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AN INVESTIGATION OF ANTIGENIC DIFFERENCES AMONG CONDYLAR, EPIPHYSEAL, AND NASAL SEPTAL CARTILAGES

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Abstract

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The history of modern craniofacial biology is characterized by a marked concern for the role that the major craniofacial cartilages play in the growth of the craniofacial skeleton. It was once widely assumed by many investigators that these cartilages acted as "growth centers" in a manner analogous to cartilaginous epiphyseal plates. In recent years, however, attention has turned toward the apparent differences in growth properties that exist among these various cartilages. By inference from the results of investigations designed to compare these cartilages, in general, and from the results of transplantation studies, in particular, several investigators have concluded that differences exist in the fundamental properties of the various cartilages. The verity of this contention has not yet been proven, and whether or not there is a molecular basis for the suggested differences has never been subjected to experimental verification.

The present study used both indirect immunofluorescent labeling and the polyacrylamide gel disc electrophoresis to investigate antigenic and chemical differences among the cartilages of the

nasal septum, condyle, and epiphysis. The cartilages were obtained by careful dissection from six month old calves, reduced and alkylated with 2-mercaptoethanol and iodoacetic acid respectively, and their soluble extracts used as antigens for the production of specific antisera in rabbits. Non-specific antibodies were removed from the antisera by adsorption against bovine liver and spleen. Examination for antigenic differences among the various cartilages was conducted in three separate experiments, in which samples of each of the various antisera were adsorbed against nasal septal, epiphyseal, and condylar cartilage respectively and applied to tissue sections of each of the three cartilages. The antigen-antibody complexes were subsequently labeled with fluorescein-conjugated anti-rabbit globulin and examined microscopically.

In addition, limited characterization of the soluble fractions was accomplished by sodium dodecyl sulfate gel electrophoresis. The resulting band patterns of each of the cartilaginous fractions were stained with a protein-specific dye and compared.

Microscopic examination of the immunofluorescent labeled sections of the three cartilages revealed that fluorescent staining of both the epiphyseal and nasal septal cartilages was limited to the territorial matrix, whereas the entire territorial and interterritorial matrix of the condylar cartilage fluoresced strongly. Moreover, regional differences in staining were observed within the nasal septal cartilage.

The antisera of each of the three cartilages demonstrated an

ability to cross-react with the other cartilages. Adsorption of each of the antisera against the other two cartilages indicated that condylar cartilage possesses at least one unique antigenic determinant.

Limited characterization of the soluble fractions of each of the cartilages by gel electrophoresis revealed that, with minor variations, nasal septal and epiphyseal are essentially similar. By contrast, the electrophoretic patterns of the soluble fraction of the condylar cartilage were clearly different from both the epiphyseal and nasal septal cartilages.

The results of the study may be summarized:

1. The use of the indirect immunofluorescent technique revealed antigenic differences among the condylar and the epiphyseal and nasal septal cartilages. In addition, regional differences in the location of antigenic determinants were revealed within the nasal septal cartilage.

2. Condylar, epiphyseal, and nasal septal cartilage possess common antigenic determinants as evidenced by the ability of each of the antisera to cross-react with the various cartilages.

3. Condylar cartilage possesses at least one antigenic determinant distinct from those of both the epiphyseal and nasal septal cartilage.

4. Electrophoretic analysis revealed compositional differences among the condylar, epiphyseal, and nasal septal cartilaginous constituents, with the differences in condylar cartilage appearing to be most extensive.

5. The results are consistent with the assumption that condylar cartilage is fundamentally different from other craniofacial and appendicular cartilages.

6. Therefore, it appears that all cartilages may not behave in the same manner, and that generalized conclusions for all cartilaginous tissues cannot be supported.

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for her considerate kindness and friendship

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INTRODUCTION

Throughout much of the course of prenatal development, the major tissue of the craniofacial skeleton is cartilage. Although in most areas it is gradually replaced by bone, cartilage remains a major skeletal constituent of the head throughout much of the early postnatal life of the individual. By inference from the growth of the epiphyseal plates of the appendicular skeleton, many craniofacial biologists have argued that the various cephalic cartilages serve to effect the bodily translation of certain associated bones within the craniofacial complex. Accordingly, it is generally assumed that condylar, synchondrosal, and nasal septal cartilage have an "intrinsic" growth potential that is only minimally affected by local environmental influences. To test this hypothesis, several investigators have transplanted these cartilages to presumably "neutral" sites (brain, subcutaneous tissue, etc.) and have been surprised by the variability of the results obtained. As a result, it has become popular to "explain" the differences among experiments in terms of differences in the fundamental properties of the various cartilages involved. The evidence supporting this interpretation is somewhat circumstantial and decidedly "after the fact."

Nevertheless, as a direct extension of these findings, several investigators have implied that these differences in results reflect the relative importance of each of the various cartilages to the growth

of its associated skeletal structures. Unfortunately, there has been little experimentation to determine if there are, in fact, differences in the various cartilages at the molecular level.

Accordingly, the present investigation will examine and compare the cartilage of the nasal septum, condyle, and epiphysis to determine if there is a molecular basis for the apparent differential response of these cartilages to various experimental conditions. The experiment will involve the use of certain chemical, immunochemical, and immunohistochemical techniques designed to determine if chemical and antigenic differences exist among these cartilages.

REVIEW OF THE LITERATURE

The main cartilages in the postnatal craniofacial complex are the nasal septum, the synchondroses of the cranial base, and the condylar cartilage of the mandible. It has been claimed that each of these function in a manner similar to the epiphyseal "growth cartilage" Baume, 1961; Sicher and Dubrul, 1970). Proponents of this classical view emphasize the role that these cartilages play as "growth centers," structures whose volumetric and linear expansion effect the downward and forward translation of their associated skeletal tissues. With respect to cartilage, the term "growth center" implies a "place of endochondral ossification with tissue-separating force" (Baume, 1961).

In contrast, a newer theory of craniofacial growth holds that all cartilaginous growth is a secondary and compensatory response to functional demands for increased respiratory volume and for surrounding soft tissue expansion. This theory, as enunciated by Moss and coworkers (1960, 1962, 1964, 1968, 1969, 1970, 1972) views the head as a region within which a variety of functions are carried out (i.e., respiration, olfaction, vision, hearing, etc.). The tissues, organs, spaces, and skeletal parts necessary to carry out a particular function are termed a "functional cranial component." Soft tissues of a functional cranial component are classified as either "capsular" or "periosteal" functional matrices, depending on their relation to transformative and translative growth. Skeletal units are classified as either

"macroskeletal" or "microskeletal" units, depending on their relation to functional matrices.

Thus, the expansion of capsular matrices (brain, eyeball, spaces, etc.) is seen as responsible for all translative growth (relocation in space), while the action of periosteal functional matrices (muscles, blood vessels, nerves, etc.) is seen as responsible for transformative changes (alterations in size and shape) of the skeletal unit (Moss and Young, 1960; Moss and Salentijn, 1969; Moss, 1969).

Based on the assumption that condylectomy experiments had demonstrated only a secondary or compensatory role for the growth of condylar cartilage, Moss (1972) outlined the conceptual framework of the method of functional cranial analysis, including its unified view of the role of all craniofacial "growth cartilages" (p.481):

> In a word, what was true at one sutural area was true for all, and what was true for one cranial cartilage equally was presumed to be true for the others.

Clearly then, two theories have evolved that historically have been involved with the role that the craniofacial cartilages assume in the growth of the craniofacial complex. The classical theory suggests that all cartilages act as "growth centers" serving to direct and effect translatory growth, whereas the newer theory holds that the growth of craniofacial cartilages is entirely secondary to the growth of "functional matrices." Both theories are unified in their view of the growth of craniofacial cartilages and, as such, are attractive to

students of craniofacial biology. However, both theories are currently quite controversial in their unitary claims. Moreover, neither theory offers a convincing explanation for what controls the proliferation of the craniofacial cartilages at the cellular level. The answer to this question, it would seem, would be the key to determining if the craniofacial cartilages do, indeed, possess an "intrinsic" growth potential.

Evidence Implicating the Somatomedins as a New Class of Growth-Regulating Hormones

The anterior pituitary gland secretes six major polypeptide hormones. Of the six, somatotropin (also referred to as growth hormone or GH) has been implicated as the pituitary hormone most directly responsible for the regulation of skeletal growth.

The effects of GH on cartilage have been investigated extensively both in vivo and in vitro. The most important reason for choosing this tissue is that radioactive sulfate ($^{35}SO_4$) is preferentially accumulated by the glycosaminoglycans of cartilage, and this accumulation can readily be monitored both in vivo and in vitro.

When the incorporation of $^{35}SO_4$ by costal cartilage segments from normal and hypophysectomized (hypox) rats are compared in vitro, significant differences are observed. If short incubations with $^{35}SO_4$ are carried out immediately following removal from the animal, the sulfate accumulation by normal cartilage is 2-3 times that of the hypox cartilage (Daughaday et al., 1968). However, within 24 hours after GH treatment of hypox rats, sulfate incorporation by costal cartilage in vitro is restored to normal (Salmon and Daughaday,

1957). In vivo, the stimulation of sulfate incorporation by growth hormone in epiphyseal and nasal septal cartilage was found by Murphy and co-workers (1956) to be essentially the same as the in vitro tests with costal cartilage. Only physiologic amounts of GH are required for sulfate incorporation into cartilage glycosaminoglycans, and the effects cannot be reproduced with physiologic amounts of any other pituitary hormone.

In an attempt to develop a more sensitive bioassay for growth hormone, Salmon and Daughaday (1957), made an interesting observation that has led to a re-evaluation of growth hormone as the primary mediator responsible for cell proliferation in cartilaginous tissues. Having established that serum containing normal levels of GH stimulated radioactive sulfate uptake by cartilage in vitro (whereas serum from hypox children failed to do so), these investigators demonstrated that the serum of hypox children acquired the ability to incorporate sulfate in cartilage when growth hormone was administered to these hypox children. However, when the serum of the hypox children was enriched by GH in vitro, no sulfate uptake was observed (Daughaday et al., 1959; Daughaday and Kipnis, 1966; Daughaday, 1971). These observations led to the hypothesis that there is a substance in serum stimulated by, but distinct from, GH that mediates its growth-promoting effects.

Subsequent studies have indicated that normal plasma contains a number of growth hormone-dependent peptides which stimulate the cellular proliferation of both skeletal and extraskeletal tissues (Salmon and DuVall, 1970; Uthne, 1973; Pierson and Temin, 1972;

Morell and Froesch, 1973; Temin et al., 1972). Consequently, the term "somatomedin" was introduced to include all growth hormone-dependent substances that stimulate growth in responsive tissues (Daughaday et al., 1972).

Correlations Between Growth Rate and Somatomedin Levels in Plasma

Somatomedin has been found in the plasma of normal individuals of all ages. Significantly, reduced plasma levels of somatomedin have been reported consistently in hypopituitarism (Daughaday et al., 1959; Parker and Daughaday, 1968; Kogut et al., 1963; Almqvist et al., 1961; Brasel et al., 1965). As measured by a variety of bioassay techniques, plasma somatomedin levels in hypopituitary animals (including humans) average one third to one fourth of those of normal animals. Moreover, partial or complete restoration of somatomedin levels to normal is achieved by GH therapy (Daughaday et al., 1969).

Elevations in plasma somatomedin are found frequently in acromegalic patients (Daughaday et al., 1959; Almqvist et al., 1961), and rats bearing GH secretory tumors also show corresponding elevations in somatomedin levels (Peake et al., 1968).

Growth arrest is common in children with Cushing's syndrome and in children receiving large doses of corticosteroids. Elders (1973) has shown that these children also have low plasma somatomedin levels.

Wiedemann and Schwarz (1972) have demonstrated that estrogen therapy normalizes elevated plasma somatomedin levels in acromegalic

patients without lowering basal plasma growth hormone levels. This finding may explain the attenuation of growth observed in tall girls receiving high dosage estrogen therapy (Wettenhall and Roche, 1965).

A unique form of familial dwarfism occurring in some Asiatic Jews has been described by Laron et al. (1966). Clinically these patients resemble hypopituitary dwarfs; however, unlike hypopituitary patients, these subjects have low somatomedin levels that do not respond to administration of exogenous GH (Laron et al., 1971). Van Wyk et al. (1975) have suggested that the basic defect in these dwarfs is insensitivity to GH with concomitantly defective somatomedin generation.

Origin of Somatomedin

Since somatomedin does not appear to be stored in any particular organ and since plasma appears to be the greatest source of somatomedin, the question of whether it is synthesized de novo or is, instead, a cleavage product of growth hormone remains unsettled. Furthermore, the mechanism by which growth hormone induces the production of somatomedin remains largely unknown.

One of the initial hypotheses advanced to explain the origin of somatomedin is that GH is secreted in an inactive form in a manner analagous to the release of insulin from proinsulin. Since the somatomedin molecule appears to be about one third the size of GH, the possibility exists that somatomedin may be the result of a cleavage of the GH molecule itself. However, the observation that specific

antibodies to GH do not antagonize the action of somatomedin argues against this hypothesis, because this finding requires that the cleavage of GH would result in the loss of all the antigenic determinants in the original molecule (Daughaday and Kipnis, 1966).

Another possibility is that proinsulin serves as a prohormone for both insulin and somatomedin. Indeed, the insulin-like effects of somatomedin are striking (Daughaday, 1971), and it has been reported that the highly specific insulin receptor sites on the cell membranes of certain tissue cells (i.e., chondrocytes, adipocytes, liver membranes) are incapable of distinguishing between insulin and somatomedin (Hintz et al., 1972). Prior to the observations of Hintz and co-workers, only insulin and proinsulin had been shown to compete with radioactive insulin for binding to insulin receptor sites. The possibility that somatomedin is derived from proinsulin would not negate the regulatory role of GH, inasmuch as insulin levels are reported to be low in hypopituitarism, high in acromegaly, and to rise following the administration of GH (Daughaday and Kipnis, 1966; Underwood et al., 1973).

Biochemical Phenomena Induced in Cartilage by Somatomedin

Although general anabolic effects of GH have been difficult to demonstrate on isolated tissues, this has not been the case for somatomedin. The effects of somatomedin on several processes in cartilage metabolism have been well-documented. A primary role of somatomedin in amino acid uptake was demonstrated by Adamson and Anast

(1966) in experiments with embryonic chick cartilage in vitro. Salmon and DuVall (1970) have shown that somatomedin induces both the incorporation of $^{35}SO_4$ and labeled leucine (H^3-leucine) into proteoglycan. Moreover, the inhibition of protein synthesis by puromycin also blocks the incorporation of sulfate by somatomedin (Salmon et al., 1967). These observations are consistent with the concept that the proteoglycan macromolecule is synthesized intracellularly as a unit, and then associated with collagenous fibrils extracellularly to form the cartilaginous matrix. Daughaday and Mariz (1962) have demonstrated that somatomedin stimulates the formation of collagen in cartilage in vitro, whereas GH is inactive in this respect. The effect of somatomedin on the nucleic acid metabolism of cartilage has also been investigated. In thymidine uptake studies, Daughaday and Reeder (1966), observed that GH injection stimulates DNA replication in hypox rats whereas GH added in vitro had no effect on isolated cartilage from hypox rats. The investigators concluded that this process was controlled by somatomedin. Furthermore, Salmon and DuVall (1970) have demonstrated that somatomedin stimulates the uptake of uridine into cartilage RNA.

The Somatomedin Hypothesis

A considerable body of evidence has been reviewed which indicates that the effects of GH on cartilage are mediated indirectly by the induction of a circulating secondary mediator. On the basis of their growth-promoting activity, there is convincing evidence that

somatomedin and insulin act in a similar manner, and the demonstration that somatomedin, at physiologic concentrations, crossreacts with insulin receptors on the plasma membranes of chondrocytes and other cells strongly suggests that both somatomedin and insulin act by way of a common mechanism.

