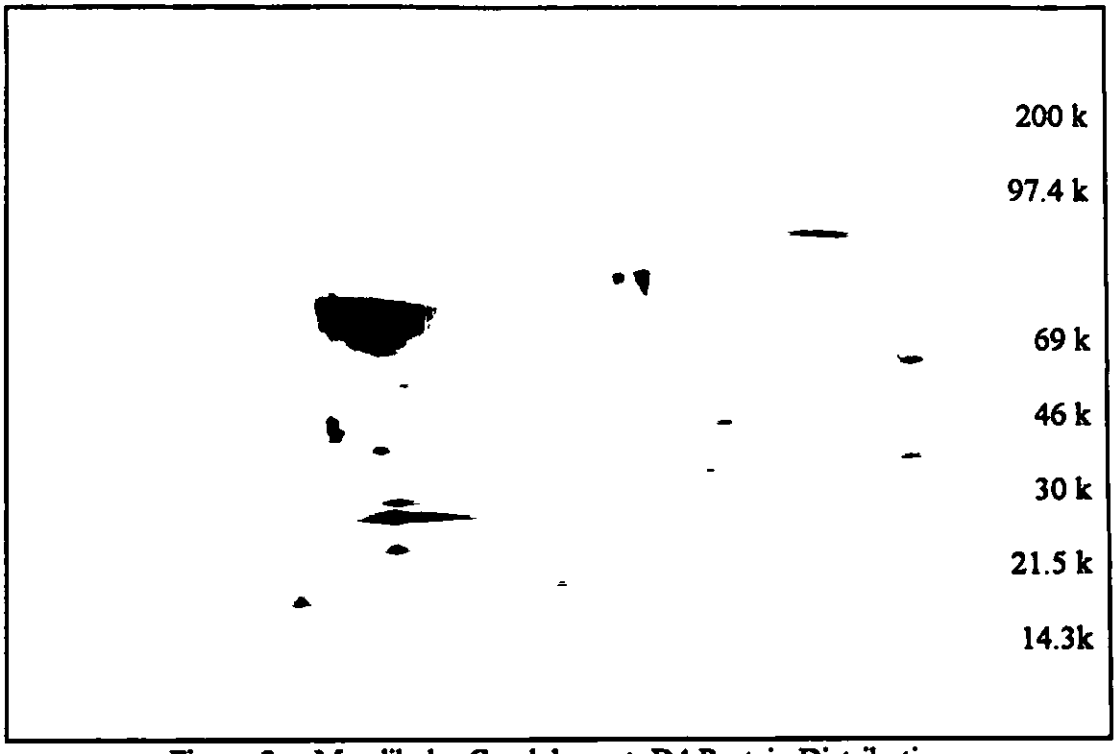


Figure 8.-- Mandibular Condyle spnt. D4 Protein Distribution
15 Ks rat nasal septal-mandibular condyle-syncondrosal
3-16% acrylamide gel/stain: silver stain
electrophoresis was for 4 hours at 200 ma

Table 18.-- Mandibular Condyle Supernatants 2D. Gel D4 Fraction
Protein P I. and M.W.

Protein Fraction	Prot No.	Prot. P I.	Prot. M.W
M.C.SPNT. D4	1	6 8 - 6.3	97 4
	2	6.8	25
	3	6.7	69
	4	6.7	25
	5	6.7	21.5
	6	6.7	60
	7	6.5	46
	8	6.5	45
	9	6.5	30
	10	6.3	70
	11	6.3	21.5
	12	6.3	14.3
	13	6	25
	14	6.1	25
	15	5.8 - 5.3	97.4
	16	5.08	50
	17	5.15	50

