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**LA THÈSE A ÉTÉ  
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GLYCOSAMINOGLYCAN SYNTHESIS AND  
THE INITIATION OF SECONDARY CHONDROGENESIS  
IN THE EMBRYO CHICK

by

Garry Thomas Ross .

Approved:

Submitted in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy

at Dalhousie University, Halifax, Nova Scotia

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# ABSTRACT

The development of secondary cartilage on the quadratojugal of stage 35 to stage 38 embryo chicks, in both normally developing embryos and in embryos which had been paralyzed, was surveyed biochemically. The activity of the cartilage specific enzymes UDPG-dehydrogenase and UDPG-4-epimerase, the concentration of UTP, the relative amounts of intermediates in the synthetic pathway of UDPNAGal, and the incorporation of  $[^3\text{H}]$ -glucosamine and  $[^{35}\text{S}]$ -sulphate have been examined. It has been shown that an increase in the activity of the cartilage specific enzymes precedes the synthesis of the major glycosaminoglycan of cartilage and that the uptake of  $[^3\text{H}]$ -glucosamine is initiated earlier in development than is the uptake of  $[^{35}\text{S}]$ -sulphate. Further, it has been demonstrated that paralysis of the embryo suppresses both the activity of the cartilage specific enzymes and the incorporation of the radiolabelled precursors as well as inhibiting the histological differentiation of secondary cartilage. These results are interpreted as indicating that levels of cartilage specific enzymes are not stabilized in differentiating chondroblasts and that a chondrogenic bias is acquired by germinal cells around the hook of the quadratojugal at a stage of development prior to the stage at which secondary cartilage is induced.

#### ABBREVIATIONS AND CONVENTIONS

Stage "n" paralysed embryos: embryos examined at stage "n", 24 hours following injection of paralysing agent at approximately stage "n-1".

AB: stained with Alcian Blue and Mayers Haemalum

ABCR: stained with the Alcian Blue-Chlorantine Red technique

GN-6-P: glucosamine-6-phosphate

NAD: nicotinamide adenine dinucleotide

NADP: nicotinamide adenine dinucleotide phosphate

NAG-1-P: N-acetylglucosamine-1-phosphate

NAG-6-P: N-acetylglucosamine-6-phosphate

UDP: uridine diphosphate

UDPG: uridine diphosphoglucose

UDPGNAc: uridine diphospho-N-acetylglucosamine

UDPNAGal: uridine diphospho-N-acetylgalactosamine

UTP: uridine triphosphate



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## INTRODUCTION

The cardinal aim of modern developmentalists is the elucidation of the cellular control mechanisms involved in the series of sequential inductions which give rise to the development of a multicellular organism from a single-celled zygote. This task is somewhat frustrated during the early stages of development by the virtual totipotency of all cells of the embryo. However, investigations conducted during the differentiation of both morphologically and biochemically defined tissues, a stage of development when any given cell of the embryo has a more limited range of response to an inductive stimulus, have greatly increased our knowledge of these control mechanisms. The formation of secondary cartilage on the quadratojugal of the embryo chick is one such system in which the germinal cells have a limited developmental potential and in which the differentiated tissue has been well characterized. While factors involved in the induction of this tissue have been examined (see page 2), the biochemical aspects of the response of germinal cells to these stimuli (i.e. cellular synthetic control mechanisms) have not been well documented. This investigation was conducted in order to discover possible control mechanisms involved in the evocation of secondary cartilage on the quadratojugal of the embryo chick.

### A. SECONDARY CARTILAGE: ITS NATURE AND FACTORS CONTROLLING ITS OCCURENCE

Secondary cartilage appears later in development than does the primary cartilagenous skeleton and is that cartilage which is associated with membranous bones (De Beer, 1937). Secondary cartilage has been demonstrated to form along the cranial and facial sutures of mammals (Pritchard et al., 1956; Moss, 1958), on the mandibular condyle in mammals

2

(Durkin et al., 1973) and on avian membrane bones (Murray, 1963; Murray and Smiles, 1965; Hall, 1967a, 1968a). Secondary cartilage, unlike articular cartilage, grows by division of undifferentiated cells (Petrovic, 1972) and is capable of adaptive and remodelling responses (Durkin et al., 1973). However, the biochemical nature of mammalian secondary cartilage has been shown to be similar to that of articular cartilage (Heeley et al., 1970) and Hall (1968b, 1968c) has shown that mammalian and avian secondary cartilage are histochemically similar. Hence, studies conducted on avian secondary cartilage would provide a useful model for the elucidation of factors controlling the development of articular cartilage in general.

Considerable information is available concerning both the time of appearance of secondary cartilage on the embryonic chick quadratojugal (a membrane bone in the upper jaw of the bird) and environmental factors involved in the evocation of this cartilage. Murray and Smiles (1965) and Hall (1968a) have shown that secondary cartilage is induced on the hook of the quadratojugal of the embryo chick between the 9th and 10th day of incubation. These authors have shown that the cartilage arises from a pool of germinal cells around the tip of the hook, located at the posterior end of the bone, and that this germinal pool gives rise exclusively to osteoblasts prior to 9 days of incubation. The switch of germinal cells from osteogenesis to chondrogenesis is induced by mechanical stimuli (Murray, 1963; Hall, 1967a, 1967b), in particular intermittent pressure and tension plus movement (Hall, 1968a, 1972a). Further, these authors have shown that the secondary cartilage forms around the bony hook, that it is hypertrophic in nature nearest to the bony shaft, and that the cartilage matrix subsequently calcifies.

Paralysis of the embryo prior to 10 days of incubation inhibits the formation of secondary cartilage while paralysis after 10 days of incubation switches the chondrogenic germinal cells back to a purely osteogenic pool (Murray, 1957; Hall, 1972a). Immobilization of the lower jaw has similarly been found to inhibit growth of mammalian condylar cartilage while hyperpropulsion stimulated growth of this cartilage (Petrovic, 1972).