It is now well established that the expression of hormonal activity of many hormones on their respective target cells is mediated through cyclic adenosine monophosphate (cyclic AMP) (Robison, 1972). Specifically, cyclic AMP is a "second messenger" between a hormone and its effects within a cell (Robison et al., 1971). Formation of cyclic AMP occurs when an enzyme located within the cell membrane, adenylate cyclase, responds to the arrival of certain hormones at the membrane. The enzyme transforms molecules of adenosine triphosphate (ATP) within the cell into cyclic AMP (Birnbaumer et al., 1970).

In recent years, the role that cyclic AMP may play in regulating cellular proliferation has attracted considerable attention. In general, the currently held view is that cyclic AMP (or agents that stimulate its intracellular accumulation) inhibits the initiation of the proliferative process, while lowering intracellular cyclic AMP levels appears to be an important factor in allowing cellular proliferation to begin (Froehlich and Rachmeler, 1972, 1974; Eker, 1974). Many catabolic hormones such as epinephrine, parathyroid hromone, and glucagon act on their target cell membranes by stimulating adenylate cyclase activity, thereby producing an increase in the intracellular level of cyclic AMP (MacManus et al., 1970; Exton et al., 1971; Kelley

and Butcher, 1974). In contrast, growth-promoting hormones such as insulin act by diminishing cellular cyclic AMP through inhibition of adenylate cyclase activity (Otten et al., 1972; Anderson et al., 1973; DeAsua et al., 1973). Recently, somatomedin has been shown to be effective in inhibiting the adenylate cyclase activity of sub-cellular membranes of cartilage cells (Tell et al., 1973).

If the hypothesis that somatomedin induces all cartilaginous growth is to remain viable, then further investigations designed to test this hypothesis await the successful isolation of sufficient purified fractions of somatomedin. If the somatomedins should prove to promote cellular proliferation of all major craniofacial cartilages in vivo, they would potentially afford the first evidence, at the cellular level, that all craniofacial cartilages possess an "intrinsic" growth potential. However, in view of the considerable body of evidence indicating that specific growth-promoting substances exist for a wide variety of tissues, the problem of the observed differential response of condylar, synchondrosal, nasal septal, and epiphyseal cartilages to certain experimental situations raises some basic questions: Is there one growth-promoting somatomedin for all cartilages or several such substances for the individual cartilages? Are the differences in response due to differences in experimental conditions or to intrinsic differences in the chemical structure of the various cartilages? In view of the evidence reviewed here, the question related to the somatomedins cannot, as yet, be answered. However, in recent years considerable attention has been given to comparing these cartilages.

Investigations Comparing the Epiphyseal, Synchondrosal, Condylar, and Nasal Septal Cartilages

Structural Organization

Upon histologic examination, it becomes immediately obvious that the craniofacial and appendicular cartilages are not totally analogous. An important difference is the orientation of the chondrocytes. The epiphyseal cartilaginous plate assumes a zonal arrangement, characterized by parallel rows of cells throughout its zones of young, mature, and hypertrophic chondrocytes (Baume, 1961). The structural similarity between basicranial synchondroses and epiphyseal cartilaginous plates is striking, with the former differing from the latter mainly in synchondrosal cartilage's bipolar orientation (Baume, 1961; Graber, 1975). The cartilage of the nasal septum displays a cellular orientation of nearly-parallel rows of chondrocytes only at the septo-ethmoidal synchondrosis (Scott, 1953; Baume, 1961). The short rows of cells radiate from the synchondrosis in a manner similar to that observed in both epiphyseal and synchondrosal cartilages. Throughout the body of the septum, however, a great number of small, isolated chondrocytes are scattered randomly and separated from one another by cartilaginous matrix (Scott, 1953; Baume, 1961).

In contrast, the type of structural organization present in the aforementioned cartilages is lacking in the condylar cartilage. Specifically, the distribution of the cells of the condylar cartilage is more random in appearance (Petrovic et al., 1975). Moreover, the

zone of mature chondrocytes in the condylar cartilage is very narrow, with the newly-formed cartilage cells becoming hypertrophic almost immediately (Blackwood, 1966).

Another important structural distinction of the condylar cartilage is the presence of an "intermediate" zone of young cells, distinct from chondrocytes, and located immediately below the fibrous layer forming the articular surface of the condyle. These young cells, referred to by Petrovic and co-workers (1975) as "skeletoblasts," are stem cells apparently capable of differentiation into preosteoblasts or prechondroblasts, depending upon the influence of certain specific factors. According to Petrovic's group, these cells are not yet synthesizing cartilaginous matrix and are largely responsible for the proliferative growth of the condylar cartilage.

Embryonic Origin

The embryonic development of the craniofacial and appendicular cartilages has been studied by several investigators. The epiphyseal cartilaginous plates of long bones, the basicranial synchondroses, and the nasal septal cartilage are derivatives of the primary cartilaginous skeleton (Scott, 1953; Ford, 1956, 1958; Baume, 1961; Hamilton, Boyd, and Mossman, 1962). The primary cartilage of the mandible in man is Meckel's cartilage, a derivative of the first branchial arch (Symons, 1951). As first shown by Symons (1952), condylar cartilage originates from mesenchymal tissue independently from, but lateral to, Meckel's cartilage. Consequently, condylar cartilage is referred to as a

"secondary cartilage" (DeBeer, 1937) since it is not derived from the primary cartilaginous skeleton.

The Effect of Growth Hormone

Petrovic and co-workers (1975) have shown that the *in vivo* administration of bovine somatotrophic (growth) hormone to young rats significantly increases the rate of growth of condylar, epiphyseal, and synchondrosal cartilage. However, the proliferative response of the condylar cartilage to growth hormone ceases upon resection of the lateral pterygoid muscle (Petrovic and Stutzmann, 1972; Stutzmann and Petrovic, 1974). Gasson and Petrovic (1972) have demonstrated that growth hormone administered to a young rat produces an accelerated elongation of the upper jaw. After resection of the nasal septal cartilage, the supplementary increase in growth stimulated by somatotrophic hormone administration decreased significantly (Gasson *et al.*, 1972).

Nutritional Studies

The Effect of Zinc Deficiency

The importance of trace elements in biological growth and function is widely accepted and, among these, the need for zinc is recognized as essential for normal growth and development in animals and man. Although little is known about the specific action of zinc in biological systems, there is experimental evidence that zinc is essential for the synthesis of DNA, RNA, and protein. Bergman (1970)

has shown that the morphological changes induced experimentally in rats placed on a zinc-deficient diet included a significant reduction in thickness of the epiphyseal and condylar cartilages. In a follow-up investigation under identical experimental conditions, Myrberg et al. (1972) have shown that zinc deficiency also reduced the size and number of cartilage cells both in the nasal septum and the presphenoid and spheno-occipital synchondroses of rats.

The Effect of Extreme Levels of Vitamin A

The effects of both hypo- and hypervitaminosis A on the condylar, epiphyseal, and synchondrosal cartilages of the rat have been investigated by Baume (1970). Histological examination of the cartilages after long-term vitamin A deficiency (average 304 days) revealed complete arrest of chondrogenic activity of the condylar cartilage whereas the cartilages of the epiphyseal plate and basioccipital synchondroses maintained some chondrogenic activity (as evidenced by the columnar arrangement of chondrocytes and some active cartilage erosion). High doses of vitamin A (25,000 international units (I.U.)) over a period of 14 days (average age at autopsy, 54 days) resulted in complete atrophy of condylar cartilage with the fibrous superficial layer lying in direct contact with cancellous bone. By contrast, the epiphyseal and synchondrosal cartilage responded with accelerated growth and maturation of the chondrocytes and a subsequent increase in the thickness of both cartilages.

The Effect of Papain Administration

The plant enzyme, papain, was first reported by Hulth (1958) to interfere with the growth of epiphyseal cartilage, and has been used by several investigators to examine the contribution of various cartilages to the growth of the craniofacial skeleton. The inhibitory effect of papain on the growth of cartilage is believed to be the consequence of a loss of the glycosaminoglycan, chondroitin sulfate, from the cartilaginois matrix (Spicer and Bryant, 1958). Thomas et al. (1960) observed that the epiphyseal cartilaginous plates were reduced in thickness following papain injection in rabbits. Ronning (1968) found from craniometric measurements that papain injections resulted in a considerable reduction in the cranial and mandibular lengths of young rats during, and for some time after, the papain treatment. The reduction in cranial and mandibular lengths were presumed to be the result of the inhibitory effect of papain on the growth of the synchondrosal and condylar cartilages, respectively. Recently, Kvinnsland (1974) has reported a significant reduction in upper facial length following papain administration in young mice. In view of the known effects of papain on cartilage, Kvinnsland concluded that interference with the growth of the nasal septum and the synchondroses resulted in the reduction in antero-posterior dimensions.

Transplantation and Organ Culture Studies

Several investigators have hypothesized that epiphyseal, condylar, synchondrosal, and nasal septal cartilage have an "intrinsic"

growth potential, capable of producing a tissue-separating force that is only minimally affected by local environmental influences. To test this assumption, the capacity of these cartilages to grow has been investigated in both transplantation and organ culture studies.

Felts (1961) found that isologous implants of epiphyseal cartilage, when placed subcutaneously in rats, grew approximately as in situ. It is generally thought that such growth is largely under "intrinsic genetic" control (van Limborgh, 1971). Transplant experiments with condylar and synchondrosal cartilage have led to dissimilar conclusions. Condylar and synchondrosal cartilage, when transplanted homologously, grew poorly and infrequently in comparison to epiphyseal cartilage (Koski, 1968, 1971; Koski and Rönning, 1969, 1970; Rönning and Koski, 1969). As a result of these studies, it was concluded by these investigators, that craniofacial cartilages lacked an "intrinsic" growth potential. In contrast, growth has been observed with autologous transplants of condyle (Duterloo, 1967) and with synchondrosal cartilage grown in organ culture (Servoss, 1973). Bremers (1973) has shown that condylar cartilage is also able to grow in organ culture. Septal cartilage has been found to grow after homologous (Wiese, 1974) and autologous (Kvinnsland, 1973, 1974) transplantation and in organ culture (Petrovic et al., 1968). Accordingly, it has been concluded by these investigators that the nasal septum serves as an autonomous growth center.

The Chemical Nature of Cartilage

Connective tissues generally consist of a variety of characteristic macromolecules (i.e., collagen, elastin, connective tissue polysaccharides, etc.). The chemical, physical, and biological properties of each connective tissue depend on molecular configuration, macromolecular proportions, and structural interrelationships (van der Waals forces, hydrogen bonding, etc.) of the various chemical and structural constituents. Cartilage consists predominantly of water, cells (chondrocytes), and an intercellular matrix (collagen and proteoglycan).

A major component of the intercellular matrix is proteoglycan, a macromolecule consisting of a protein core that is associated with several glycosaminoglycans (acid mucopolysaccharides) (Hascall and Heinegård, 1974). Recently, the proteoglycan of cartilage has been the subject of intensive study (Schubert and Hamerman, 1968; Heinegård and Hascall, 1974), and much of its structure and many of its properties have been identified. In cartilage, the glycosaminoglycan, which consists mainly of chondroitin sulfate (CS) and keratin sulfate (KS), imparts to cartilage its polyanionic character, polymorphism, and microheterogeneity (Hascall and Heinegård, 1974). The biological basis of the polymorphism is unknown (i.e., whether it is genetically determined or, instead, the result of random molecular associations); however, in different cartilages both the amounts of CS and KS and their relative proportions differ. For example, in bovine nasal septal cartilage, the CS/KS ratio exceeds 10 (Doganges and Schubert, 1964)

whereas in human costal and articular cartilage it is approximately 1.5 (Rosenberg et al., 1965). Hardingham and Muir (1972) have demonstrated that, in cartilage, CS and KS containing proteoglycans interact specifically with hyaluronic acid, a third glycosaminoglycan. This interaction appears to be fundamental to the organization of proteoglycans within the intercellular matrices.

Shatton and Schubert (1954) presented the first evidence that CS chains are bonded covalently to non-collagenous proteins to form the macromolecules currently known as proteoglycans. Later, Mathews and Lozaityte (1958) suggested that the basic proteoglycan molecule consisted of a protein backbone to which polysaccharide chains are bound at intervals, forming a rod-like macromolecule with a molecular weight of about 4×10^6 daltons. Partridge and co-workers (1961) subsequently refined the model, demonstrating that the macromolecule contained 20 to 100 CS chains (each with a molecular weight of approximately 2.8×10^4 daltons), and provided the first evidence that a second polysaccharide, keratin sulfate, was bound to the same molecule. Efforts by several investigators (Gregory et al., 1964; Anderson et al., 1965; Rodén and Smith, 1966), clarified the nature of the CS linkage, indicating that the CS chains are bound to the protein core at their reducing ends by a neutral sugar oligosaccharide. However, the results of efforts to determine the nature of the KS linkage have been inconclusive.

The application of both dissociative extraction methods (Sajdera and Hascall, 1969) and cesium chloride (CsCl) density

gradient centrifugation techniques (Franek and Dunstone, 1967; Hascall and Sajdera, 1969) have provided means for the isolation and purification of proteoglycan macromolecules obtained from cartilages. Working with isolated proteoglycan molecules from bovine nasal septal cartilage, the average proteoglycan molecule was found to contain about 100 CS chains (Luscombe and Phelps, 1967) and 30 to 60 KS chains (Hopwood and Robinson, 1974; Hascall and Riolo, 1972). These macromolecules occupy large domains in solution, suggesting that the CS chains extend away from the protein core in all directions, as in a "lampbrush" arrangement. Recently, Matthews (1971) has provided evidence indicating that the CS chains are arranged primarily in pairs or closely-spaced groups that are separated by as few as 10 amino acid residues.

The studies of Heinegård and Hascall (1974) suggest that the protein core varies in length, depending upon the number of associated CS chains. Electron microscopic studies (Lohmander and Thyberg, 1975) have demonstrated that the portion of the protein core that interacts with hyaluronic acid has very little attached polysaccharide, and that this region contains two glycoproteins (link proteins A and B). Link protein A can be isolated and has been shown to interact with hyaluronic acid (Heinegård and Hascall, 1974). The essential function of link protein A in aggregate stability has also been demonstrated (Hascall and Heinegård, 1974). However, link protein B has not, as yet, been isolated. Consequently, whether it interacts with hyaluronic acid directly or with link protein A remains undetermined (Gregory,

1974). Accordingly, the data presented are consistent with the currently proposed structure for proteoglycan molecules illustrated in Figure 1.