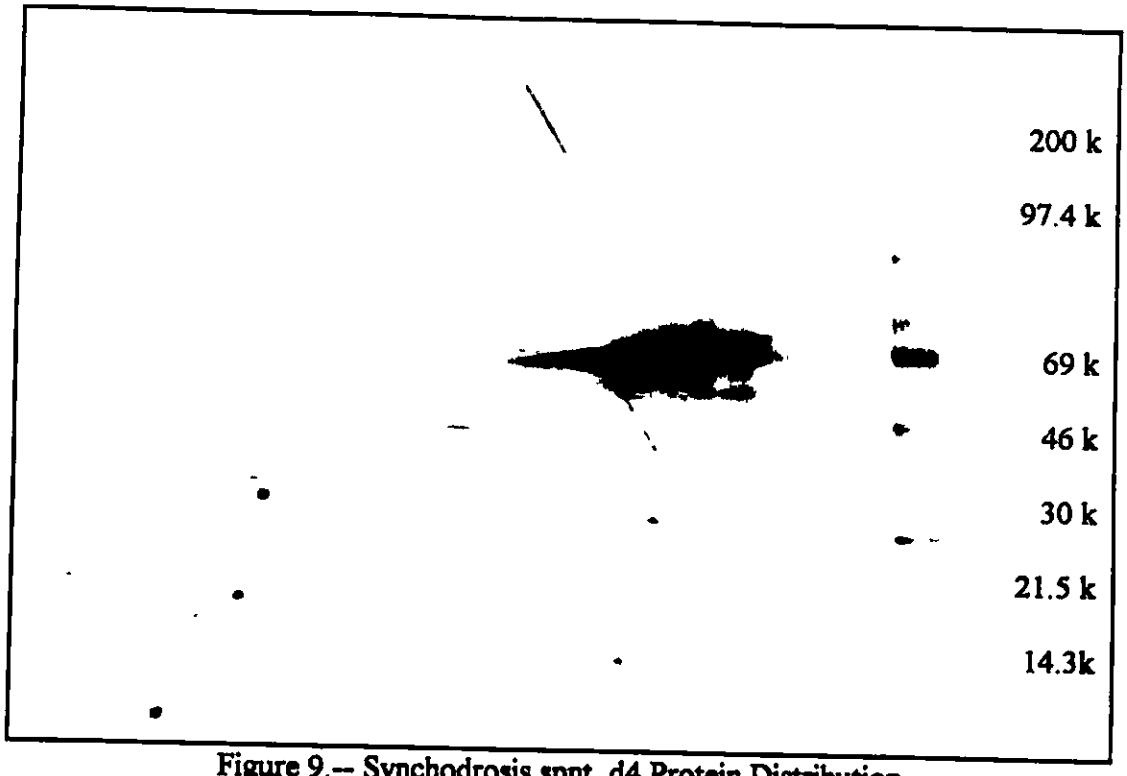


Figure 9.-- Synchodrosis spnt. d4 Protein Distribution
15 Ks rat nasal septal-mandibular condyle-syncondrosal
3-16% acrylamide gel/stain: silver stain
electrophoresis was for 4 hours at 200 ma

Table 19 -- Sphenosynchondrosal Supernatant 2D. Gel D4 Fraction
Protein P.I. and M.W.

Protein Fraction	Prot. No	Prot P.I.	Prot M.W
SYNC SPNT. D4	1	6 8	40
	2	6.3	46
	3	6.8	21 5
	4	6.1 - 4.95	69
	5	5.5	25
	6	5.3	30