Several attempts to isolate both the mode of action of the mechanical stimuli and possible mechanisms controlling the differentiation of avian secondary cartilage, have been undertaken. Hall (1969) has shown that low oxygen tensions allow the formation of secondary cartilage from 9 day embryo chick quadratojugals in culture and that the switch from bone differentiation to cartilage differentiation involves a change in the rate of both collagen and glycosaminoglycan synthesis (Hall, 1968c). Generally, increased oxygen tension, blood supply and rate of collagen synthesis favors the differentiation of bone while increased rate of glycosaminoglycan synthesis, mechanical stress and decreased oxygen tension favors secondary cartilage formation on the quadratojugal of the embryonic chick (Hall, 1970a, 1970b). It has also been shown that following immobilization of the quadratojugal in organ culture, the cartilage matrix shows a loss of hexosamines (Hall, 1972a) while chemical inhibitors of either the sulphation of chondroitin or the synthesis of mature, insoluble collagen reduce hexosamine synthesis, without affecting total collagen synthesis, and inhibit secondary chondrogenesis (Hall, 1970c, 1972b).

These and other investigations have shed considerable light on the histological and histochemical aspects of secondary cartilage differentiation as well as on the alterations in these aspects caused by various

inhibitors of this cartilage. However, no information is available regarding the biosynthetic pathway of cartilage matrix synthesis in cells undergoing secondary chondrogenesis. While histochemical studies have provided indirect evidence on the functioning of this pathway, it is necessary to examine the activity of the various synthetic enzymes in order to further isolate the control mechanisms involved in the evocation of secondary cartilage.

#### B. BIOSYNTHESIS OF CARTILAGE

Cartilage is a mesodermally derived connective tissue whose structure is determined by the composition of the 'non-living', extracellular matrix. This matrix may be divided into two general components: 1) amorphous component in which is embedded 2) the fibrous component. The main constituents of the amorphous component are glycosaminoglycans and proteoglycans. The main fibrous component is collagen.

The structure and biosynthesis of glycosaminoglycans and proteoglycans have been extensively investigated and several recent reviews of this topic are available (Muir, 1964; Barrett, 1968; Roden, 1970; Levitt and Dorfman, 1974). In general, glycosaminoglycans are long chain heteropolysaccharides composed of repeating disaccharide units. The disaccharide unit consists of a hexosamine (which may or may not be sulphated) and a non-nitrogenous sugar linked by a glycosidic bond. Examples of sulphated glycosaminoglycans are chondroitin-4-sulphate, chondroitin-6-sulphate, and keratan sulphate, while hyaluronic acid is a non-sulphated glycosaminoglycan. The disaccharide unit of the chondroitin sulphates is composed of N-acetyl-galactosamine and glucuronic acid; that of keratan sulphate is composed of N-acetylglucosamine and galactose; that of hyaluronic acid is composed

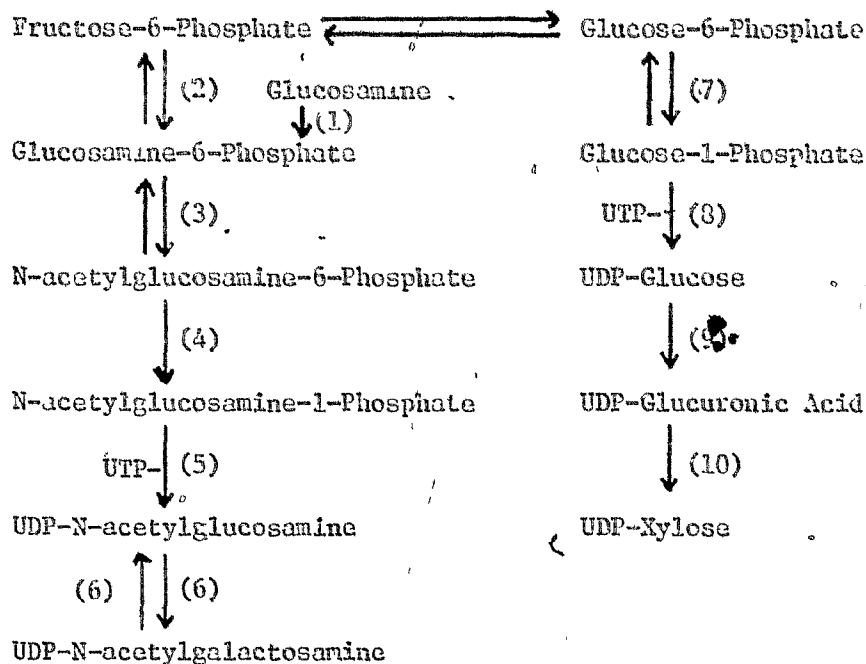
of N-acetylglucosamine and glucuronic acid. The glycosaminoglycans comprise about 10% of the dry weight of adult cartilage with the chondroitin sulphates constituting the major portion of the glycosaminoglycans, but small amounts of keratan sulphate and hyaluronic acid are also present (Anderson et al., 1964; Levitt and Dorfman, 1974). Glycosaminoglycans are usually covalently linked to protein and this complex is known as a proteoglycan (Roden, 1970).

The chondroitin sulphate glycosaminoglycan is linked to the protein core through a trisaccharide link composed of one molecule xylose and two molecules galactose (Lindahl and Roden, 1966; Roden and Smith, 1966). The xylose is bound through a covalent glycosidic bond, to the hydroxyl group of a serine residue in the core protein and approximately one-half of the available serine residues bear carbohydrate substituents (Muir, 1958; Baker et al., 1972; Kao et al., 1972; Hopwood and Robinson, 1975). While synthesis of the core protein no doubt normally precedes glycosaminoglycan chain initiation during the synthesis of proteoglycans (de la Haba and Holtzer, 1965; Telser et al., 1965; Jeffrey and Rienits, 1972), recent evidence indicates that chondroitin sulphate polymerization can be initiated in the absence of protein acceptor (Okayama et al., 1973; Schwartz et al., 1974; Robinson et al., 1975).