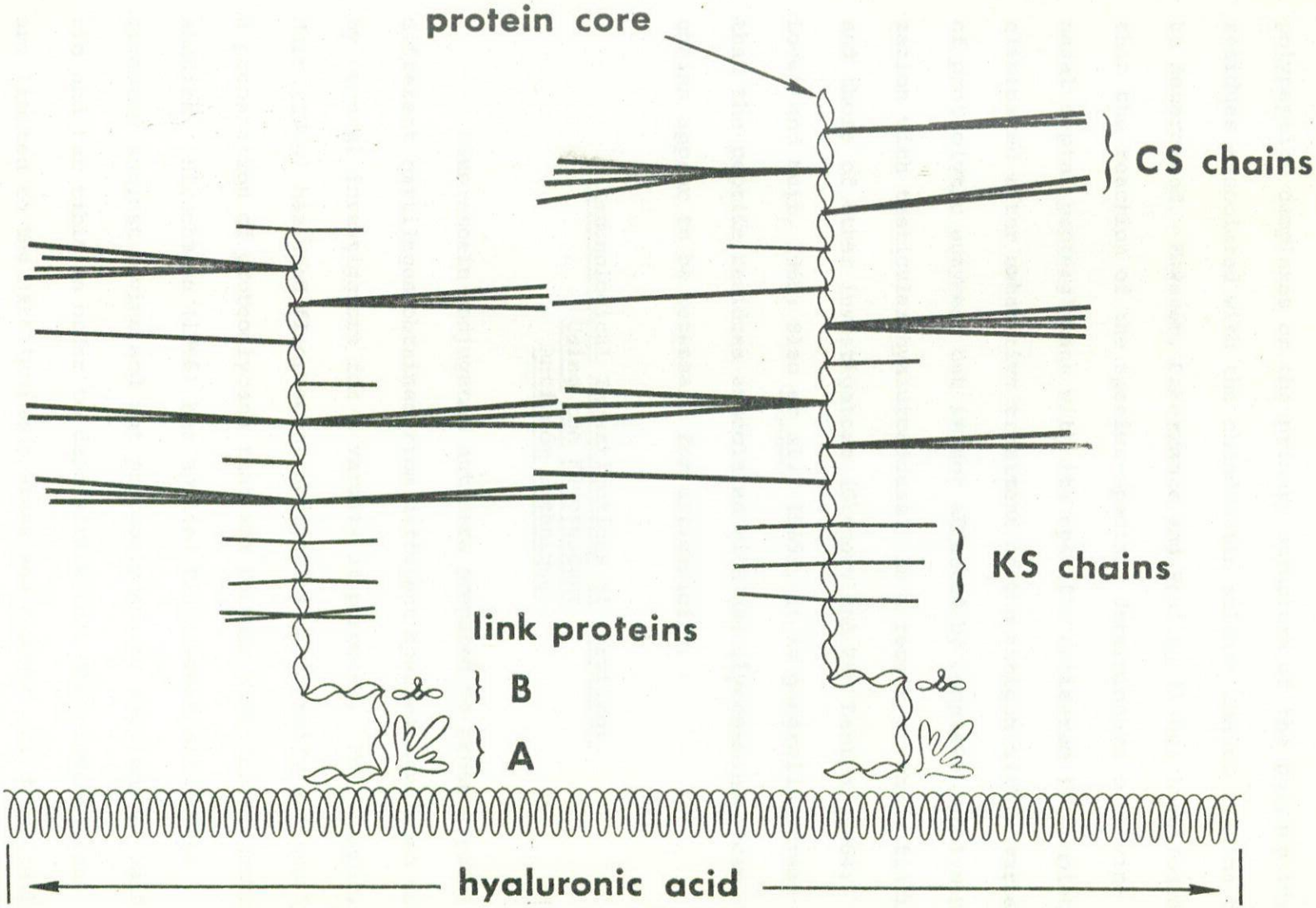
The Antigenicity of Cartilage Proteoglycans

In recent years, the antigenic properties of cartilage proteoglycans isolated from hyaline cartilage of different species have been investigated by several laboratories (White et al., 1963; DiFerrante, 1964; Loewi, 1964). Injection into rabbits of human, bovine, or porcine cartilage proteoglycans in Freund's adjuvant has consistently produced antibodies capable of reacting with the immunizing preparation.

Through the use of passive hemagglutination and hemagglutination inhibition studies, DiFerrante (1964) has demonstrated the cross-reactivity of antisera directed against cartilage proteoglycans of different species. By using the technique of indirect hemoagglutination, DiFerrante and Pauling (1964) have shown that two different types of antibodies are produced with proteoglycans extracted from bovine nasal septa. One type, directed against the glycopeptide residues near the keratin sulfate portion of the proteoglycan complex, appears to be species specific (Gregory and Van Lenten, 1964); the other type, directed against the chondroitin sulfate chains (or the glycopeptide residues associated with them), is not species specific.

Whether the antigenic determinants for the cross-reactive antibodies depend on the tertiary structure of the chondroitin sulfate-

Figure 1.--Schematic model for the structure of proteoglycan molecules (after Hascall and Heinegård, 1975).


protein core
CS chains
KS chains
link proteins
B
A
hyaluronic acid

polypeptide complexes or the primary structure of the polypeptide residues associated with the chondroitin sulfate chains remains to be determined. However, DiFerrante and Pauling (1964) have found that the reaction of the species-specific determinants of bovine nasal septal proteoglycans with its specific antiserum is completely eliminated after exhaustive treatment of the antigen with a variety of proteolytic enzymes, but is not affected by complete depolymerization with testicular hyaluronidase. As a result of these findings and those of other investigators (Gregory and Van Lenten, 1964; Loewi and Muir, 1965; Blau et al., 1965), it is generally agreed that the peptide residues associated with the glycosaminoglycan chains appear to be necessary for antigenicity.

Immunological Investigations of Cartilage Using the Fluorescent Antibody Technique

Fluorescein conjugated antisera prepared to proteoglycans of different cartilages obtained from different species have been used by several investigators for a variety of purposes. For example, Muir (1964) has used fluorescent antibodies to identify and purify a preparation of proteoglycans that was further used for structural studies. Hirschmann (1966) has applied fluorescent antibodies directed against bovine and rat proteoglycans to sections of calf rib and rat tibia in order to demonstrate that the proteoglycans are limited to the cartilaginous areas and removed (or drastically altered) in the process of endochondral ossification. Loewi and

Muir (1964) have used immunofluorescent antisera to porcine tracheal proteoglycans to localize the antigenic sites in tracheal cartilage and have further demonstrated the cross-reactivity of the antibody by its ability to stain the basement membranes of various soft tissues and the cartilages of other species.

In view of the aforementioned results that indicate that at least one of the major craniofacial cartilages possesses both non-specific and species-specific antigenic determinants; and because the immunofluorescent labeling technique affords the opportunity to observe both the location and distribution of the specific reaction of antibody with its antigenic determinants within the tissues, the immunofluorescent labeling-technique would seem to constitute a simple method for uncovering the possible existence of a "tissue-specific" antigenic determinant for a particular cartilage.

STATEMENT OF THESIS

In recent years, a considerable effort has been made by several investigators to emphasize the apparent differences that exist among the craniofacial cartilages, at least in response to various experimental conditions. By inference from the results of these experiments, and, in particular, the results of transplantation studies, several investigators have implied that these observed differences in results are due to differences in the cartilages themselves. A review of the literature, however, has indicated that no attempts have been made thus far to investigate whether differences actually exist among these cartilages beyond the histological level.

Therefore, the purpose of the present investigation is to determine if chemical and antigenic differences exist among the condylar, epiphyseal, and nasal septal cartilages of the calf. If no differences are revealed, then the once-popular belief that "cartilage is cartilage" would be further substantiated, at least at the immunological level. However, if differences are uncovered, then the evidence that has been accumulating recently suggesting that the variability in response of the various cartilages to identical experimental conditions reflects an intrinsic difference in the cartilage themselves would find support at the immunological level.

To examine this question, the condylar, epiphyseal, and nasal septal cartilages of the calf will be dissected free of all adjacent connective tissue, and their proteoglycans extracted and injected into rabbits to obtain specific antisera. The antisera will be absorbed against bovine liver and spleen to remove non-specific antibodies to bovine tissue and serum proteins, and the respective absorbed antisera will be applied to each cartilage followed by the application of fluorescein conjugated anti-rabbit globulin. In this way, the distribution of the antigenic determinants of each of the aforementioned cartilages will be identified and the extent of the cross-reactivity of their respective antisera will be established.

To determine the existence of an antigenic determinant unique to any particular cartilage, a portion of each antisera will be adsorbed against one of each of the various cartilages and subsequently applied to tissue sections of all three cartilages. If adsorption with cartilage eliminates all staining ability of each of the three antisera, then this result would suggest that there are no unique antigenic determinants. However, if adsorption of one of the antisera against any of the other cartilages eliminates the ability of that antiserum to stain these cartilages but permits it to stain its own cartilage, then this result would provide support for the suggestion that there are immunological differences among cartilages within the same species.

MATERIALS AND METHODS

Preparation of Cartilaginous Tissues

Twenty-four heads and forty epiphyses of long bones from six month old calves were obtained from an abattoir within one hour of slaughter. The condylar, epiphyseal, and nasal septal cartilages were dissected free of adhering non-cartilaginous tissue (Plates I-III) and rinsed with 0.15M phosphate buffer solution (PBS), pH 7.0. Slices of each cartilage approximately 0.5 mm thick were fixed in cold 95% ethanol and embedded in paraffin (Sainte-Marie, 1962). The paraffin-embedded cartilage was stored in a dessicator at 4^{o} C until used as substrates for immunofluorescent staining. To ensure that the cartilage used for antibody production was free of non-cartilaginous tissue contaminants, control sections of each cartilaginous fragment were checked by histological examination (Plate IV).

Proteoglycans (PG) were extracted in PBS by high speed homogenization (Virtis 45, Gardiner, New York) in an icebath (modified from Sajdera and Hascall, 1969). The extracts of each cartilage were centrifuged twice at 27,000 X g. for one hour at 0^{o} C and the supernatants (PG-S) removed. The PG-S of each cartilage was dialyzed exhaustively against distilled water and lyophilized. The lyophilized material was stored at -20^{o} C.

The sediment of each cartilage was divided in half. One half

PLATE I

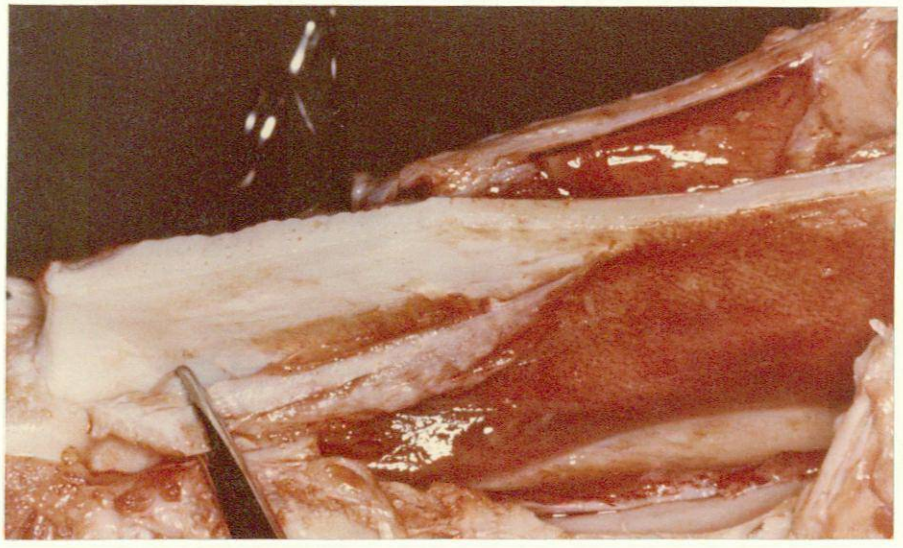
Dissection of Bovine Nasal Septum

A. Head of six-month old calf

B. Dissection of nasal septum

C. Nasal septum dissected free of non-cartilaginous tissue






0
10
20
50
60
70
90
100
110
120

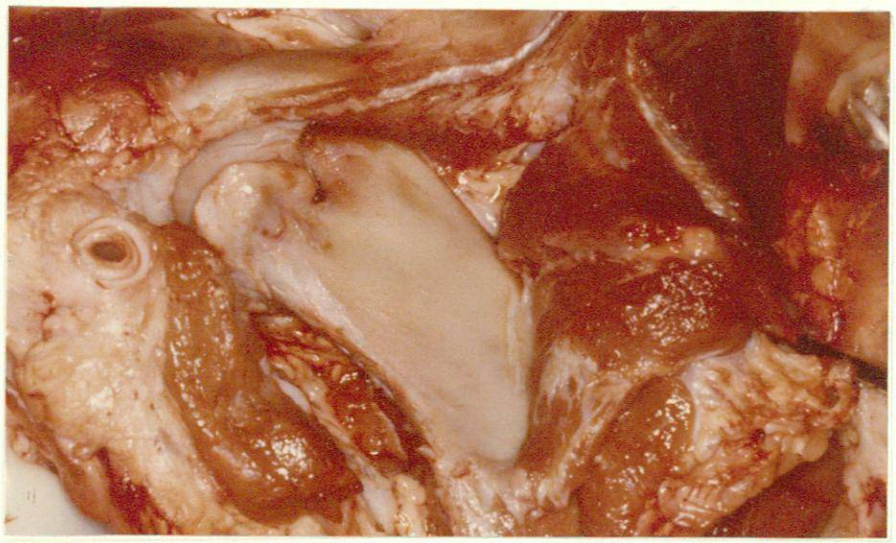
PLATE II

Dissection of Bovine Condylar Cartilage

A. Condyle of bovine mandible exposed

B. Condylar cartilage and bovine condylar head

C. Condylar cartilage dissected free of non-cartilaginous tissue





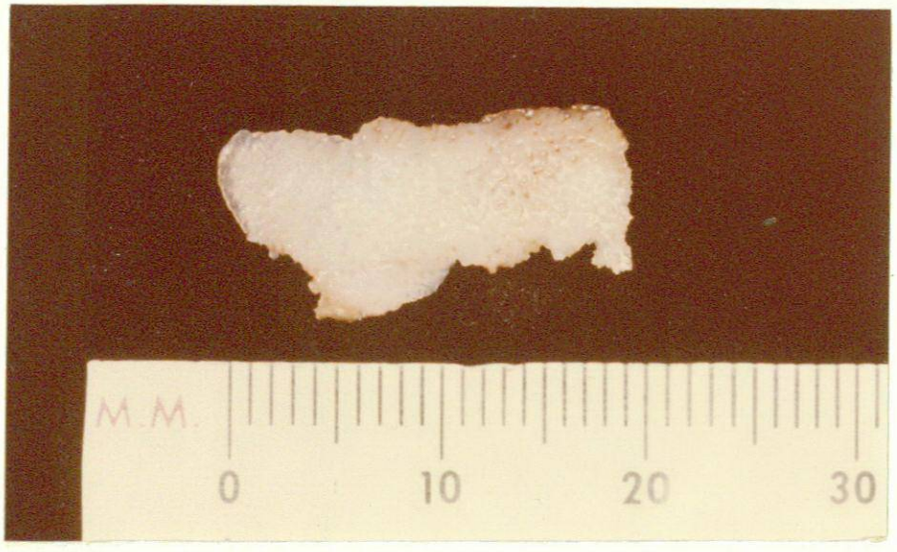

M.M.
0
10
20
30

PLATE III

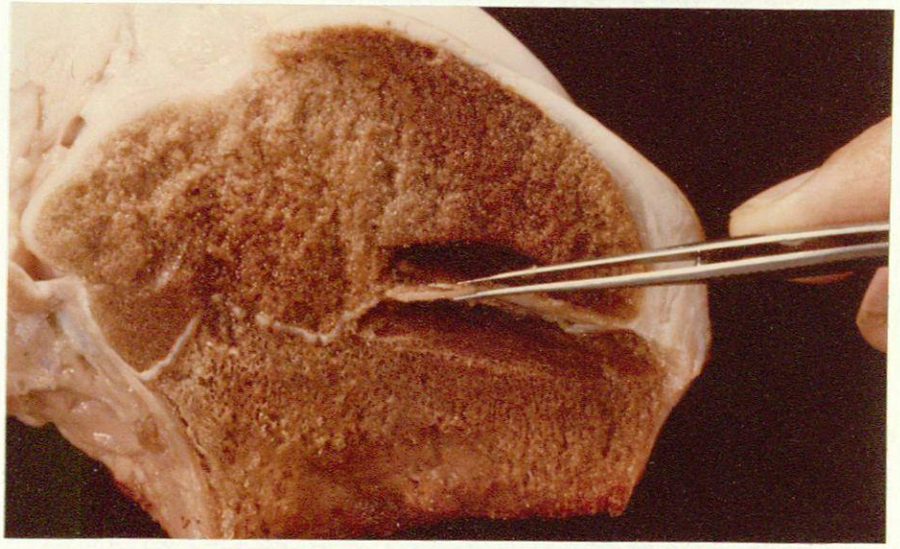
Dissection of Bovine Epiphyseal Cartilage