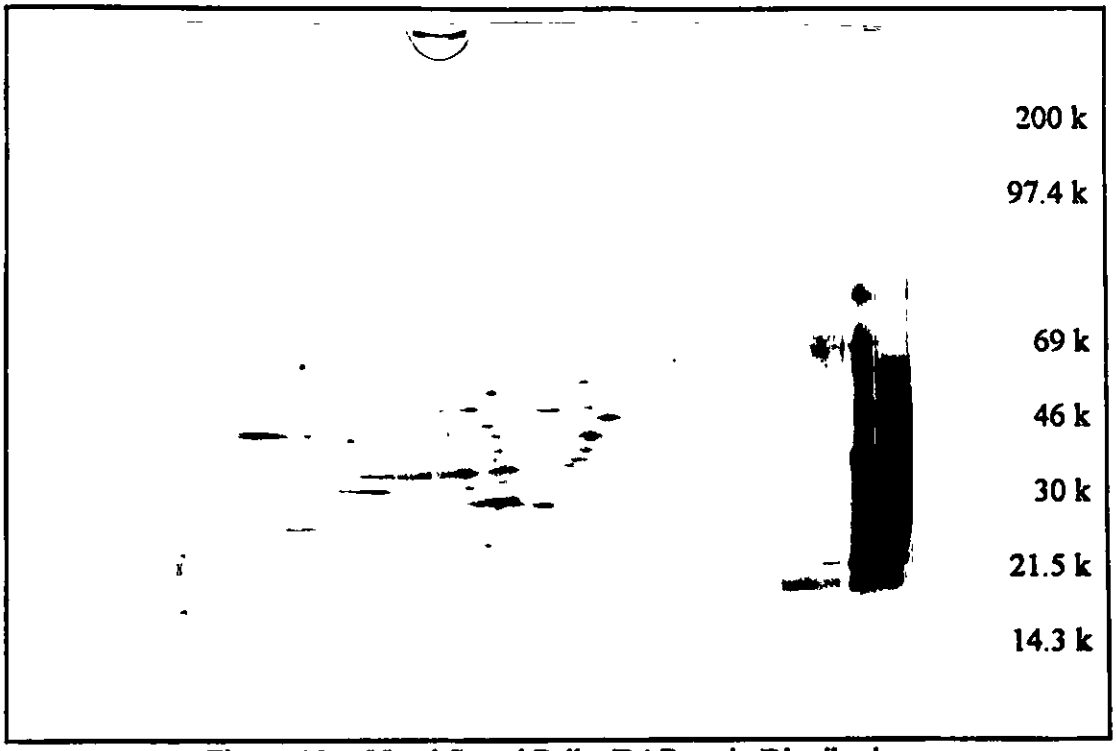


Figure 10.-- Nasal Septal Pellet D4 Protein Distribution
15 Ks rat nasal septal-mandibular condyle-syncondrosal
3-16% acrylamide gel/stain: silver stain
electrophoresis was for 4 hours at 200 ma

Table 20.-- Nasal Septal Pellet D4 Fraction 2D Gel Protein P.I. and M.W.

Protein Fraction	Prot. No	Prot. P.I.	Prot. M.W.
N S. Pellet D4	1	6.9	21.5
	2	6 8	46
	3	6.5	32
	4	6 2	40
	5	6.1	50
	6	6.1	40
	7	6.1	30
	8	6.1	25
	9	5.8	69
	10	5.5	69
	11	5.4	46
	12	5.5	14.3
	13	5.4	14.3