Perlman et al. (1964) and Silbert (1964) have shown the requisite precursors of chondroitin sulphate synthesis to be the UDP derivatives of N-acetylgalactosamine and glucuronic acid. It has also been shown that uridine nucleotide sugars are the precursors of all glycosaminoglycan synthesis (Roden, 1970). The biosynthetic pathway of uridine nucleotide precursors is shown in Figure I. The initiation and polymerization of the chondroitin sulphate chain is carried out on the membranes of the

SYNTHETIC PATHWAYS OF THE UDP-MONOSACCHARIDE

PRECURSORS OF THE GLYCOSAMINOGLYCANS



- (1) Hexokinase
- (2) L-Glutamine-D-fructose-6-Phosphate Amidotransferase
- (3) Phosphoglucosamine transacetylase
- (4) Acetylglucosamine phosphomutase
- (5) UDP-N-acetylglucosamine pyrophosphorylase
- (6) UDP-N-acetylglucosamine-4-epimerase
- (7) Phosphoglucomutase
- (8) Glucose-1-phosphate uridylyltransferase
- (9) UDP-glucose dehydrogenase
- (10) UDP-glucuronate decarboxylase

FIGURE 1

endoplasmic reticulum, initiation occurring in the rough endoplasmic reticulum and chain elongation proceeding as the molecule passes from the rough into the smooth endoplasmic reticulum and Golgi apparatus (Horwitz and Dorfman, 1968). Sulphation of the chondroitin has been shown to occur during chain elongation and to take place to a large extent in the smooth endoplasmic reticulum (Horwitz and Dorfman, 1968; DeLuca et al., 1973; Richmond et al., 1973). Olsson (1972) has demonstrated that hyaluronic acid synthesis in myeloid cells of rabbit bone marrow also occurs in the endoplasmic reticulum.

The synthesis of the protein-glycosaminoglycan link region and the sequential alternate transfer of monosaccharides from their UDP derivatives to the ends of growing chains is catalyzed by a series of six glycosyl transferases which usually occur as a multienzyme complex bound to the membranes of the endoplasmic reticulum (Robinson et al., 1966; Telser et al., 1966; Helting and Roden, 1968; Stoolmiller et al., 1972). The sulphation of chondroitin sulphate is accomplished by specific sulfotransferases which transfer the sulphate ester groups from the activated intermediate 3'-phosphadenosine-5'-phosphosulphate (PAPS) (Roden et al., 1972; DeLuca et al., 1973). Several factors controlling chondroitin sulphate synthesis have been studied.

An obvious potential point of control of the synthesis of cartilage matrix is at the level of the glycosyl transferases involved in the synthesis of chondroitin sulphate. Caplan and Stoolmiller (1973) have shown that the synthesis of Xylosyltransferase and N-acetylgalactosaminyltransferase may be separately regulated. It has been suggested that the interaction between Xylosyltransferase and the first galactosyltransferase may act to position the multienzyme complex on the endoplasmic reticulum



(Schwartz, 1974; Schwartz, Roden and Dorfman, 1974). It has also been shown that the interaction between these two enzymes may be selective and that chondroitin sulphate synthesis may be regulated by the association and dissociation of Xylosyltransferase with the multienzyme complex (Stoolmiller et al., 1972; Speciale et al., 1974). However, Levitt and Dorfman (1973) found only a slight reduction in the activity of Xylosyltransferase and N-acetylgalactosaminyltransferase following bromodeoxyuridine inhibition of chondrogenesis in embryonic chick limb bud cell cultures, and Nevo and Dorfman (1972) found no change in these two enzymes under conditions of stimulated glycosaminoglycan synthesis in embryo epiphyseal cartilage cell cultures.

Control of cartilage synthesis may also be mediated through control of synthesis of the uridine nucleotide sugar precursors. Abbott and Holtzer (1968), have shown that bromodeoxyuridine inhibition of chondrogenesis in cultured chondrocytes reduced the incorporation of [ $^{14}\text{C}$ ]-glucose into chondroitin sulphate. Inhibition of chondrogenesis, with this agent, in cultured chick limb bud cells and in chick embryo chondrocytes has also been shown to suppress the activity of UDPG-dehydrogenase and of UDPGNac-4-epimerase (Marzullo, 1972; Levitt and Dorfman, 1973). Increasing activity of these two enzymes has been shown to accompany both cartilage development (Medoff, 1967; Marzullo and Desiderio, 1972) and the post partum surge in chondrogenesis in achondroplastic mice (Johnson and Hunt, 1974). A general instability in the synthetic pathway of uridine nucleotide sugars in young chondrocytes but not in mature cells has been demonstrated during embryonic cartilage differentiation (Lash, 1968; Ellison and Lash, 1971; Levitt and Dorfman, 1972; Marzullo, 1972).

Regulation of some of these precursor synthetic enzymes has been shown to occur via feedback inhibition of uridine nucleotide sugars

themselves. Kornfeld (1967) has shown that L-glutamine-D-fructose-6-phosphate amidotransferase, the first enzyme in the synthesis of uridine nucleotide sugars (see Figure 1) is inhibited by UDPGNac. This enzyme is also inhibited by 5'-adenylate, a regulating factor of glycolysis, (Ariambide et al., 1968) and by glutamylaminoacetonitrile, a competitive inhibitor of glutamine for the enzyme (McCallum and Arbutnot, 1969). It has also been shown that UDPGNac can regulate the activity of the glucuronyltransferase enzyme (Vessey et al., 1973). The allosteric inhibition of UDPG-dehydrogenase by UDP-xylose has been demonstrated in a variety of systems (Gainey and Phelps, 1972; Balduini et al., 1973).