A. Epiphyseal plate of cartilage from humerus of six-month old calf

B. Dissection of epiphyseal cartilage

C. Epiphyseal cartilage dissected free of non-cartilaginous tissue






M M
0
10
20
30
40
50
60

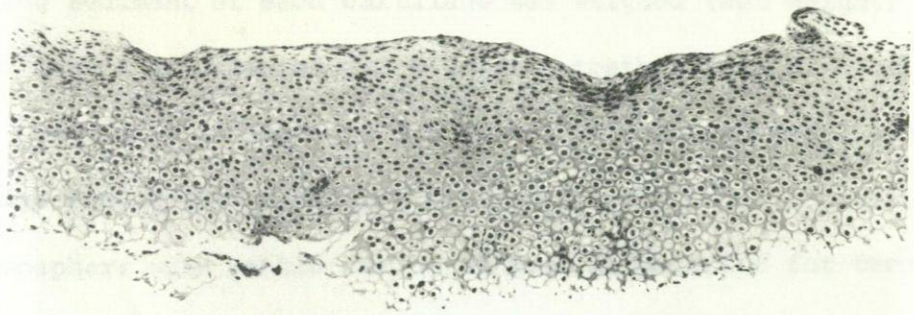
PLATE IV

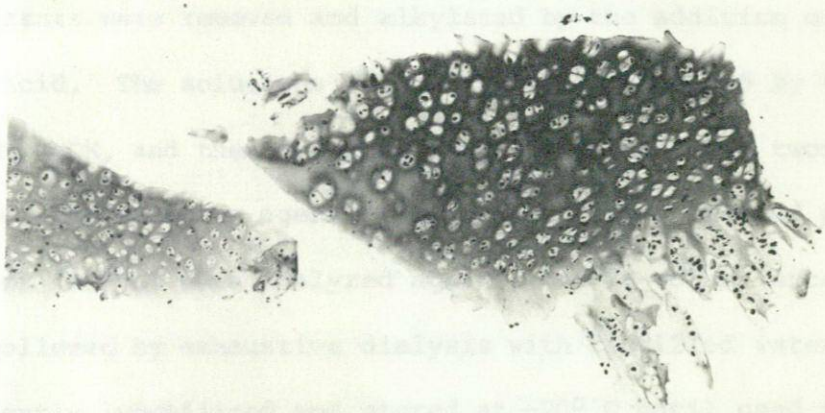
Histological Examination of Dissected Cartilages (magnification 62X, Kodak Plus-X Pan professional film, 1.5 sec. exposure)

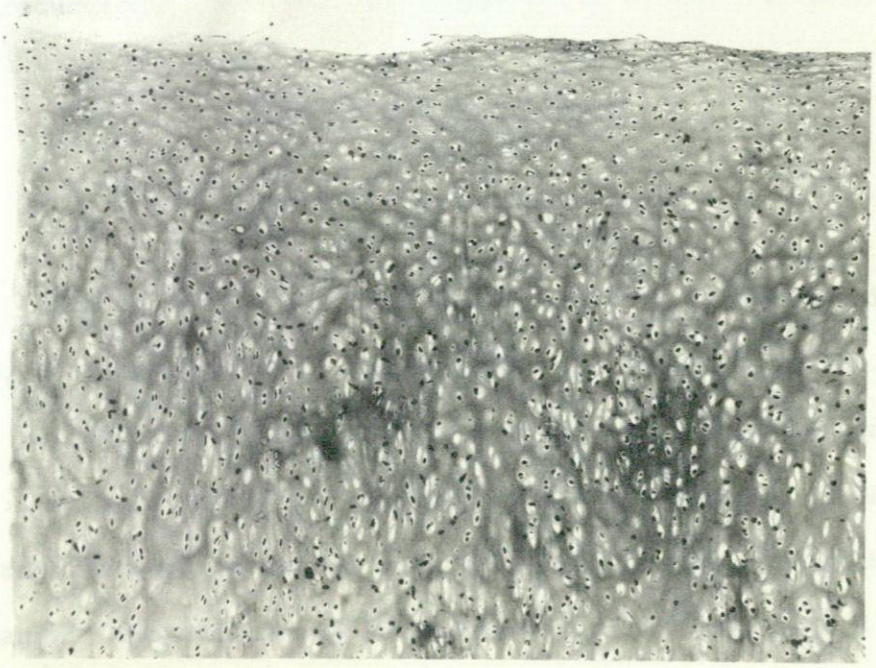
A. Condylar Cartilage

B. Epiphyseal Cartilage

C. Nasal Septal Cartilage







was stored at 0° C and used later for adsorption of antisera. The remaining sediment of each cartilage was weighed (wet weight) and reacted with a solution of 0.3 M 2-mercaptoethanol in 0.5M Tris-HCl buffer containing 8M urea, pH 8.5 (10 ml of solution per 1 gram of cartilage sediment). Reduction was carried out under a positive nitrogen atmosphere with gentle mixing at room temperature for twenty-four hours. The reduction procedure was repeated twice, at which time the supernatants were removed and alkylated by the addition of 0.6M iodoacetic acid. The solutions were maintained at pH 8.5 by titration with 10M NaOH, and then quenched by the addition of a twofold molar excess of the reducing agent. The reduced and alkylated proteoglycan solutions (PG-RA) were dialyzed against one 20 volume excess of 0.15 NaCl, followed by exhaustive dialysis with distilled water, and were subsequently lyophilized and stored at -20° C until used for antibody production.

Preparation of Antisera

Immunization Procedures

Each of the New Zealand white rabbits were anesthetized (1.5 mg Acepromazine/kg of body weight) and injected in the region of the popleteal lymph nodes with 1 mg of antigen (PG-RA) in 0.5 ml of a 1:1 mixture of PBS in complete Freund's adjuvant. Groups of three rabbits were immunized with the PG-RA from one of each of the cartilages. Two additional intramuscular injections of 0.5 mg of antigen in PBS were given at three week intervals. Control bleedings

were made on each rabbit prior to the start of the immunization sequence.

Serum Collection and Storage

Heart punctures were performed on the fasting rabbits eight weeks after the initial inoculation. Twenty-five milliliters of blood were withdrawn from each rabbit. The antisera were separated from whole blood, clarified, and collected (Campbell et al., 1964), and stored at -96° C until used for immunofluorescent staining.

Purification of the Antisera

Non-Specific Adsorption

Bovine liver and spleen were used as adsorbents to remove non-specific interfering antibodies. The adsorbents were prepared by Kawamara's method (1969) for preparation of acetone dried powders. The antisera were adsorbed twice for periods of one hour at room temperature using 100 mg of liver and 50 mg of spleen per milliliter of serum. Following the last adsorption, the mixture was centrifuged at 27,000 X g. for 20 minutes at 0° C, the serum carefully pipetted off to avoid contamination with the adsorbent, and stored at -96° C until needed. Half of each of the non-specifically adsorbed antisera was then subjected to specific adsorption.

Specific Adsorption

Homogenized condylar, epiphyseal, and nasal septal cartilage were used as adsorbents to remove antibodies common to the respective

cartilages. Each non-specifically adsorbed antiserum was adsorbed twice for periods of one hour and two hours, respectively, at room temperature using approximately 25 mg of cartilage at each time period per ml of serum. Techniques for the centrifugation, collection, and storage of adsorbed antisera were the same as those used for non-specific adsorption. Specific adsorption was carried out such that 2 ml of each antiserum were adsorbed with nasal septal cartilage, another 2 ml of each antiserum were adsorbed with condylar cartilage, and another 2 ml of each antiserum were adsorbed with epiphyseal cartilage.

Experimental Design

Indirect Fluorescent Labeling

According to Long and co-workers (1968), regional variations in chondrocyte activity exist in the cartilaginous nasal septum of the growing rabbit. Accordingly, tissue sections were removed from the anterior, middle and posterior portions of the nasal septum (Figure 2), fixed according to the aforementioned procedures, and used as substrates for the indirect immunofluorescent technique of Coons and Kaplan (1950) (Figure 3). After comparison of the results, and, in view of the accessibility of the anterior portion of the nasal septum, all further immunofluorescent staining of nasal septal cartilage was performed on sections from the anterior region.

The indirect fluorescent antibody technique was applied to cold-ethanol fixed sections of condylar, epiphyseal, and nasal septal

Figure 2.--Diagram illustrating areas from which tissue sections were removed from the nasal septum and compared through immunofluorescent staining.

AS, anterior section
MS, middle section
PS, posterior section

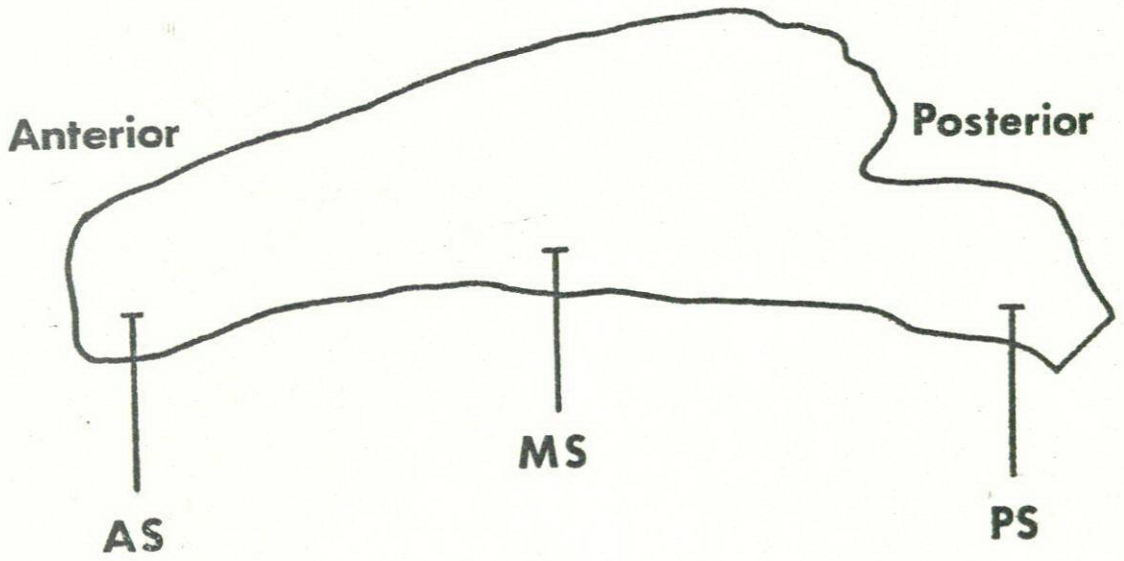
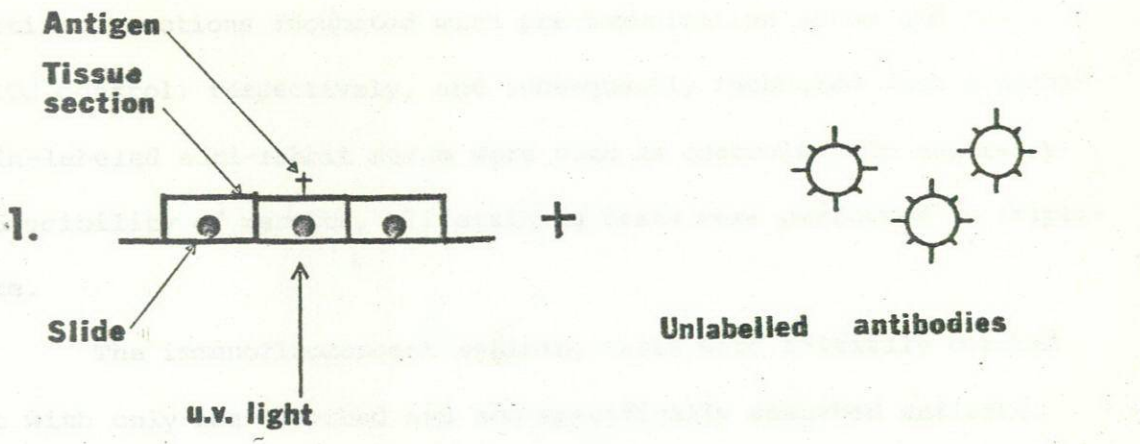

Anterior
Posterior
AS
MS
PS

Figure 3.--Schematic diagram illustrating the indirect immunofluorescent labeling technique of Coons and Kaplan (1950).


Antigen
Tissue section
1.
Slide
u.v. light
Unlabelled antibodies
2.
Fluorescein-labelled antibodies
3.

cartilage to test the ability of each of the antisera to stain its respective cartilage and to cross-react and stain the other cartilages. Cartilage sections incubated with pre-immunization serum and PBS (FITC control) respectively, and subsequently incubated with fluorescein-labeled anti-rabbit serum were used as controls. To ensure reproducibility of results, all staining tests were performed in triplicate.

The immunofluorescent staining tests were initially carried out with only non-adsorbed and non-specifically adsorbed antisera. Following the observation and comparison of the staining character of each cartilage, the tests were repeated using antisera which had been non-specifically and specifically adsorbed. Table I illustrates the assignment of test antisera to the various cartilage sections.

Experimental Procedure

Immunohistochemical Characterization

Indirect Fluorescent Labeling

Paraffin-embedded sections (6μ) of each of the cartilages were transferred to microscopic slides and deparaffinized. Two drops of antiserum were applied to each tissue and the sections were incubated at 37° C in a moist chamber for one hour. The sections were then washed with PBS (3 X 10 minutes), rinsed immediately with distilled water, and allowed to air dry. Two drops of sheep anti-rabbit serum conjugated with fluorescein isothiocyanate (FITC, Gibco

TABLE I

ASSIGNMENTS OF TEST ANTISERA

Tissue Sections	Antisera											
	Non-Specifically Adsorbed			Non-Specifically and Specifically Adsorbed								
				A*			B*			C*		
Condylar Cartilage	ACCA	AECA	ANSA	ACCA	AECA	ANSA	ACCA	AECA	ANSA	ACCA	AECA	ANSA
Epiphyseal Cartilage	ACCA	AECA	ANSA	ACCA	AECA	ANSA	ACCA	AECA	ANSA	ACCA	AECA	ANSA
Nasal Septal Cartilage	ACCA	AECA	ANSA	ACCA	AECA	ANSA	ACCA	AECA	ANSA	ACCA	AECA	ANSA

*A, Antisera specifically adsorbed with condylar cartilage.
*B, Antisera specifically adsorbed with epiphyseal cartilage.
*C, Antisera specifically adsorbed with nasal septal cartilage.

ACCA--anti-condylar cartilage antiserum.
AECA--anti-epiphyseal cartilage antiserum.
ANSA--anti-nasal septum cartilage antiserum.