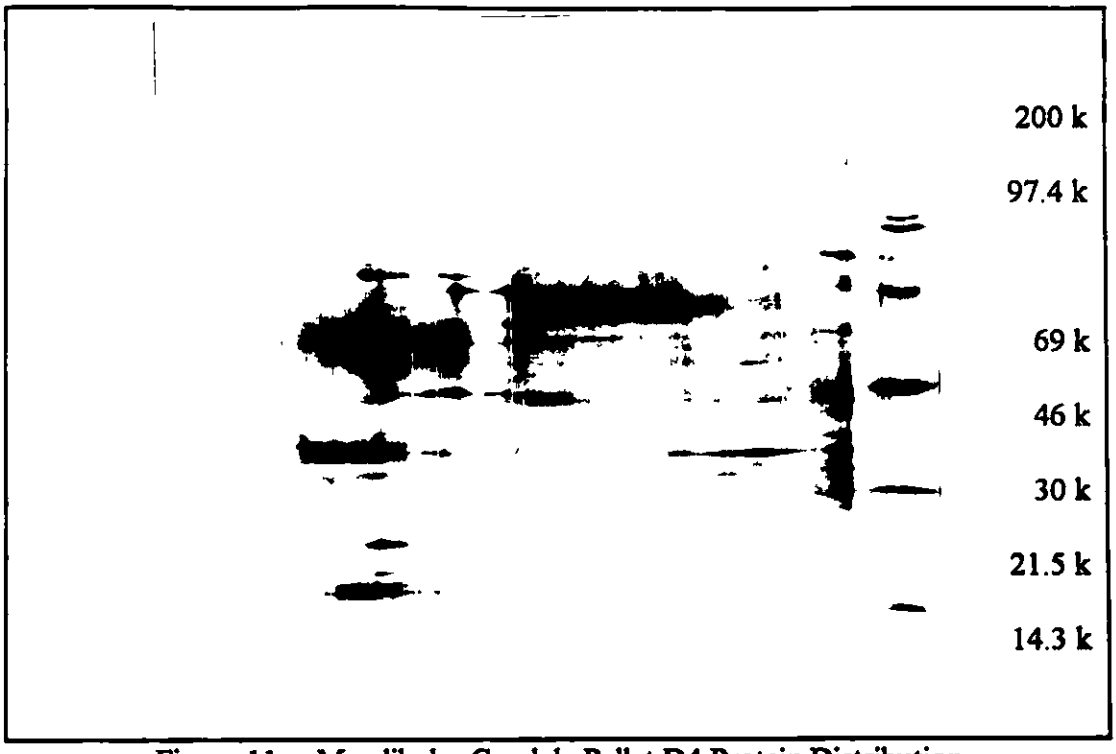


Figure 11.-- Mandibular Condyle Pellet D4 Protein Distribution
15 Ks rat nasal septal-mandibular condyle-syncondrosal
3-16% acrylamide gel/stain: silver stain
electrophoresis was for 4 hours at 200 ma

Table 21.-- Mandibular Condyle Pellet D4 Fraction 2D Gel Protein P.I. and M W

Protein Fraction	Prot No.	Prot. P I.	Prot. M.W.
M.C. PELLET D4	1	6.5	97.4
	2	6.7 - 6.3	69
	3	6.8 - 6.3	40
	4	6.5	21.5
	5	6.7 - 6.3	21.5
	6	6.3	97.4
	7	6.4	46
	8	6.1	69
	9	5.8 - 5.08	69
	10	4.95	40