There is evidence to suggest that control of matrix synthesis is influenced by environmental factors in the immediate vicinity of the chondrocyte. Conflicting evidence exists supporting theories of both negative and positive feedback control by matrix macromolecules. Loss of matrix macromolecules following hyaluronidase digestion or lysosomal activity in organ cultures has been shown to stimulate synthesis of chondroitin sulphate (Fitton-Jackson, 1970; Hardingham et al., 1972; Deshmukh and Hemrick, 1976). Conversely chondroitin sulphate added to chondrocyte cultures also stimulated chondroitin sulphate synthesis (Nevo and Dorfman, 1972; Kosher et al., 1973; Schwartz and Dorfman, 1975) while the accumulation of matrix macromolecules has been shown to both stimulate further synthesis and to be the critical factor in tissues which induce chondrogenesis during embryonic development (O'Hare, 1972a, 1972b; Gordon and Lash, 1974; Solursh and Meier, 1974; Kosher and Lash, 1975; Solursh and Karp, 1975). In addition, exogenous D-glucosamine has been found to stimulate chondroitin sulphate synthesis and to increase cellular pools of UDP-N-acetylhexosamines (Kim and Conrad, 1974). Other

environmental factors such as vitamins (Solursh and Meier, 1973; Vasan and Liss, 1975) and critical ion concentrations (Lash et al., 1973; Prince et al., 1974) have also been shown to affect the synthesis of chondroitin sulphate. It has been postulated that control in general of cartilage synthesis may be mediated through receptors on the chondrocyte cell surface (Chacko et al., 1969; Nevo and Dorfman, 1972).

### c. RATIONALE OF THE APPROACH OF THIS INVESTIGATION

Knowledge of the level(s) at which metabolic control is exerted on the synthesis of chondroitin sulphate is essential to an understanding of the mechanism of induction and cytodifferentiation of secondary cartilage on the quadratojugal of the embryo chick. The existence of a unique, cartilage specific type of collagen molecule (Miller and Matukas, 1969; Starwich and Niani, 1971; Trelstad et al., 1972) and the loss of the ability to produce this molecule in dedifferentiated chondrocytes in vitro (Schultz et al., 1973) would suggest that the control of cartilage differentiation is exerted at the level of gene regulation. Similarly, genetic regulation is suggested by the presence in cartilagenous tissue of a cartilage specific proteochondroitin sulphate (Goetinck et al., 1974) and by the suppression of this molecule in nanomelic chicks and in bromodeoxyuridine inhibited chondrocytes in vitro (Palmoski and Goetinck, 1972; Pennypacker and Goetinck, 1976). However, the cells giving rise to the secondary cartilage on the chick quadratojugal initially were giving rise to bone, and small amounts of chondroitin sulphate are normally found in bone matrix. It has also been shown that the ability to synthesize glycosaminoglycans is almost universal amongst the tissues of the early chick embryo (Lash, 1968; Mayne et al., 1971; Manasek et al., 1973). Thus, while the induction of secondary cartilage no doubt involves

the turning on of at least one new gene (collagen), the synthesis of cartilage matrix glycosaminoglycans may represent no more than the amplification of an enzyme pathway already present in the undifferentiated cells. However, this amplification is also subject to control and may be turned up or down.

Possible points of control in a synthetic pathway, as well as the nature of the control mechanism, are indicated by several parameters. Feedback inhibition of an enzyme is often found when the cell has but a single use for the product of the reaction catalyzed by that enzyme. These feedback controls frequently act via allosteric mechanisms, in particular allosteric  $K_m$  modifying mechanisms. Non-equilibrium type reactions are also indicative of likely points of control. The enzymes UDPG-dehydrogenase and UDPGNac-4-epimerase, two enzymes in the synthetic pathway of uridine nucleotide sugars, are suggested by these criteria as possible points of control in the synthesis of chondroitin sulphate. Another possible control of this synthesis is the availability of UTP, an essential coenzyme in the synthesis of UDPGNac.

The kinetic parameters of the enzyme UDPG-dehydrogenase from various tissues have been described (Gainey and Phelps, 1972; Balduini et al., 1973). The suitability of this enzyme as a synthetic control point is indicated by its strong feedback inhibition by UDP-xylose, by the fact that this inhibition occurs via allosteric mechanisms, and by the fact that, although the product of this reaction, UDP-glucuronic acid, has restricted direct metabolic usefulness, the reaction catalyzed by UDP-glucose dehydrogenase is an irreversible reaction (Ridley et al., 1975). The kinetic parameters of UDPGNac-4-epimerase have also been demonstrated (Glaser, 1959; Jacobson and Davidson, 1963). Although it has been suggested that

this enzyme is not rate limiting during chondroitin sulphate synthesis. (Hindley, and Phelps, 1972) it is noted that the normal equilibrium of this enzyme reaction favors UDPGNac. Alteration of this equilibrium to favor UDPNAGal might be expected to stimulate chondroitin sulphate synthesis.

That these two enzymatic steps are potential points of control in the evocation of secondary cartilage is further emphasized by the findings of Marzullo and Desiderio (1972) and Marzullo (1972) that the activities of UDPG-dehydrogenase and UDPG-4-epimerase were higher in cartilage than in any other tissue capable of synthesizing glycosaminoglycans. Since cartilage contains very little keratan sulphate, it has been suggested (Marzullo and Desiderio, 1972) that the UDPGNac-4-epimerase enzyme has a broad substrate specificity and that the suitability of UDPGal as a substrate for this enzyme resulted in the large apparent rise in UDPG-4-epimerase measured in vitro in homogenates of developing cartilage. The significance of UTP as a potential regulating factor in chondrogenesis is indicated by the findings of Lash (1968) that tissues of the early embryo capable of synthesizing only small amounts of glycosaminoglycans tended to accumulate NAG-1-P (see Figure 1).