Grand Island, New York), were applied to each section, and the slides were incubated for thirty-five minutes at 37° C in a moist chamber. The sections were washed and dried as previously described, mounted in a mixture of buffered saline and glycerol, and examined microscopically (Ultraphot II, Zeiss fluorescence microscope with high-pressure mercury lamp, BG 12 exciting filter, and a yellow barrier filter, Oberkochen/Wuertt., West Germany). The indirect fluorescent labeling tests were performed in the following order and may be summarized:

1. Non-specifically adsorbed anti-condylar cartilage antiserum (ACCA), anti-epiphyseal cartilage antiserum (AECA), and anti-nasal septum antiserum (ANSA) were each tested against sections of condylar, epiphyseal, and nasal septal cartilages.
2. Non-specifically adsorbed ACCA, AECA, and ANSA, specifically adsorbed with condylar cartilage were tested against sections of condylar, epiphyseal, and nasal septal cartilages.
3. Non-specifically adsorbed ACCA, AECA, and ANSA, specifically adsorbed with epiphyseal cartilage were tested against sections of condylar, epiphyseal, and nasal septal cartilages.
4. Non-specifically adsorbed ACCA, AECA, and ANSA, specifically adsorbed with nasal septal cartilage were tested against sections of condylar, epiphyseal, and nasal septal cartilages.

Polyacrylamide Gel Disc Electrophoresis

Limited characterization of the proteoglycans (PG) extracted

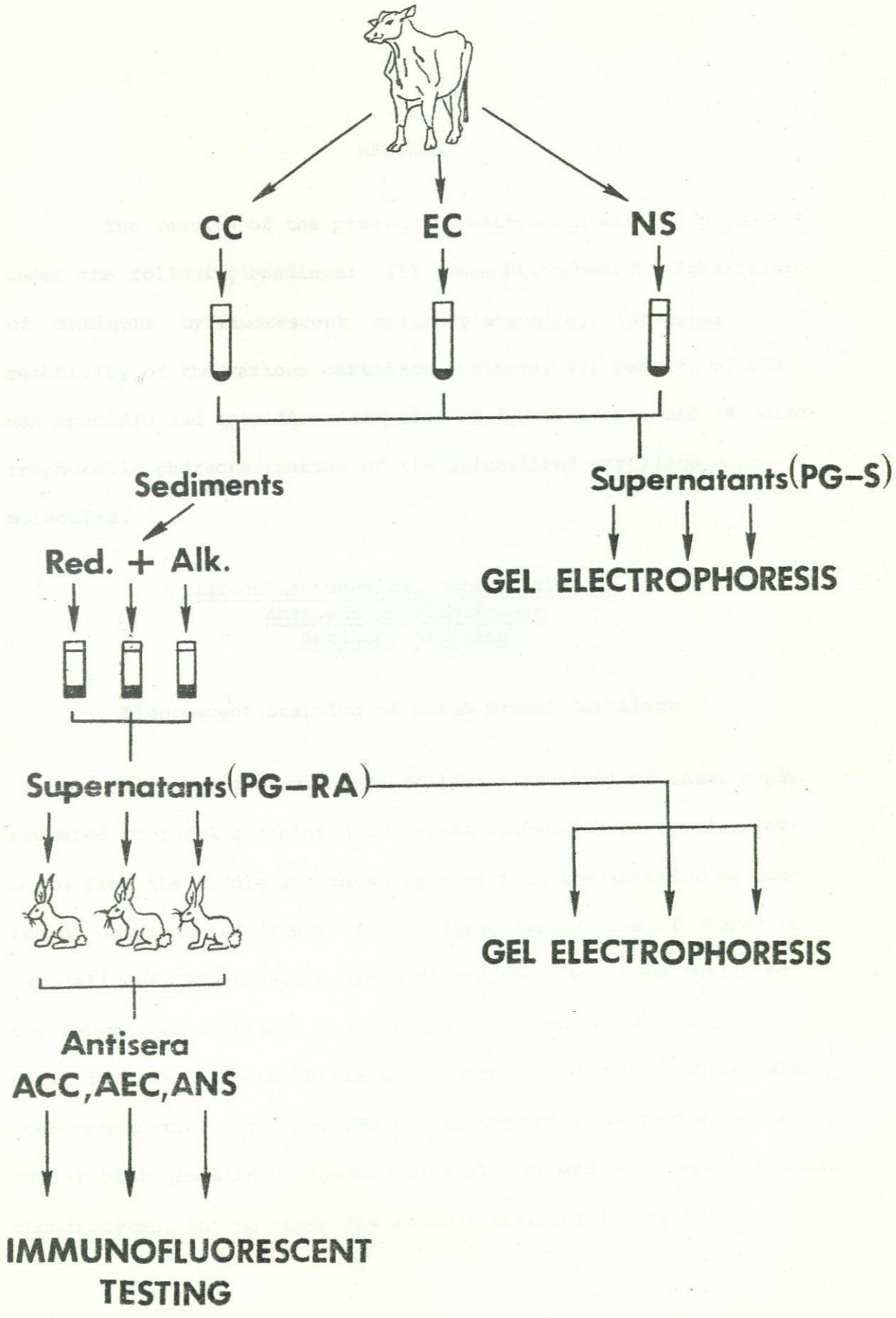
from the three cartilages (PG-RA and PG-S) was accomplished by sodium dodecyl sulfate (SDS) gel electrophoresis. Proteoglycans migrate on these gels as sharp bands, the mobilities of which are dependent on their molecular weight (Weber and Osborn, 1969). A sample of collagen was included as a reference standard.

The gel technique was conducted according to a procedure for SDS disc gel systems modified from Neville (1971) (Lillie, personal communication, 1976). The gels were stained for twelve hours in 0.05% Coomassie Blue R250 in 45% methanol and 5% acetic acid, and were destained by diffusion against 7.5% acetic acid and 5% methanol for forty-eight hours.

A schematic representation of the general experimental method is presented in Figure 4.

Figure 4.--Schematic representation of experimental procedure.

CC, condylar cartilage
EC, epiphyseal cartilage
NS, nasal septal cartilage
ACC, anti-condylar cartilage antiserum
AEC, anti-epiphyseal cartilage antiserum
ANS, anti-nasal septal cartilage antiserum
Red. + Alk., reduced and alkylated


CC
EC
NS
Sediments
Supernatants(PG-S)
Red. + Alk.
GEL ELECTROPHORESIS
Supernatants(PG-RA)
GEL ELECTROPHORESIS
Antisera
ACC, AEC, ANS
IMMUNOFLUORESCENT
TESTING

RESULTS

The results of the present investigation will be presented under the following headings: (1) immunohistochemical localization of antigens by fluorescent antibody staining, (2) cross-reactivity of the various cartilage antisera, (3) results of the non-specific and specific adsorption of the antisera, and (4) electrophoretic characterization of the solubilized cartilage macromolecules.

Immunohistochemical Localization of Antigens by Fluorescent Antibody Staining

Fluorescent Staining of Nasal Septal Cartilage

Microscopic examination of tissue sections of nasal septum revealed regional staining differences between those sections removed from the middle and those removed from the anterior or posterior nasal septum (Table II). A dense narrow zone of characteristically brilliant yellow-green fluorescence was seen outlining the lacunae of cartilage in sections from both the anterior and posterior nasal septum (Plate V). Control sections incubated with pre-immunization serum and PBS and subsequently reacted with sheep anti-rabbit globulin conjugated with FITC showed occasional stained chondrocytes, but no other fluorescent staining (Plate VI).

TABLE II

COMPARISON OF FLUORESCENT STAINING ABILITY
OF SECTIONS OF NASAL SEPTUM

Tissue Section Location	Anti-Serum		FITC Control
	Pre-Immunization Control	Non-Specifically Adsorbed*	
Anterior	-	+	-
Middle	-	±	-
Posterior	-	+	-

*Anti-nasal septum antiserum adsorbed with liver and spleen only.

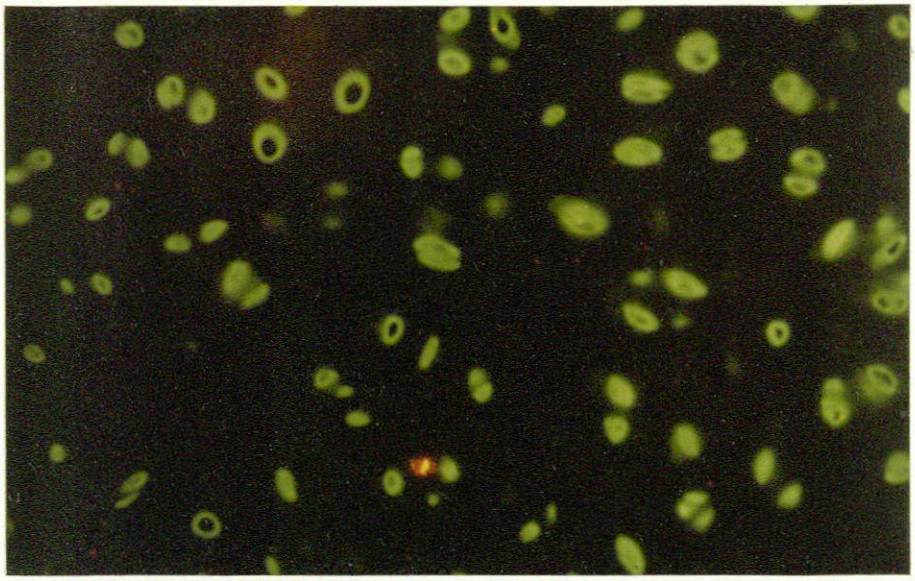
PLATE V

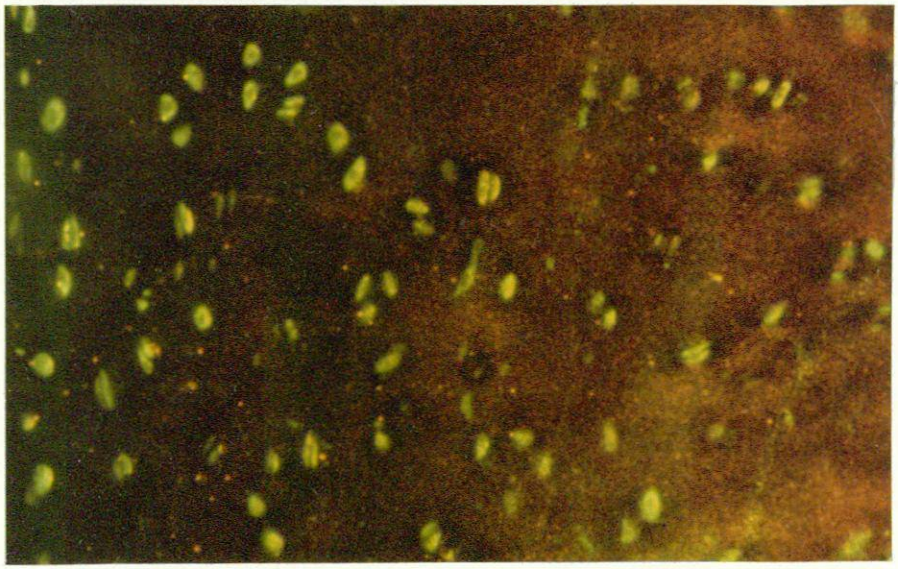
Regional Staining Differences within the Nasal Septum (magnification 146X, 35 mm Kodak daylight high speed Ektachrome ER film, 30 sec. exposure)

A. Nasal Septum, Anterior Region

B. Nasal Septum, Middle Region

C. Nasal Septum, Posterior Region





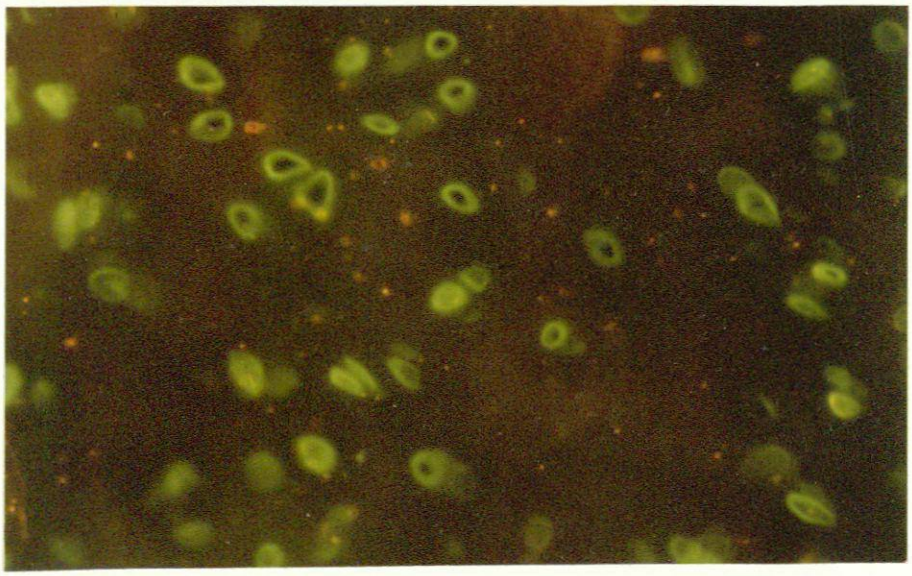


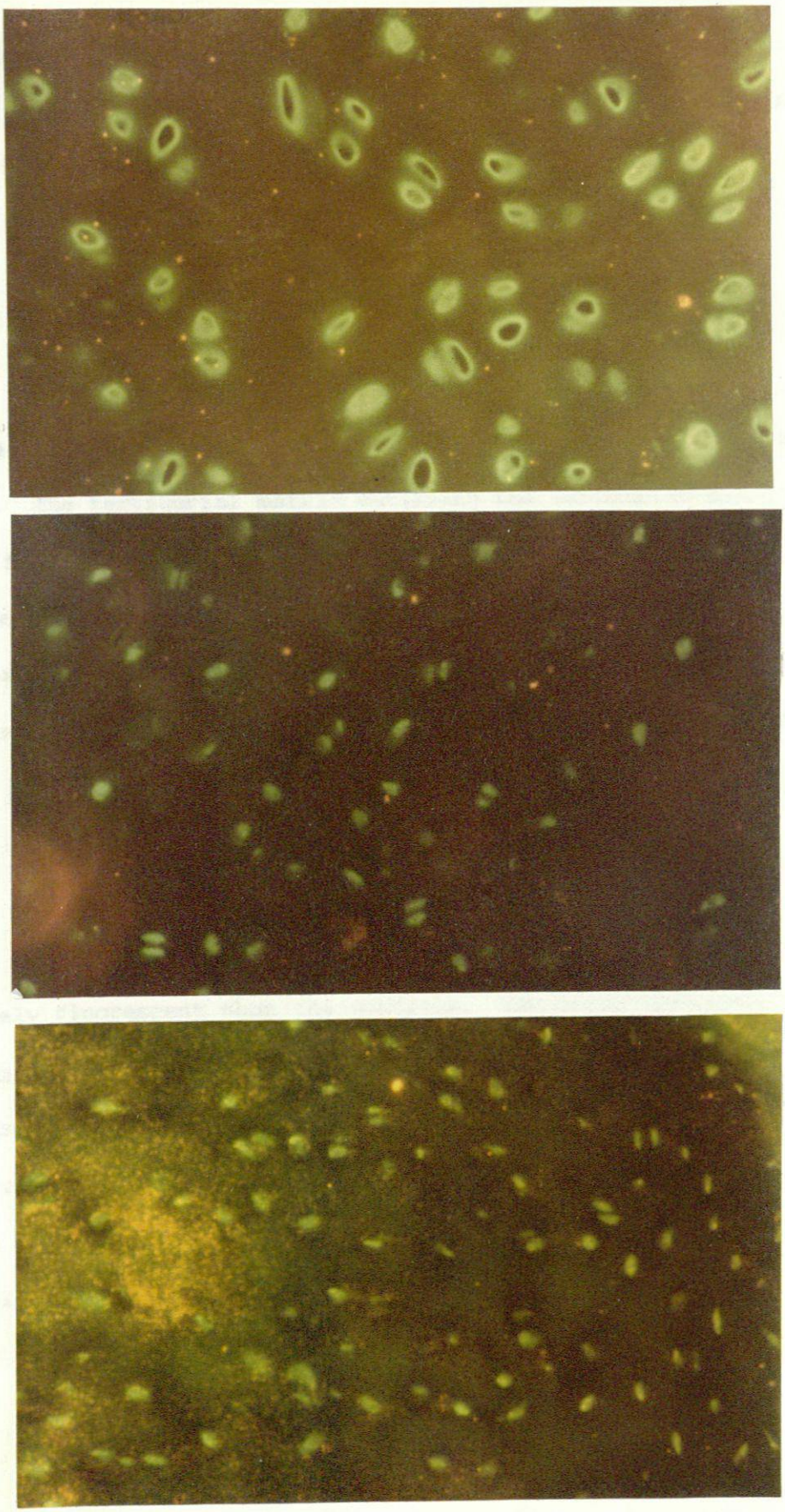
PLATE VI

Fluorescent Staining of Nasal Septum (magnification 146X, 35 mm Kodak daylight high speed Ektachrome ER film, 30 sec. exposure)

A. Characteristic staining of perilacunar interritorial matrix, anterior region

B. Pre-immunization serum control

C. FITC Control



Examination of stained sections from the middle region of the nasal septum, more closely resembled the controls described above than sections of the anterior and posterior septum. The lacunae of the middle sections showed only occasional outline staining (Plate V).