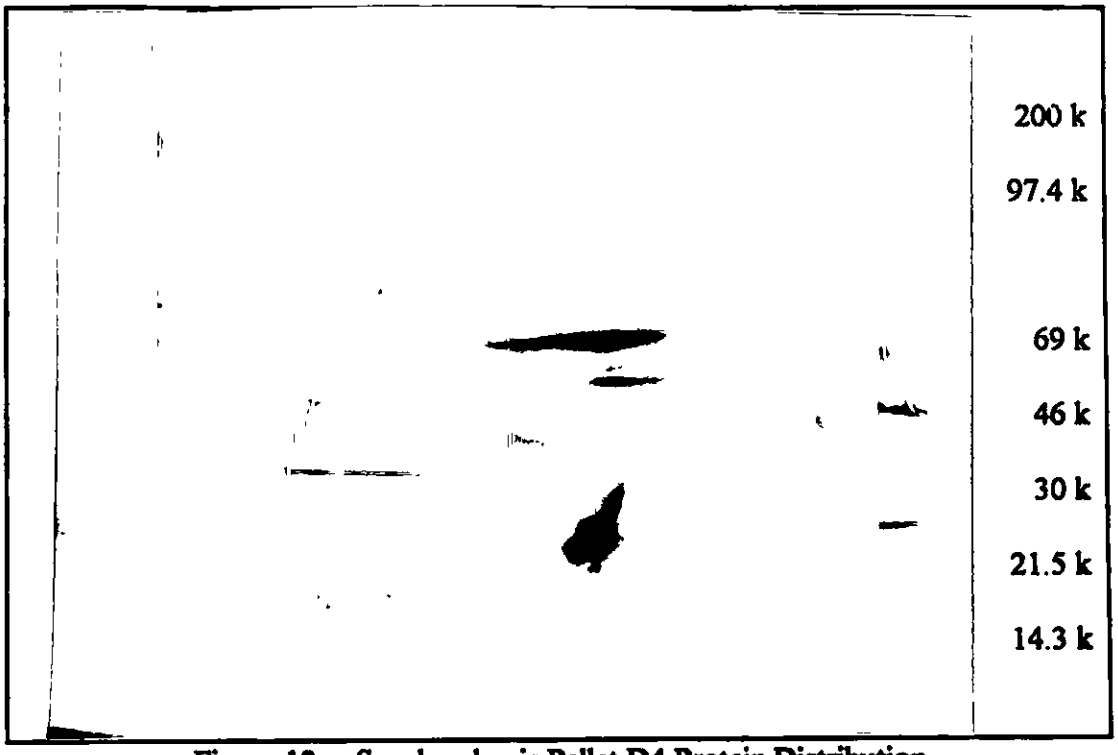


Figure 12.-- Synchondrosis Pellet D4 Protein Distribution
15 Ks rat nasal septal-mandibular condyle-syncondrosal
3-16% acrylamide gel/stain: silver stain
electrophoresis was for 4 hours at 200 ma

Table 22.-- Sphenosynchondrosal pellet D4 Fraction 2D Gel

Protein Fraction	Prot No	Prot. P.I.	Prot. M.W.
SYNC. Pellet D4	1	7	69
	2	6.8 - 6.1	40
	3	6.5 - 6.3	50
	4	6.1 - 5.15	69
	5	5.8 - 5.15	60

DISCUSSION

Protein sequence determination and antibody production are important parts of modern molecular cell biology. Native and denatured proteins are used as antigens to produce antibodies, which recognize the original native proteins. These proteins used to purify the antigens in preparative scale as well as determine the amount of antigens in the cell. It is established procedure that partially determined amino acid sequences are frequently used to assist in the isolation of cDNA coding for a particular protein (Suggs, 1981).

Unfortunately, the proteins of interest in this study are available only in limited amounts. Because of this fact, attempts have been made to develop an efficient purification technique to determine protein sequence and to produce antibodies. A currently available method uses mainly sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Hunkapiller, 1983). For the study, eighty rats were ordered with one to two grams of protein obtained from each rat. Two dimensional gel electrophoresis was used to separate the proteins in the nasal septum mandibular condyle, and sphenosynchondrosal cartilages.

The two dimensional gel electrophoresis was chosen instead of one dimensional gel because Smithies, (1955) demonstrated that a starch gel could serve as a molecular sieve through which zone electrophoresis of proteins could be carried out. Initially, the main objective of the two-dimensional gel electrophoresis technique was to monitor visually the homogeneity or heterogeneity of a protein preparation and to

follow the appearance or disappearance of a protein "band" throughout a given experimental procedure. One-dimensional gels were found to be quite adequate when relatively simple protein samples were analyzed. But, as more complex systems demanded greater resolving power, new two dimensional systems were developed to efficiently clear hundreds or even thousands of polpeptides found in the same sample. However, the task of correlating a protein or "spot" with a known function has often been difficult. The two-dimensional gel techniques rely on maintaining at least one of the following prerequisites: 1) retention of polypeptide's specific activity throughout electrophoresis, 2) possible renaturation of a denatured polypeptide, 3) covalent crosslinkage of the protein in question to a detectable ligand prior to electrophoresis. Gel electrophoresis in one direction, followed by a gel electrophoresis in an orthogonal direction (two-dimensional electrophoresis), has been used to increase the number of electrophoretically distinguishable proteins and determine whether or not proteins that form a single band after electrophoresis in one direction have a uniform electrophoretic mobility, (Wieman and Sicher, 1955). The gel used for the first electrophoresis to be referred to as the first dimension gel must be carried and placed at the origin of a slab gel for the second electrophoresis. Currently, most gel electrophoretic separations are performed on slab gels. Mauer (1974) stated a disadvantage that upon staining and destaining all protein samples migrated in the different channels and were treated in exactly the same way: when there are strong differences in the amounts of protein loaded in the different channels, it may turn out that some lanes are overloaded while

others became invisible after normal destaining The two-dimensional gel system is satisfactory for resolving peptides longer than 30 amino acids (Davie, 1982). However, peptides shorter than of 30 amino acids migrated as smears on the second-dimension gel. Based on the numerous experiments that were carried out with this study various concentration of proteins were tested to choose the best resolution which obtained using 20 grams of protein (full strength).