The identification of points of metabolic control is facilitated by studies of the metabolic pathway under conditions of inhibition as well as during normal differentiation. However, if points of control functional during normal development are to be recognized, direct chemical intervention into the metabolic pathway is undesirable. Ideally, removal of an inductive stimulus would be expected to greatly aid the search for normal points of control of tissue differentiation. The development of secondary cartilage on embryo chick quadratojugals is a system in which removal

of the inductive stimulus is possible. Paralysis of the embryo inhibits secondary cartilage by inhibiting mechanical stimulation, the normal inductive stimulus of this tissue.

In order to gain an insight into the mechanism of action of the known inducers of secondary cartilage differentiation, on the quadratojugal of the embryo chick, this investigation has examined the biosynthetic pathway of chondroitin sulphate precursor synthesis in the tissue, both in normally developing and in paralysed embryos. The enzymes UDPG-dehydrogenase and UDPG-4-epimerase were studied directly while other enzymes in the pathway were studied indirectly via chromatographic and autoradiographic techniques. The paralyzing agent used in this study is Decamethonium iodide. Drachman (1963) has shown that this agent works at the motor end plates in skeletal muscle. Hall (1975) has shown that single injections of this agent into the air space of developing chick eggs produces long term paralysis in the embryo, and that paralyzing doses of this drug produce no changes in body weight or normal mortality rates over short periods of development following administration of the drug. Thus, Decamethonium iodide constitutes a useful tool in the search for levels of control of the development of secondary cartilage on the quadratojugal of embryo chicks, as changes in the synthetic pathways of differentiating chondrocytes observed following administration of this drug are expected to result solely from the absence of the tissue's normal inductive stimuli.

## METHODS AND MATERIALS

### A. GENERAL

Embryos of the common fowl (Gallus domesticus) were used. Eggs, obtained from a commercial hatchery, were incubated without rotation in a forced-draft incubator, at  $37 \pm 1^\circ\text{C}$  and  $57 \pm 2\%$  relative humidity. The quadratojugal of stage 35 to stage 38 embryos (Hamburger and Hamilton, 1951; Appendix I) was used for all experiments. This bone is a membrane (dermal) bone and comprises the most posterior component of the upper jaw. It is located just beneath the skin and can be found passing posteriorly, from just below the eye, to articulate with the quadrate which constitutes the most posterior portion of the hinge of the jaw (Figure 2). Bones used for routine histological examination were fixed in absolute ethanol, cleared in xylene, embedded in paraffin (M.P.  $53-55^\circ\text{C}$ ), serially sectioned and stained with a technique employing alcian blue and chlorantine fast red (adapted from Lison, 1954; Appendix II). Protein determinations were performed according to the method of Lowry et al. (1951), crystallized ovalbumin in concentrations ranging from 0.03 mg/ml to 0.25 mg/ml acting as the standard. A single standard Beer-Lambert line was used for all determinations and the accuracy of the line checked at each use with two concentrations of crystallized ovalbumin. When these check points failed to fall within  $\pm 1\%$  of the standard line, a new Beer-Lambert line was prepared. Homogenization was carried out, in a hand operated ground glass homogenizer with a ground glass pestle, in a 3 ml volume of the appropriate extraction medium (see below).

Figure 2 Right lateral view of an embryo chick head, about stage 38,  
showing the position of the quadratojugal bone. Diagrammatic.

Figure 3 Expanded view of the hook of the quadratojugal showing the  
positions of the secondary cartilage pads and the pool of  
undifferentiated 'germinal' cells, and the point at which  
the bone was cut prior to collection of the hooks.  
Diagrammatic.