Fluorescent Staining of Epiphyseal Cartilage

The results with sections of epiphyseal cartilage were similar to those obtained with sections of anterior or posterior nasal septum. The territorial matrix outlining the lacunae of all cells showed moderate to strong fluorescence. Complete loss of chondrocytes within all sections of epiphyseal cartilage was observed. Consequently, no staining was observed within the lacunar spaces. No fluorescent staining was observed in either the pre-immunization or the FITC controls (Plate VII).

Fluorescent Staining of Condylar Cartilage

The cells of all zones of the condylar cartilage were more intensely fluorescent than the controls. The interterritorial matrix of both the zones of proliferating and maturing cartilage fluoresced strongly. The matrix outlining the cells of the hypertrophic zone showed moderate to strong fluorescence. The territorial matrix outlining the cells of the prechondroblastic zone fluoresced only slightly (Plate VIII).

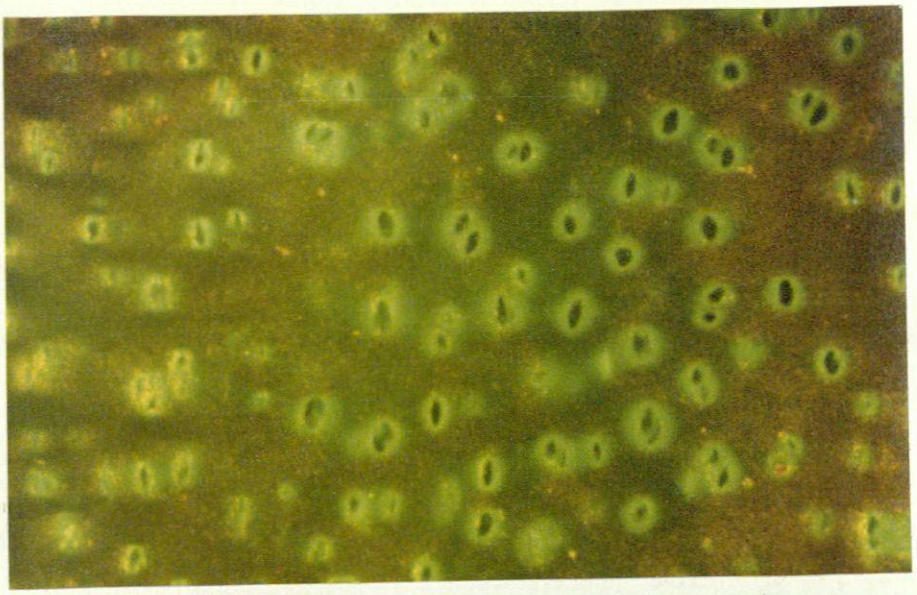
PLATE VII

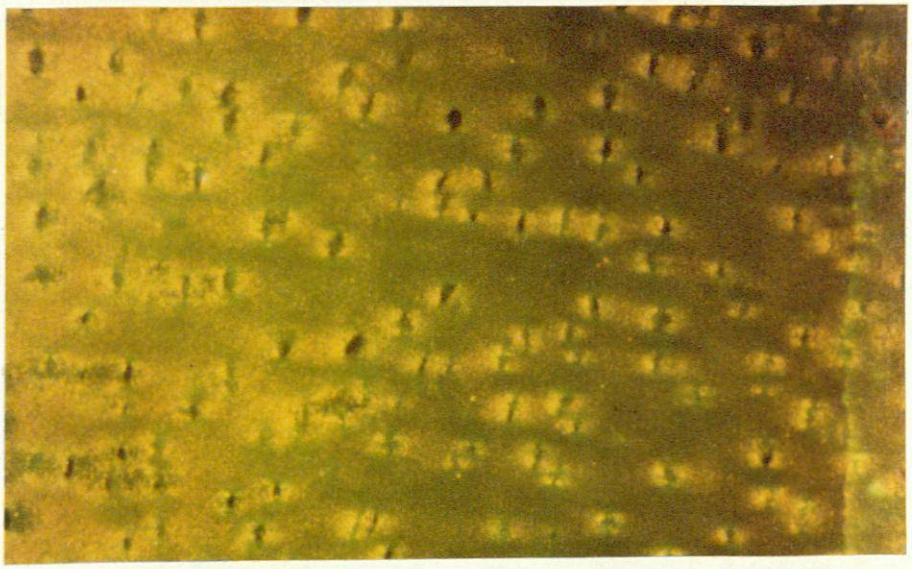
Fluorescent Staining of Epiphyseal Cartilage (magnification 146X, 35 mm Kodak daylight high speed Ektachrome ER film, 30 sec. exposure)

A. Characteristic staining of perilacunar interterritorial matrix.

B. Pre-immunization control

C. FITC control





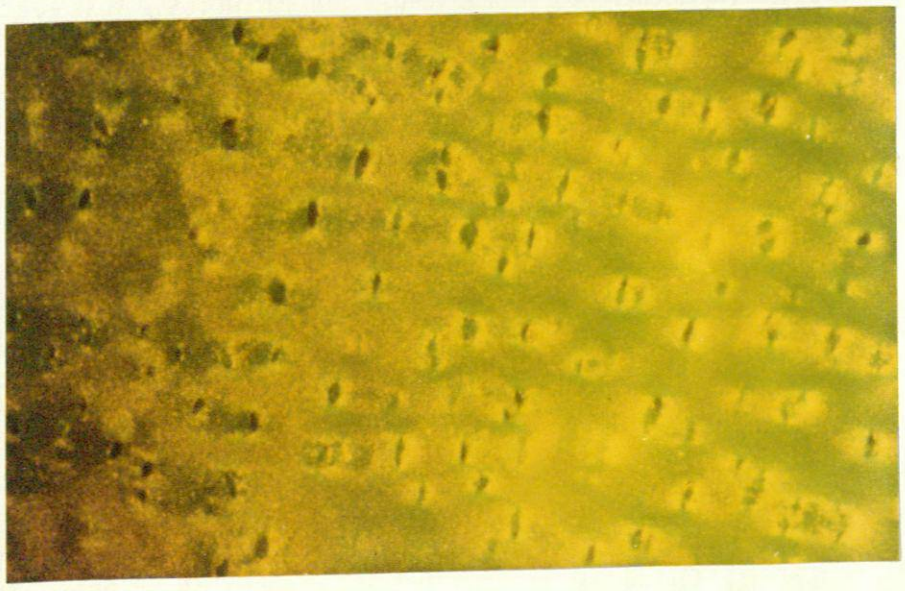


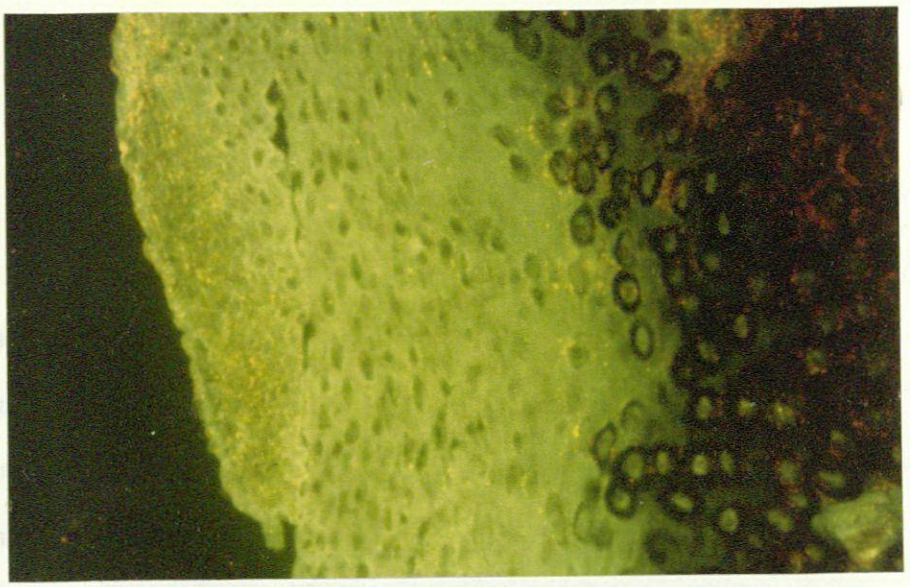
PLATE VIII

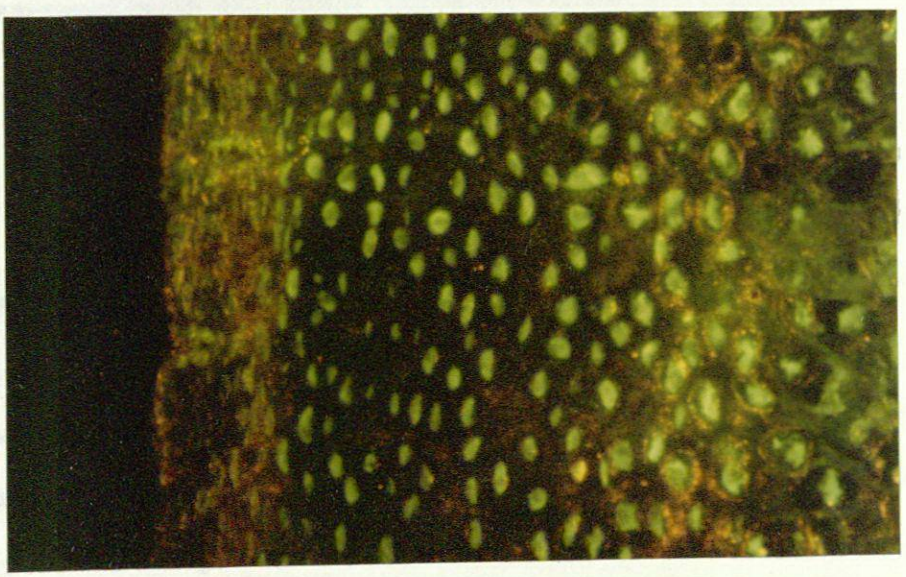
Fluorescent Staining of Condylar Cartilage (magnification 146X, 35 mm Kodak daylight high speed Ektachrome ER film, 30 sec. exposure)

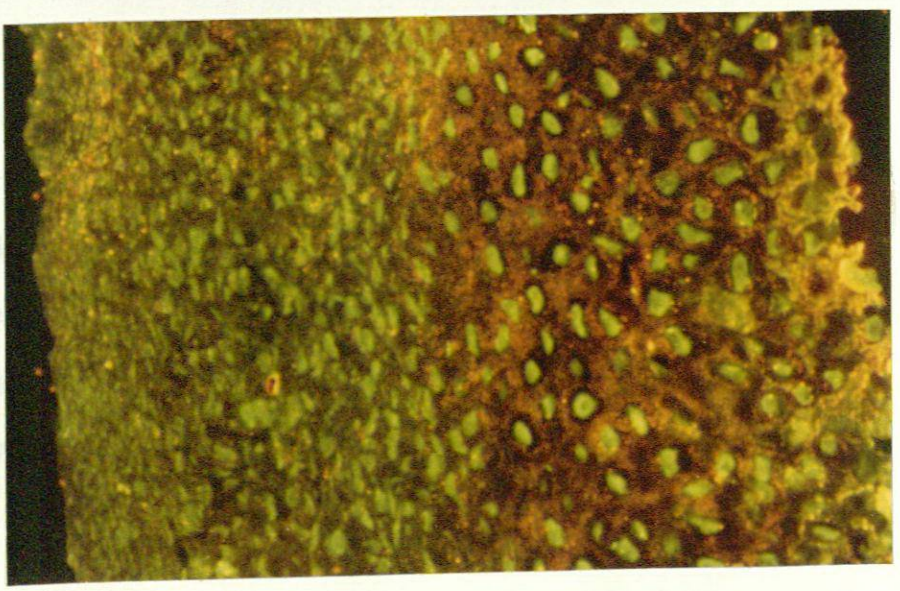
A. Characteristic staining of all interstitial matrix within zones of young and mature chondrocytes.

B. Pre-immunization control

C. FITC control







Cross-Reactivity of the Various Cartilage Antisera

A cross-reaction among all three antisera was demonstrated by the ability of each antiserum to stain condylar, epiphyseal, and nasal septal cartilage. Moreover, no differences in intensity or location of fluorescent staining was observed when one antiserum was cross-reacted with the other cartilages. Non-specific adsorption of each antiserum did not affect the ability of any of a given antiserum to cross-react with the other cartilages (Table III).

Results of the Non-Specific and Specific Adsorption of the Antisera

Preliminary tests comparing pre-adsorbed and non-specifically adsorbed antisera showed no differences in staining intensity or location. Specific adsorption of all three antisera with nasal septal cartilage eliminated all staining ability (reactivity) of both ANSA and AECA. However, ACCA lost its ability to stain nasal septal and epiphyseal cartilage but retained its ability to stain condylar cartilage. Specific adsorption of all three antisera with epiphyseal cartilage produced results identical with those obtained with adsorption of nasal septal cartilage. Again, ACCA retained its ability to stain only condylar cartilage. Specific adsorption of all three antisera with condylar cartilage, however, eliminated all staining ability of each antiserum. The results of all fluorescent staining tests are presented in Table III.

TABLE III

RESULTS OF IMMUNOFLUORESCENT STAINING
BY THE TEST ANTISERA

Tissue Sections	Antisera											
	Non-Specifically Adsorbed			Non-Specifically and Specifically Adsorbed								
				A*			B*			C*		
	ACCA	AECA	ANSA	ACCA	AECA	ANSA	ACCA	AECA	ANSA	ACCA	AECA	ANSA
Condylar Cartilage	+	+	+	-	-	-	+	-	-	+	-	-
Epiphyseal Cartilage	+	+	+	-	-	-	-	-	-	-	-	-
Nasal Septal Cartilage	+	+	+	-	-	-	-	-	-	-	-	-

*A, Antisera specifically adsorbed with condylar cartilage.
*B, Antisera specifically adsorbed with epiphyseal cartilage.
*C, Antisera specifically adsorbed with nasal septal cartilage.

ACCA--Anti-condylar cartilage antiserum.
AECA--Anti-epiphyseal cartilage antiserum.
ANSA--Anti-nasal septum antiserum.

+, Indicates positive fluorescent staining as compared with controls.
-, Indicates negative fluorescent staining as compared with controls.