It is generally agreed that a mesenchymal stem cell divides many time and gives rise to skeletal tissues: cartilage, bone, tendon, ligament, marrow stroma, and connective tissue, Caplan, (1984). Both intrinsic and extrinsic factors combine to control the molecular and cellular pattern of expression that results in specific tissues that perform specific functions based on their molecular repertoire. The sum of these various intrinsic and extrinsic signals defines the development position of the cells.

The craniofacial cartilages of interest are the nasal septum, mandibular condyle, and sphenosynchondrosis. All of which develop from the same germ layer which is mesenchymal. It will be very easy to conclude that in terms of extracellular matrix proteins one could hypothesize that all these cartilages should contain the same kinds of matrix proteins. The results showed, however, some similarities and dissimilarities among these cartilages. The dissimilarities existed between the supernatant and the pellet of the same cartilage. The real question is: does the similarity have anything to do with facial growth? It appears that all cartilages may not behave in the same manner, and that generalized conclusions relating to all cartilaginous tissues can not be

supported (Brigham, 1976).

Based on the similarities and dissimilarities of the protein in each cartilage of interest, the results obtained are consistent with the assumption that condylar cartilage is fundamentally different, from all other craniofacial cartilages. Possibly, this is because of the multiple functions condylar cartilages perform. The dissimilarities among the craniofacial cartilages may be related to the kind and nature of each cartilage; the condylar cartilage is secondary cartilage while the nasal septal and the sphenosynchondrosal cartilages are primary. Further study is indicated to enhance our understanding of this problem. Craniofacial cartilages are responsible for overall facial growth, and the dissimilarities among the proteins of the nasal septum, mandibular condyle, and sphenosynchondrosis may be the reason behind the uniqueness of this process. For example, a Class II or Class III skeletal malocclusion may be related to the activity of the dissimilar proteins in the craniofacial cartilages, which means activity (turn-on, turn-off) is compromised. It is possible to speculate that in a Class II skeletal malocclusion dissimilar proteins are activated or turned-on, allowing the nasal septum to overgrow while the dissimilar proteins in the mandibular condyle are turned-off, creating the Class II skeletal malocclusion, vice-versa in the Class III skeletal malocclusion.

Another speculation in regards to the facial type and the way that the face grows is that the similar and dissimilar proteins in craniofacial cartilages work hand-in-hand to create the unique facial type that is observed, brachycephalic or

dolicocephalic patients have completely different protein patterns, and the activity of these proteins is different but the real question is how then can one explain the offspring from brachycephalic mother and a dolichocephalic father? This requires further study. The two-dimensional gel, mandibular condylar and sphenosynchondrosal supernatants, provide greater quantity of the proteins with low molecular weights and are much higher in molecular weight than that of the nasal septal supernatants. This enables us to speculate that these low molecular weight proteins might have some kind of function which we do yet not understand. This function might have something to do with controlling the local growth of the rat face.

The results obtained showed similarities of protein distribution and location between nasal septal supernatants (Figure 1) and synchondrosal pellets (Figure 6, Table 6) except that they differed with each other in terms of microheterogeneity. Such few similarities that existed between synchondrosal supernatants and pellets of the D4 fraction would support the need of further research in identifying unique proteins. Therefore, the decision was made to use a template on the two-dimensional gel supernatant and pellet D4 fractions where proteins were the points of interest of comparison showing a needs for greater scrutiny in the future.

After finding out the full strength of protein concentration and attempting to compare the protein pattern and distribution for the three cartilages of interest, I would like to suggest using a computer program be employed to compare the protein distribution with future experiments. This will make it easier to find out which protein

is unique to a particular cartilage. By using the triangular method on two-dimensional gels, one will be able to identify the protein of interest for each craniofacial cartilage (Figure 14).

There is more than one way of staining a two-dimensional gel electrophoresis. The best way is using silver-staining because the proteins in polyacrylamide gels, developed by (Switzer, 1974) were shown to be about 100-fold more sensitive than those using conventional staining with Coomassie blue R-250. The technique, modified by Oakley (1980) is now simpler and cheaper than the original. Moreover, treatment of gels with dithiothreitol results in more reproducible patterns.