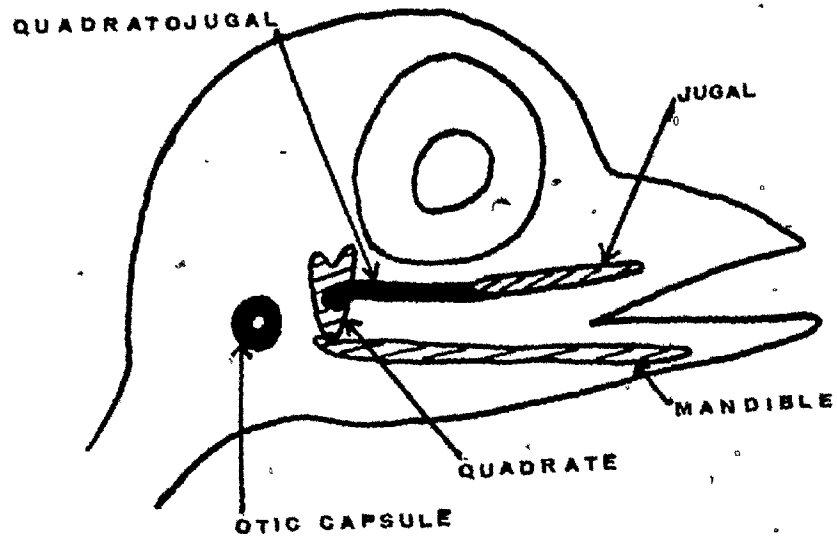


Figure 2

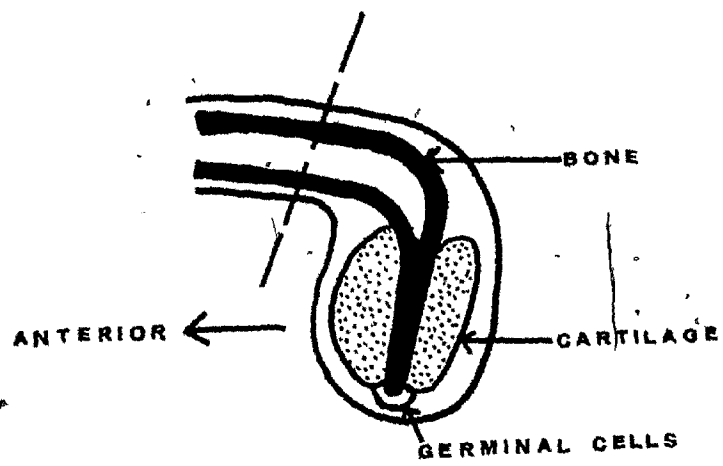


Figure 3

## B. INJECTION OF PARALYZING AGENT

Embryos were paralyzed by injection of decamethonium iodide into the all space of the egg. The drug was dissolved in sterile saline (0.85% NaCl in distilled water) and a volume of 0.2 cc/embryo injected through a sterile millipore filter (0.22 micron pore size) using a pre-sterilized Tuberculin syringe and a 21 gauge needle. The pin-hole made in the shell for injection was sealed with Scotch tape and incubation of the eggs continued. Stage 35 and stage 36 embryos received doses of 1 mg/embryo and stage 37 embryos received a dose of 0.4 mg/embryo.

Quadratojugals were removed from treated embryos 24 hours after administration of the paralyzing drug and assayed as outlined below. A few specimens from each experiment were prepared for routine histological examination.

## C. ASSAYS OF ENZYME ACTIVITY AND ENERGY LEVELS

The quadratojugals from 50 to 60 embryos of the same Hamburger-Hamilton stage were pooled for all assays. Examination of the degree of development of the nictitating membrane and eyelids of 8 to 10 randomly picked embryos ensured continuity of developmental stage in each pooled group of embryos. Quadratojugals were removed from the embryo, after reflection of the skin, by grasping the shaft with watchmaker forceps and pulling. The hooks of the quadratojugals were collected by cutting, using sharpened watchmaker forceps, at the point where the bone flexes (see Figure 3). These hooks normally contain a small core of bone, the germinal pool of cells, differentiating chondroblasts and osteoblasts and, at developmental stages later than stage 36, the secondary cartilage pads. Prior to extract preparation the quadratojugal hooks were collected and washed in saline at 4°C. All subsequent steps in extract preparation were

also performed at 4°C. Spectrophotometric measurements were conducted, in a 0.5 ml capacity quartz-iodine cuvette with a 1 cm light path, using either a Unicam SP1800 split beam spectrophotometer or a Hitachi Perkin-Elmer model 139 spectrophotometer.

#### I) UDPG-DEHYDROGENASE

Pooled quadratojugal hooks were washed in 0.1M potassium phosphate buffer, pH 6.5, containing  $10^{-2}$ M mercaptoethanol and  $10^{-3}$ M EDTA, and homogenized in 1 ml of this same buffer. The homogenate was spun for 15 minutes at 2,000 g and the supernatant then spun for 1 hour at 78,000 g. The resulting supernatant was dialysed overnight in 0.02M potassium phosphate buffer, pH 6.5, containing  $10^{-2}$ M mercaptoethanol and  $10^{-3}$ M EDTA (Medoff, 1967). The dialysate served as the enzyme extract. An aliquote of this extract was used for protein determination.

Enzyme activity was assayed in varying amounts of extract, using UDPG as a substrate, by measurement of the increase in optical density at 340 nm due to the reduction of NAD (Medoff, 1967; Appendix III). The Unicam SP1800 split beam spectrophotometer, with a full scale excursion of 0.2 optical density units, was used. Assay mixture containing boiled enzyme extract served as the reference standard. Enzyme amounts were recorded as units/mg total protein of extract.

#### II) UDPG-4-EPIMERASE

Pooled quadratojugal hooks were washed in 0.1M Tris buffer, pH 8.0, and homogenized in 1 ml of the same buffer. The homogenate was spun for 15 minutes at 2,000 g and the supernatant used as the crude enzyme extract. All assays were performed the same day as the extract was prepared. An aliquote of the extract was used for protein determination.

Enzyme activity was measured by following spectrophotometrically, at 340 nm, the reduction of NAD on a substrate of UDP-galactose with added UDPG-dehydrogenase (Pontis and Leloir, 1962; Appendix IV). The reaction was followed in the Hitachi Perkin-Elmer spectrophotometer, model 139, and enzyme amounts recorded as units/mg total protein of extract. Assay mixture containing boiled enzyme extract served as the reference standard. Negative controls (i.e. assay mixture containing enzyme extract but minus UDP-galactose) produced no measurable reaction.

### III) ESTIMATION OF URIDINE TRIPHOSPHATE

Crude extracts identical to that used in the UDPG-4-epimerase assay were prepared. All estimates were performed on either freshly prepared extracts or extracts stored for not more than 1 week at -20°C.

The assay technique was adapted from Mills, Ondarza and Smith (1954) and is based on the interconversion of ADP and UTP to ATP and UDP by nucleoside diphosphokinase (Berg and Joklik, 1953). Reduction of NAD on substrates of ADP and glucose with added nucleoside diphosphokinase, hexokinase and glucose-6-phosphate dehydrogenase was followed spectrophotometrically at 340 nm in the Hitachi Perkin-Elmer spectrophotometer, model 139. The total change in absorbancy, produced by the reaction running to completion, was used to estimate the UTP level in the extracts (Appendix V). These levels were recorded as micromoles UTP/mg total protein of extract. The reaction was standardized by addition of known amounts of UTP.