Electrophoretic Characterization of the Extracted Cartilage Components

When PBS-soluble fractions (PG-S) of each cartilage were subjected to electrophoresis on SDS-polyacrylamide gels and stained with Coomassie Blue R250, four major protein bands (A, B, C, and D) were observed (Plate IX, XB) for both the epiphyseal cartilage (E) and the nasal septal cartilage (F). Similarly prepared fractions from condylar cartilage revealed the same four major bands; however, bands A, B, and C stained more prominently. Moreover, a series of lower molecular weight molecules appeared between bands B and C. Using interstitial collagen as a molecular weight standard (G, guinea pig dermal collagen, provided by Dr. John H. Lillie, University of Michigan, Ann Arbor, Michigan) in the same system, the approximate molecular weight of component D was estimated to be 1.75×10^5 daltons.

Samples from supernatants of cartilage treated with 2-mercaptoethanol in 8M urea (PG-RA) and subjected to the same gel system revealed four major protein bands that were similar though not identical to those of the PG-S gels. In addition, a series of less prominent bands were observed in all three gels (Plate IX, XA). Bands a, b and c of the epiphyseal cartilage (B) and bands a, b, and d of the nasal septal cartilage (C) were less prominent than their corresponding components in the PG-S gels. Again, the protein bands of the condylar cartilage (A) were more prominent than those of the epiphyseal (B) and nasal septal (C) cartilages, and an extensive

PLATE IX

Protein Band Patterns of Samples Subjected to Electrophoresis on SDS-Polyacrylamide Gels

1. Samples Treated with .2-Mercaptoethanol (PG-RA)

   A--Condylar Cartilage

   B--Epiphyseal Cartilage

   C--Nasal Septal Cartilage

2. Samples Treated with PBS (PG-S)

   D--Condylar Cartilage

   E--Epiphyseal Cartilage

   F--Nasal Septal Cartilage

   G--Collagen Reference Standard

   H--Blank

   O--Origin

   T--Tracking Dye

   O T indicates direction of migration of the protein bands in all gels.

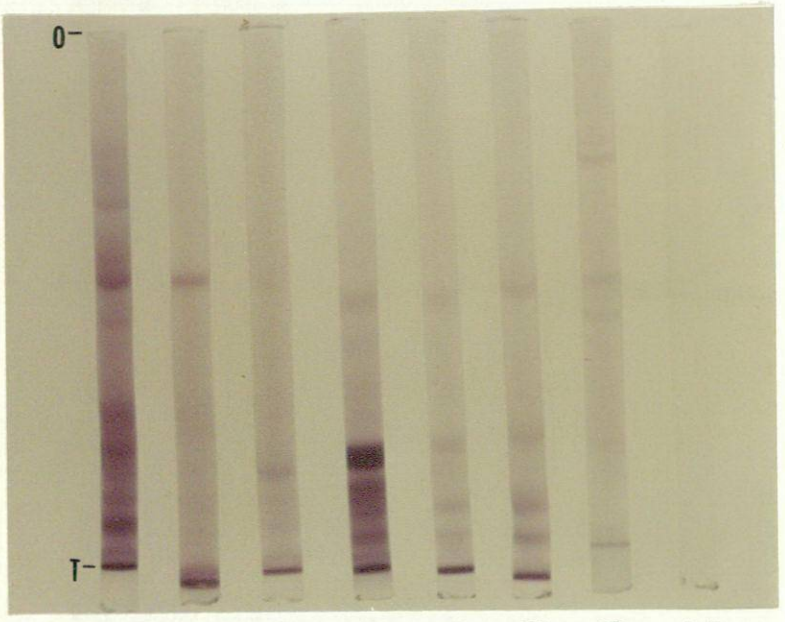

O
T
A B C D E F G H

PLATE X

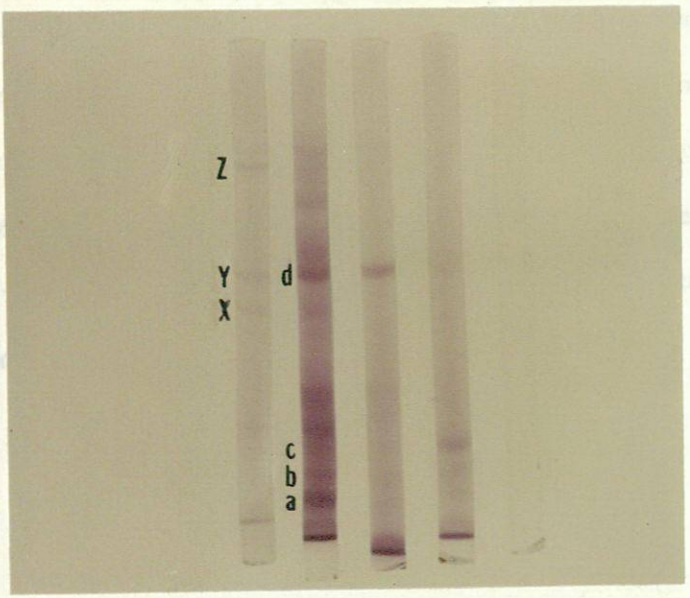
Band Patterns on SDS-Polyacrylamide Gels

A. Major Protein Bands (a, b, c, d) of PG-RA Acrylamide Gels Compared with Protein Bands of Collagen Standard (X, Y, Z).

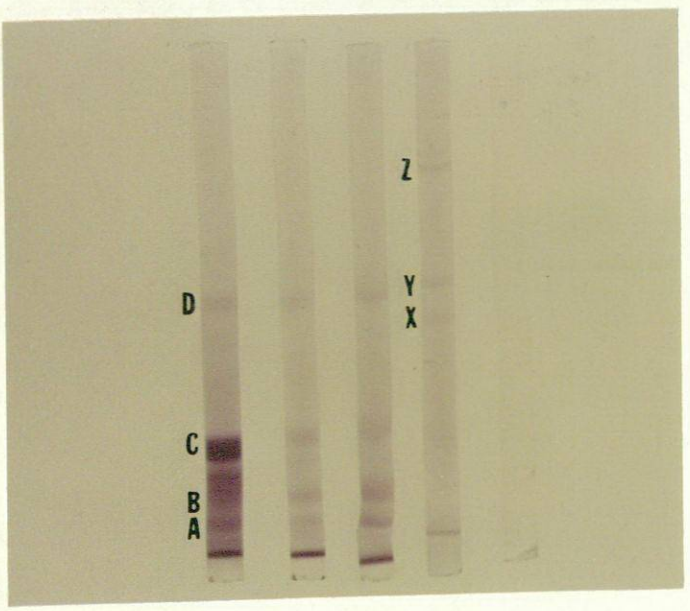
X, Alpha chain, molecular weight 10^5 daltons.
Y, Beta dimer, molecular weight 2×10^5 daltons.
Z, Tropocollagen unit, molecular weight 3×10^5 daltons.

A, Condylar Cartilage (PG-RA).
B, Epiphyseal Cartilage (PG-RA).
C, Nasal Septal Cartilage (PG-RA).
D, Condylar Cartilage (PG-S).
E, Epiphyseal Cartilage (PG-S).
F, Nasal Septal Cartilage (PG-S).
G, Collagen Reference Standard.
H, Blank.

B. Major Protein Bands (A, B, C, D) of PG-S Acrylamide Gels Compared with Protein Bands of Collagen Standard (X, Y, Z).



G A B C H



D E F G H

series of smaller peptides were observed between components a and d. In addition, a series of protein bands with a molecular weight larger than component d (approximately 2 X 10^5 daltons as compared with the collagen standard) were observed in the condylar cartilage gel (A); and comparison with the Z component of the collagen standard suggests that the condylar cartilage gel (A) contains protein fractions with molecular weights as high as 3 X 10^5 daltons.

DISCUSSION

The purpose of this investigation was to determine if, within the same species, chemical and antigenic differences exist among the cartilages of the nasal septum, epiphysis, and condyle. The primary question to be examined was whether or not a molecular basis exists for the observed differential response of certain craniofacial and appendicular cartilages to identical experimental conditions.

The experiments were designed to preclude the possibility that Forssman antigen (a widely distributed group of heterophil antigens) or other heterophil (non-specific) antigens might be responsible for the observed reaction of the antisera with the various cartilages: (1) the immunizing preparation was of bovine origin, and bovine tissues do not possess Forssman antigen (Carpenter, 1956), (2) non-specific adsorption of the antisera with liver and spleen would eliminate all antibodies to heterophil antigens (Kawamura, 1969), and (3) the use of pooled tissue sections (tissues from different calves) would eliminate the possibility of an antibody to an antigenic determinant unique to an individual animal.

After a number of dissections, both the nasal septal and condylar cartilages could be removed and freed of adhering connective tissue with relative ease. This was not the case for epiphyseal cartilage. Removal of adhering bony elements often resulted in "shredding" of the thin epiphyseal cartilage, often yielding only

20-30% of the total amount of epiphyseal cartilage, hence the necessity for dissecting 40 epiphyseal cartilaginous plates in order to acquire the amount of cartilage necessary to conduct this experiment. In spite of the difficulties with the epiphyseal cartilage, visual examination proved adequate to determine whether or not the cartilage fragments used in the immunizing preparations were free of all non-cartilaginous connective tissues. In all control histological examinations, no contaminating connective tissue elements were detected (Plate IV).

In preliminary tests, frozen tissue sections were originally used as substrates for immunofluorescent staining. However, difficulties were encountered with keeping the tissue sections (especially the nasal septum) on the glass microscopic slides through the numerous PBS and distilled water rinses. Consequently, frozen tissue sections were abandoned for paraffin-embedded sections (Sainte-Marie, 1962), a change which greatly minimized, but did not completely eliminate, the loss of tissue from slides during the rinsing procedures.

Although both the amount and type of FITC conjugate used throughout the immunofluorescent staining procedures was consistent, a slight variation in the characteristic yellow-green color was observed throughout the photographed FITC-stained tissue sections. According to the manufacturer (Gibco, Grand Island, New York), this was to be expected.

Characteristic differences in fluorescent staining were

observed between condylar and nasal septal and epiphyseal cartilages. The nasal septal and epiphyseal cartilages exhibited similar staining characteristics, with the staining limited to the territorial matrix immediately outlining the lacunar spaces (Plates VII-VIII).

In contrast, the entire territorial and interterritorial matrix throughout the zones of young and mature chondrocytes of condylar cartilage fluoresced strongly (Plate VI). One possible explanation for this observed difference is that the results of the immunofluorescent staining technique actually reflect the state of aggregation of the cartilage proteoglycans within the cartilage matrix, and thus the relative age difference between the cartilaginous matrix of the condylar and nasal septal and epiphyseal cartilages. Concomitant with the age of the cartilaginous matrix would be an increase in collagen content of the matrix and thus an increase in interstructural bonding between the collagen and cartilage proteoglycans. This theoretical increase in aggregate complexity could possibly "mask" the antigenic sites of the older cartilage proteoglycans, whereas the antigenic sites of the newly-synthesized cartilaginous matrix immediately surrounding the lacunar spaces would not be fully aggregated and thus loosely bound to collagen. Consequently, the antigenic sites within the perilacunar matrix would be readily susceptible to the formation of antigen-antibody complexes. In order to explain the observation that the entire interterritorial matrix of the condylar cartilage fluoresced, this hypothesis would require that the cartilaginous matrix of the condylar cartilage does not

form the complex interstructural relationships to the extent as the matrices of epiphyseal and nasal septal cartilages, and thus does not age in the same manner as the other two cartilages. This theoretical lack of complexity of the interterritorial matrix of condylar cartilage could be due to a relatively lower collagen content.

Moreover, this hypothesis could be extended to explain the observed staining differences between the middle and anterior and posterior regions within the nasal septum (Plate V). Accordingly, the middle sections of nasal septum that demonstrated a minimal fluorescent staining in comparison with controls would reflect a more advanced stage of aging in which the production of cartilaginous matrix by the chondrocytes in this region is negligible. Moreover, the dimunition in size of the chondrocytes of the middle sections of nasal septum, as compared with the size of the chondrocytes in the anterior and posterior sections (Plate V), may be a degenerative change characteristic of senescent cartilage (Quintarelli et al., 1975).

The above explanation for the observed staining characteristics of the various cartilages is in accord with the findings of Quintarelli and co-workers (1975) who have shown that the great output of proteoglycans by chondrocytes in the costal cartilage of rabbits at the neonatal stage was reduced drastically one month after birth. They observed a rapid deterioration of the chondrocytes that was accompanied by a significant reduction in proteoglycan synthesis, and a concomitant, increased insolubility of the organic matrix to

dissociative solvents. This change in the aggregational properties of the matrix was accounted for by a great increase in collagen content which had taken place within the first month of age. Moreover, the effect of aging on the extractability of cartilage proteoglycans with dissociative solvents was underlined by extraction values of about 75 percent in the newborn to only 7 percent in 4 year old animals.

The explanation based on aging, in view of the differences in staining observed among sections of nasal septum (Plate V), is also in accord with the findings of Long and co-workers (1968) that active proliferation of chondrocytes in the nasal septum of the growing rabbit is most pronounced in the anteroinferior and posterior zones and least pronounced in the middle region.

In addition, the results obtained with condylar cartilage may support the contention that the chondrocytes of the condylar cartilage advance through the zone of mineralization without actually degenerating (Holtzer et al., 1970; Silbermann and Frommer, 1972). According to this concept, the chondrocytes of the condylar cartilage appear to survive the hypoxic conditions that are reported to prevail within the mineralizing zone of endochondral ossification through dedifferentiation. The dedifferentiated chondrocytes apparently redifferentiate into more specialized cells, possible "osteoprogenitor" cells in the vicinity of the ossifying front (Silbermann and Frommer, 1972). This is in direct contrast with the fate of the chondrocytes in epiphyseal cartilage, which have been reported to degenerate as they reach the

calcified zone and subsequently die (Scott and Pease, 1956; Olson and Low, 1971).

The observation that the FITC-conjugated antiserum against condylar cartilage (ACCA) retained its ability to stain condylar cartilage after adsorption against epiphyseal and nasal septal cartilage whereas similar treatment of AECA and ANSA eliminated their staining ability indicates that condylar cartilage possesses at least one antigenic determinant distinct from (and in addition to) those of either epiphyseal or nasal septal cartilage. By inference from the studies of DiFerrante (1964), DiFerrante and Pauling (1964), and Loewi and Muir (1965), it is possible to speculate that the unique antigenic determinant(s) in condylar cartilage may be the result of differences in the tertiary structure of the proteoglycan complex or the result of differences in the primary structure (amino acid sequence) of the protein moiety within the proteoglycan complex.

If the unique antigenicity of condylar cartilage proteoglycans depends on its tertiary structure, then treatment with 2-mercaptoethanol would be expected to disrupt the tertiary structure, resulting in a heterogenous population of proteoglycan subunits only part of which would retain the structural requirements necessary to induce the appropriate antibody response. Formation of proteoglycan subunits appears to be born out by the additional protein bands observed in the SDS-acrylamide gel of the condylar cartilage fraction that was reduced with 2-mercaptoethanol (Plate IX,A). Consequently, it is more likely that the unique antigenicity is due to differences in

the primary structure of the protein moiety of the proteoglycans, or, more specifically, to differences in amino acid sequence within the protein subunits.

Whatever the nature of the unique antigen in condylar cartilage, the results of this investigation appear to indicate that nasal septal and epiphyseal cartilage are immunologically indistinguishable and chemically similar, whereas condylar cartilage possesses a unique antigenic determinant(s) distinct from both the nasal septal and epiphyseal cartilages.