The second part of this study was designed to bind the human proteoglycans with the proteins from the nasal septum, mandibular condyle, and sphenosynchondrosis. The rationale behind the binding study was based on the fact that extracellular matrix proteins have the potential to alter the function of proteoglycans by binding them. The results were negative because there was either no binding at all, binding occured but weak to the degree where we cannot detect the proteins on the transfer paper, species differences, or age differences, because the proteoglycan samples were from sixty-five year old patients. To achieve the binding , associative gradient of A1A1 was prepared then treated by dissociative CsCl gradient to obtain A1A1D1. This experiment gave negative results meaning this could be due to the fact that a highly purified species of proteoglycan was used.

The negative binding between the human proteoglycans and the rat craniofacial

cartilages may also be related to the introduction of proteoglycan residues into proteins, or species differences which may alter their electrophoretic mobility.

In conclusion, the use of two-dimensional gel electrophoresis SDS technique revealed the many differences among the condylar, synchondrosal, and nasal septal cartilages ECMP exist. In addition, regional differences in the location of the proteins were revealed only within the mandibular condylar cartilage. Condylar, sphenosynchondrosal, and nasal septal cartilages possess common proteins evidenced in the various samples. Condylar cartilage possesses at least one unique protein distinct from those of both the sphenosynchondrosal and nasal septal cartilages. Electrophoresis analysis revealed compositional differences among the three cartilages with the differences in condylar cartilages appearing to be the most extensive. These results were consistent with the assumption that condylar cartilages are fundamentally different from other craniofacial cartilages. Therefore, it appeared that all cartilages may not behave in the same manner, and that a generalized conclusion for all craniofacial cartilages cannot be supported.

APPENDIX

Optimizing Protein Transfer

Gel Composition	Decrease the total monomer in the gel formulation to create a more porous gel Gradient gels are often more effective than single percentage gels' for resolving a wide range of proteins.
Transfer time	An initial test run should be performed to determine the time required for complete transfer. Times vary from 30 minutes to overnight; overnight transfers should be performed at low voltages and with the super cooling coil to minimize heating.
Power setting	Initial test runs should be performed to evaluate the transfer efficiency at various field strengths (V/cm). Temperature increases may change buffer resistance and subsequently the power delivered, as well as the state of protein denaturation, thus affect transfer efficiency.
Buffer strength	Dilution of the transfer buffer results in decreased current drain at any given voltage. This will allow the use of higher voltages without excessive heating. Reuse of transfer buffers is not recommended since buffers lose their ability to

	maintain a stable pH during transfer.
Buffer type and PH	Different buffer types may yield different transfer efficiencies. Generally, this buffers allow more efficient transfer than acetate or phosphate buffers. Proteins near their isoelectric point will transfer poorly. Use a more basic or acidic buffer to increase protein mobility. Do not adjust the pH of transfer buffers. Adjustment of buffer pH will result in increased buffer conductivity, manifested by a higher than expected initial current drain and decreased resistance.
SDS	The addition pH SDS to the transfer buffer can increase protein mobility, but may also reduce the binding efficiency to nitrocellulose. SDS will increase the relative current, power and heating, and also may affect the antigenicity of some proteins. Elimination of alcohol in the transfer buffer results in increased protein mobility but diminishes the binding of SDS proteins to nitrocellulose.
Membrane selection	The properties of membrane should be considered in selection of the transfer conditions, e.g. the use of PVDF or positively charged nylon membrane allows efficient transfer in the presence of SDS.

Equilibration	Rinse the gel and membrane buffer prior to transfer to help prevent shrinking or swelling of either component during transfer, and to eliminate contaminating electrophoresis buffer salts and other reactants such as urea or SDS. If not removed, the salts will increase the conductivity of the transfer buffer and the amount of heat generated during the run.
Gel-membrane contact	Poor contact due to air bubbles at the gel-membrane interfere will reduce the efficiency of protein transfer. The use of a test tube or pipette as a " rolling pin" and proper selection of filter paper spacers will help assure good contact.
Stirring	The use of a stir bar inside the transfer cell will help to maintain uniform conductivity and temperature during the transfer

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