### D. CHROMATOGRAPHIC SEPARATION OF PHOSPHORYLATED AND UDP DERIVATIVES OF ACETYLHEXOSAMINES

Quadratojugal hooks pooled from 50' to 60 embryos of the same Hamburger-Hamilton stage were used for each assay. The pooled hooks were

rinsed in distilled water then homogenized in 2 to 2.5 ml of distilled water. The homogenate was spun at 2,000 g for 15 minutes and the supernatant lyophilized to dryness on a Virtis lyophilizer. The lyophilizate was stored at  $-20^{\circ}\text{C}$  and redissolved in 0.2 ml distilled water immediately before use.

Thin layer chromatography plates were prepared by hand, the layer thickness being that of double thickness masking tape. Slurries of 20 g silica gel G and 40 ml distilled water were mixed by hand in a beaker and then spread with a glass rod on 20 x 20 cm glass plates. The plates were then air dried for 10 minutes, placed in an oven at  $100^{\circ}\text{C}$  for 20 minutes and stored in a cabinet. Prepared plates were scraped to an  $83^{\circ}$  wedge, 2.5 cm at the base. The solvent consisted of 52 parts n-propanol and 48 parts concentrated ammonium hydroxide (V/V) (Marzullo and Lash, 1967). Sample volumes of 0.1 ml reconstituted lyophilizate were spotted 1.5 cm from the base of scraped plates and the chromatogram developed at room temperature until the solvent front had advanced 13-14 cm. A 2% glucose solution was used as a reference standard.

Developed chromatograms were dried for 10 minutes in an oven at  $100^{\circ}\text{C}$ . The separated compounds were visualized by spraying the plates with benzidine reagent (Appendix VI) and heating to 100 to  $110^{\circ}\text{C}$  for 20 to 30 minutes. The plates were then scanned, using transmitted light, at 430 nm on a Zeiss thin layer chromatogram scanner with recorder. Relative amounts of the separated compounds were computed on the basis of relative peak height. Chromatograms were photographed immediately following scanning.

#### E. AUTORADIOGRAPHY

Whole quadratojugal bones were used for incorporation studies. The right quadratojugals from 3 or 4 embryos were cultured for 4 hours in

pre-sterilized, 35 mm diameter, Falcon plastic petri dishes. The culture medium consisted of 1 ml sterile Dulbecco's modified Eagle medium, with added glutamine and containing no antibiotics, to which was added either 10 microcuries [ $^3\text{H}$ ]-glucosamine (specific activity 10  $\mu\text{Ci}/\text{mM}$ ) or 1 microcurie sodium- $^{35}\text{S}$ -sulphate (specific activity 128  $\text{mCi}/\text{mM}$ ).

Following culture the bones were fixed in absolute ethanol for 2 hours then cleared in xylene (2 changes, 15 minutes each) and embedded in paraffin (M.P. 53-55°C). Serially sectioned (9 microns) bones were stained with Meyers haematoxylin and alcian blue then air-dried.

Autoradiograms were prepared by coating dry sections with a thin layer of Kodak NTB3 Nuclear Tract emulsion, diluted 1:1 with double distilled deionized water, and exposing at 4°C for either 8 days ([ $^3\text{H}$ ]-glucosamine) or 16 days ( $^{35}\text{S}$ -sulphate). Exposed autoradiograms were developed for 2 minutes in Kodak D19, fixed for 5 minutes in Illofix acid fixer, washed in water for 5 minutes, dehydrated in graded ethanol, cleared in xylene and coverslipped. Grains of incorporated precursor were counted at a magnification of 800 diameters with the aid of a 10 x 10 squared micrometer eyepiece. Ten randomly chosen areas of every third section, through the full thickness of the secondary cartilage pads, or presumptive cartilage regions, were counted. Five randomly chosen areas of background immediately adjacent to the section were also counted and incorporation calculated as average grain count per unit area over tissue less average grain count per unit area of background. Tissue sections with an adjacent background count in excess of 2 grains per unit area of the micrometer eyepiece were rejected.

#### F. STATISTICAL ANALYSIS

Data obtained in this investigation was analysed using the Student's 't' test. However, it is to be noted that while pooling of quadratojugal

hooks was employed the concentration of the enzymes and intermediates under study remained small (total protein in the homogenates of pooled quadratojugal hooks varying between 0.11 and 0.19 mg/ml). Steel and Torrie (1960) have noted that, unless large real differences occur, statistical analysis requiring a 5% significance level may not detect differences between samples in small-sized experiments. These authors further state that the significance levels of 5% and 1% are arbitrary and suggest that the choice of a 10% significance level may be more appropriate for small experiments. Accordingly, since this study consisted of essentially small experiments as noted above, a 10% significance level has been chosen for the statistical analysis of data obtained during this investigation.

#### G. CHEMICALS AND SUPPLIES

Decamethonium iodide, lot number H4137, was purchased from Koch-Light laboratories.

Nucleoside diphosphokinase and NADP were purchased from Boehringer Mannheim Corporation. All other substrates and standards were purchased from Sigma Chemicals. Enzyme solutions were prepared every other day as required and stored at 4°C until used. Solutions of other assay substrates were prepared weekly and stored in small aliquotes at -20°C until used.

Silica gel G was purchased from Mondray Limited.

Tritiated glucosamine was purchased from New England Nuclear as D-glucosamine-6-<sup>3</sup>H(N) hydrochloride in ethanol water (7/3) solution (catalogue number NET-190).

Sodium-[<sup>35</sup>S]-sulfate as the anhydrous salt was purchased from Amersham/Searle Corporation (catalogue number SJ-162).

Dulbecco's Modified Eagle medium, with added glutamine and containing no antibiotics, was purchased from Grand Island Biological Company (catalogue number 168G).