Results in View of Contemporary Theories

According to Koski and associates (1968, 1969, 1970), condylar cartilage responds differently from other craniofacial and appendicular cartilages in transplantation experiments. The results of this investigation appear to further substantiate the contention that condylar cartilage is different from other cartilages, at least at the immunological level. However, the "experimental environment" of the transplant studies may be so drastically abnormal as to negate any conclusions that might be drawn concerning the growth of these cartilages. In light of the body of evidence that is accumulating on the effects of somatomedin on cartilaginous growth, it would seem more appropriate to assume that all cartilages have an "intrinsic" ability to grow, and that the expression of this capacity is modified by a number of factors, including possible chemical differences among the cartilages themselves.

From the results of condylectomy experiments, Moss (1972) concluded that the growth of condylar cartilage is secondary to the growth of orofacial matrices, and extended this interpretation to include the growth of all craniofacial cartilages. The present investigation provides support for the contention that certain cartilages are different and, therefore, may not behave in the same manner. Consequently, there is reason to suspect that Moss's unitary view of all major craniofacial cartilages is wrong. Indeed, the results indicate that it is not possible to make generalized conclusions for all similar tissues.

SUMMARY AND CONCLUSIONS

The history of modern craniofacial biology is characterized by a marked concern for the role that the major craniofacial cartilages play in the growth of the craniofacial skeleton. It was once widely assumed by many investigators that these cartilages acted as "growth centers" in a manner analogous to cartilaginous epiphyseal plates. In recent years, however, attention has turned toward the apparent differences in growth properties that exist among these various cartilages. By inference from the results of investigations designed to compare these cartilages, in general, and from the results of transplantation studies, in particular, several investigators have concluded that differences exist in the fundamental properties of the various cartilages. The verity of this contention has not yet been proven, and whether or not there is a molecular basis for the suggested differences has never been subjected to experimental verification.

The present study used both indirect immunofluorescent labeling and the polyacrylamide gel disc electrophoresis to investigate antigenic and chemical differences among the cartilages of the nasal septum, condyle, and epiphysis. The cartilages were obtained by careful dissection from six month old calves, reduced and alkylated with 2-mercaptoethanol and iodoacetic acid respectively, and their

soluble extracts used as antigens for the production of specific antisera in rabbits. Non-specific antibodies were removed from the antisera by adsorption against bovine liver and spleen. Examination for antigenic differences among the various cartilages was conducted in three separate experiments, in which samples of each of the various antisera were adsorbed against nasal septal, epiphyseal, and condylar cartilage respectively and applied to tissue sections of each of the three cartilages. The antigen-antibody complexes were subsequently labeled with fluorescein-conjugated anti-rabbit globulin and examined microscopically.

In addition, limited characterization of the soluble fractions was accomplished by sodium dodecyl sulfate gel electrophoresis. The resulting band patterns of each of the cartilaginous fractions were stained with a protein-specific dye and compared.

Microscopic examination of the immunofluorescent labeled sections of the three cartilages revealed that fluorescent staining of both the epiphyseal and nasal septal cartilages was limited to the territorial matrix, whereas the entire territorial and interterritorial matrix of the condylar cartilage fluoresced strongly. Moreover, regional differences in staining were observed within the nasal septal cartilage.

The antisera of each of the three cartilages demonstrated an ability to cross-react with the other cartilages. Adsorption of each of the antisera against the other two cartilages indicated that condylar cartilage possesses at least one unique antigenic determinant.

Limited characterization of the soluble fractions of each of the cartilages by gel electrophoresis revealed that, with minor variation, nasal septal and epiphyseal are essentially similar. By contrast, the electrophoretic patterns of the soluble fraction of the condylar cartilage were clearly different from both the epiphyseal and nasal septal cartilages.

The results of the study may be summarized:

1. The use of the indirect immunofluorescent technique revealed antigenic differences among the condylar and the epiphyseal and nasal septal cartilages. In addition, regional differences in the location of antigenic determinants were revealed within the nasal septal cartilage.

2. Condylar, epiphyseal, and nasal septal cartilage possess common antigenic determinants as evidenced by the ability of each of the antisera to cross-react with the various cartilages.

3. Condylar cartilage possesses at least one antigenic determinant distinct from those of both the epiphyseal and nasal septal cartilage.

4. Electrophoretic analysis revealed compositional differences among the condylar, epiphyseal, and nasal septal cartilaginous constituents, with the differences in condylar cartilage appearing to be most extensive.

5. The results are consistent with the assumption that condylar cartilage is fundamentally different from other craniofacial and appendicular cartilages.

6. Therefore, it appears that all cartilages may not behave in the same manner, and that generalized conclusions for all cartilaginous tissues cannot be supported.

LITERATURE CITED

Adamson, L. F., and Anast, C. S.: Amino acid, potassium, and sulfate transport and incorporation by embryonic chick cartilage: The mechanism of the stimulatory effects of serum, Biochim. Biophys. Acta 121: 10, 1966.

Almqvist, S., Ikkos, D., and Luft, R.: Studies on sulfation factor (SF) activity of human serum. Serum SF in hypopituitatism and acromegaly, Acta Endocrinol. 36: 577-595, 1961.

Anderson, B., Hoffman, P., and Meyer, K.: The O-serine linkage in peptides of chondroiton 4- or 6-sulfate, J. Biol. Chem. 240: 156-167, 1965.

Anderson, W. B., Russell, T. R., Carchman, R. A., and Pastan, I.: Interrelationship between adenylate cyclase activity, cyclic AMP phosphodiesterase activity, cyclic AMP levels, and growth of cells in culture, Proc. Natl. Acad. Sci. U.S.A. 70: 3802-3805, 1973.

Baume, L. J.: Principles of cephalofacial developmental biology, Am. J. Orthod. 47: 881-901, 1961.

Baume, L. J.: Differential response of condylar, epiphyseal, synchondrotic, and articular cartilages of the rat to varying levels of vitamin A, Am. J. Orthod. 58: 537-551, 1970.

Bergman, B.: The distribution and concentration of zinc and the effect of zinc deficiency in the mammalian body, Odont. Revy. 21: Suppl. 26., 1970.

Birnbaumer, L., Pohl, S. L., Michiel, H., Krans, J., and Rodbell, M.: The actions of hormones on the adenylate cyclase system, In: Advances in Biochemical Psychopharmacology, New York: Raven Press, 1970.

Blackwood, H. J. J.: Growth of the mandibular condyle of the rat studied with tritiated thymidine, Arch. Oral Biol. 11: 493-500, 1966.

Blau, S., Janis, R., Hamerman, D., and Sandson, J.: Cellular origin of hyaluronate protein in the human synovial membrane, Science 150: 353-355, 1965.

Brasel, J. A., Wright, J. C., Wilkins, L., and Blizzard, R. M.: An evaluation of seventy-five patients with hypopituitarism beginning in childhood, Am. J. Med. 38: 484-498, 1965.

Bremers, L. M. H.: De Condylus mandibulae in vitro, Dissertation, University of Nijmegen, The Netherlands, 1973.

Campbell, D. H., Garvey, J. S., Cremer, N. E., and Sussdorf, D. H.: Methods in Immunology, New York: W. A. Benjamin, Inc., 1964.

Carpenter, P. L.: Immunology and Serology, Philadelphia: W. B. Sanders Company, 1956.

Coons, A. H., and Kaplan, M. H.: Localization of antigen in tissue cells: Improvements in a method for the detection of antigen by means of fluorescent antibody, J. Exp. Med. 91: 1-13, 1950.

Daughaday, W. H.: Regulation of skeletal growth by sulfation factor, Adv. Intern. Med. 17: 237-263, 1971.

Daughaday, W. H., Hall, K., Raben, M. S., Salmon, W. D., and Van den Brande, J. L.: Somatomedin: A proposed designation for the "sulfation factor", Nature 235: 107, 1972.

Daughaday, W. H., Heins, J. N., Srivastava, L., and Hammar, C.: Sulfation factor: Studies of its removal from plasma and metabolic fate in cartilage, J. Lab. Clin. Med. 72: 803, 1968.

Daughaday, W. H., Laron, Z., Pertzelan, A., and Heins, J. N.: Defective sulfation factor generation: a possible etiological link in dwarfism, Trans. Assoc. Am. Physicians. 82: 129-138, 1969.

Daughaday, W. H., and Kipnis, D. M.: The growth-promoting and anti-insulin actions of somatotropin, Recent Prog. Horm. Res. 22: 49-99, 1966.

Daughaday, W. H., and Mariz, I. K.: Conversion of proline-U-C^{14} to labeled hydroxyproline by rat cartilage in vitro: Effects of hypophysectomy, growth hormone and cortisol, J. Lab. Clin. Med. 59: 741-752, 1962.

Daughaday, W. H., and Reeder, C.: Synchronous activation of DNA synthesis in hypophysectomized rat cartilage by growth hormone, J. Lab. Clin. Med. 68: 357-368, 1967.

Daughaday, W. H., Salmon, W. D., and Alexander, F.: Sulfation factor activity of sera from patients with pituitary disorders, J. Clin. Endocrinol. Metab. 19: 743-758, 1959.

DeAsua, L. J., Surian, E. S., Flawia, M. M., and Torres, H. N.: Effect of insulin on the growth pattern and adenyl cyclase activity of BHK fibroblasts, Proc. Natl. Acad. Sci. U.S.A. 70: 1388-1392, 1973.

DeBeer, G. R.: The Development of the Vertebrate Skull, London: Oxford University Press, 1937.

DiFerrante, N.: Precipitins in the rabbit produced by protein-polysaccharide from bovine nasal cartilage, Science 143: 250, 1964.

DiFerrante, N., and Pauling, M.: Properties of antibodies to a protein polysaccharide from bovine nasal cartilage, J. Lab. Clin. Med. 63: 945-952, 1964.

Doganges, P. T., and Schubert, M.: The use of lanthanum to study the degradation of a protein polysaccharide from cartilage, J. Biol. Chem. 239: 1498-1508, 1964.

Duterloo, H. S.: In vivo implantation of the mandibular condyle of the rat: An experimental investigation of the growth of the lower jaw, Dissertation, University of Nijmegen, The Netherlands, 1967.

Eker, P.: Inhibition of growth and DNA synthesis in cell cultures by cyclic AMP, J. Cell Sci. 16: 301-307, 1974.

Elders, M. J., Wingfiel, B. S., McNatt, L. M., and Hughes, E. R.: Somatomedin activity in plasma of children receiving prednisone, abstracted, Pediatr. Res. 7: 324, 1973.

Exton, J. H., Lewis, S. B., Ho, R. J., Robison, G. A., and Park, C. R.: The role of cyclic AMP in the interaction of glucagon and insulin in the control of liver metabolism, Ann. New York Acad. Sci. 185: 85-100, 1971.

Felts, W. J. L.: In vivo implantation as a technique in skeletal biology, Int. Rev. Cytol. 12: 243-302, 1961.

Ford, E. H. R.: The growth of the foetal skull, J. Anat. 90: 63-72, 1956.

Ford, E. H. R.: Growth of the human cranial base, Am. J. Orthod. 44: 498-506, 1958.

Franek, M. D., and Dunstone, J. R.: Connective tissue protein-polysaccharides. Fractionation of the protein-polysaccharides from bovine nasal cartilage, J. Biol. Chem. 242: 3460-3467, 1967.

Froehlich, J. E., and Rachmeler, M.: Effect of adenosine 3',5'-cyclic monophosphate on cell proliferation, J. Cell Biol. 55: 19-31, 1972.

Froehlich, J. E., and Rachmeler, M.: Inhibition of cell growth in the G_1 phase by adenosine 3',5'-cyclic monophosphate, J. Cell Biol. 60: 249-257, 1974.

Gasson, N., and Petrovic, A.: Mécanismes et régulation de la croissance antéro-postériere du maxillaire supérieur. Rechercheo expérimentales, chez le jeune rat, sur le role de l'hormone somatotrope et du cartilage de la cloison nasale, Orthodont. Franç. 43: 255-270, 1972.

Gasson, N., Stutzmann, J., and Petrovic, A.: Les méchanismes régulateurs de l'adjustement occlusal, interviennent-ils dans le controlê de la croissance du cartilage condylien? Expériences d'administration d'hormone somatotrope et de résection du cartilage septal chez le jeune rat, Orthodont. Franç. 46: (in press), 1975. As cited in Petrovic et al., 1975.

Graber, T. M.: Extrinsic control factors influencing craniofacial growth. In: Control Mechanisms in Craniofacial Growth, Ann Arbor: The University of Michigan, 1975.

Gregory, J. D.: Structure and aggregation of proteoglycans of cartilage, In: Protides of the Biological Fluids (H. Peeters, ed.), Oxford: Pergammon Press, 1975.

Gregory, J. D., Laurent, T. C., and Rodén, L.: Enzymatic degradation of chondromucoprotein, J. Biol. Chem. 239: 3312-3320, 1964.

Gregory, J. D., and Van Lenten, L.: Countercurrent distribution of bovine chondromucoprotein degraded with testicular hyaluronidase, Fed. Proc. 23: 483, 1964.

Hamilton, W. J., Boyd, J. D., and Mossman, H. W.: Human Embryology: Prenatal Development of Form and Function, New York: The Williams and Wilkins Co., 1962.

Hardingham, T. E., and Muir, H.: The specific interaction of hyaluronic acid with cartilage proteoglycans, Biochim. Biophys. Acta 279: 401-405, 1972.

Hascall, V. C., and Heinegård, D.: Aggregation of cartilage proteoglycans: The role of hyaluronic acid, J. Biol. Chem. 249: 4232-4241, 1974.

Hascall, V. C., and Heinegård, D.: The structure of cartilage proteoglycans. In: Extracellular Matrix Influences on Gene Expression (H. C. Slavkin and R. G. Grevlich, eds.), New York: Academic Press, 1975.

Hascall, V. C., and Riolo, R. L.: Characteristics of the protein-keratan sulfate core and of keratan sulfate prepared from bovine nasal cartilage proteoglycan, J. Biol. Chem. 247: 4529-4538, 1972.

Hascall, V. C., and Sajdera, S. W.: Protein polysaccharide complex from bovine nasal cartilage. The function of glycoprotein in the formation of aggregates, J. Biol. Chem. 244: 2384-2396, 1969.

Heinegård, D., and Hascall, V. C.: Aggregation of cartilage proteoglycans: Characteristics of the proteins isolated from trypsin digests of aggregates, J. Biol. Chem. 249: 4250-4256, 1974.

Hintz, R. L., Clemmons, D. R., Underwood, L. E., and Van Wyk, J. J.: Competitive binding of somatomedin to the insulin receptors of adipocytes, chondrocytes, and liver membranes, Proc. Natl. Acad. Sci. U.S.A. 69: 2351-2353, 1972.

Hirschman, A.: Protein-polysaccharide loss during endochondral ossification: Immunochemical evidence, Science 154: 393-395, 1966.

Holtzer, H., Chacko, S., Abbot, J., Holtzer, S., and Anderson, H.: Variable behavior of chondrocytes in vitro. In: Chemistry and Molecular Biology of the Intercellular Matrix (E. A. Balazs, ed.), New York: Academic Press, 1970.

Hopwood, J. J., and Robinson, C. H.: The alkali-labile linkage between keratan sulphate and protein, Biochem. J. 141: 57-69, 1974.

Hopwood, J. J., and Robinson, C. H.: The structure and composition of cartilage keratan sulphate, Biochem. J. 141: 517-526, 1974.

Hulth, A.: Experimental retardation of endochondral growth by papain, Acta Orthop. Scand. 28: 1-21, 1958.

Kawamura, A.: Fluorescent Antibody Technique and Their Applications, Tokyo: University of Tokyo Press, 1969.