## RESULTS

A. PARALYSING AGENT

Injection of decamethonium iodide into the air space of the egg produced complete paralysis of the embryo, as assessed by the lack of movement seen in embryos removed from the shell, but did not arrest gross development of the embryo as assessed by Hamburger-Hamilton determination of development stage 24 hours after administration of the drug. While a dose of 1 mg per embryo was required to produce paralysis in stage 35 and stage 38 embryos, paralysis was produced in stage 37 embryos with a dose of 0.4 mg per embryo. Indeed, 1 mg per embryo doses of decamethonium iodide administered to stage 37 embryos resulted in a mortality rate in excess of 90%. The apparent increased sensitivity of stage 37 embryos to the drug, as compared to stage 35 and stage 36 embryos, is not understood.

B. HISTOLOGICAL FINDINGS

Quadratojugals from stage 35 embryos (Figure 4) showed no histological evidence of cartilage. The regions where secondary cartilage was seen at later stages of development were occupied by closely associated mesenchymal-like cells. Quadratojugals from stage 36 embryos (Figure 5) showed an increase in the length of the bony hook as compared to stage 35 embryos. While secondary cartilage was not normally seen in quadratojugals from stage 36 embryos, very small quantities of cartilagenous matrix were occasionally noted surrounding a few cells in the region where the anterior cartilage pad later formed. Detection of cartilage in quadratojugals from stage 36 embryos was likely due to slight variations

Figure 4 Quadratojugal hook from a stage 35 control embryo. Bone matrix (B) is present. ABCR. X 60.

Figure 5 Quadratojugal hook from a stage 36 control embryo. Bone matrix (B) is prominent and cells around bony hook are tightly packed. ABCR. X 80.

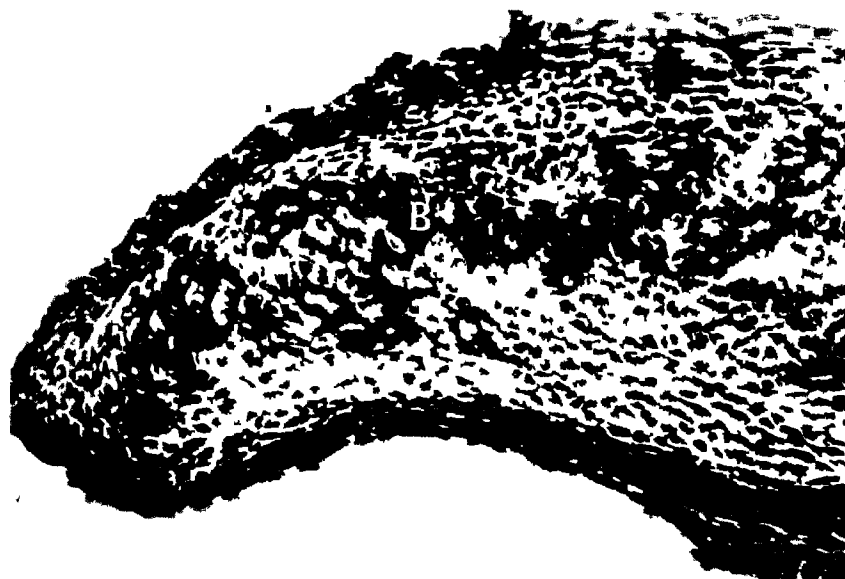


Figure 4



Figure 5

Figure 6    Quadratojugal hook from a stage 37 control embryo. Non-hypertrophic cartilage (C) is prominent on the anterior aspect of the bone (D). Anterior to the right. ABCR. X 80.

Figure 7    Quadratojugal hook from a stage 38 control embryo. Hypertrophic cartilage (HC) and non-hypertrophic cartilage (C) are present on both anterior and posterior aspects of the bone (B). ABCR. X 60.



Figure 6



Figure 7

in the staging of the embryo. The embryonic stage of development was determined by observing the degree of development of the nictitating membrane and subjective variations of  $\pm 1/4$  stage, especially in the assessment of stage 36 and stage 37 embryos, are not unexpected.

Secondary cartilage was routinely observed histologically around the tip of the quadratojugals of stage 37 embryos (Figure 6), the cartilage pad on the anterior surface of the bone being larger and more mature than that on the posterior surface. Hypertrophic chondrocytes were seen in those areas of the anterior cartilage nearest the bony shaft while the posterior pad was composed entirely of ovoid cells separated by small amounts of matrix (i.e. differentiating chondrocytes). Both anterior and posterior cartilage pads on quadratojugals in stage 38 embryos showed hypertrophic cells next to the bony shaft and the anterior pad continued to be larger than the posterior pad (Figure 7). The outer margins of both pads contained differentiating chondrocytes indicating that growth of this secondary cartilage was still continuing in stage 38 embryos.

Quadratojugals examined 24 hours after administration of the paralyzing drug routinely displayed inhibition of secondary cartilage formation. Embryos paralyzed at stage 35 developed a quadratojugal whose bony hook, at stage 36, was longer and more slender than that of similar stage controls (Figure 8). A few thin layers of closely associated mesenchymal-like cells surrounded the bony hook of the quadratojugal in these paralyzed embryos. Quadratojugals from stage 37 embryos, following paralysis at stage 36, contained very little or no cartilage and the bony hook was bulbous in shape (Figure 9). The expanded portion of the bony hook in these quadratojugals was very cellular with small amounts of

Figure 8 Quadratojugal hook from a stage 36 paralyzed embryo. Note the elongated bony hook (B) and loosely packed cells around the hook (cf. fig. 5). ABCR. X 60.

Figure 9 Quadratojugal hook from a stage 37 paralyzed embryo. Bone has formed (P) where cartilage is normally found (cf. fig. 6). ABCR. X 80.



Figure 8.



Figure 9



Figure 10 Quadratojugal hook from a stage 38 paralyzed embryo. Bone  
has ~~formed~~ (P) where cartilage is normally found (cf. fig. 7).  
ABCR. X 80.



Figure 10

matrix separating the cells, giving the impression more of calcified cartilage than of normal bone. Bulbous bony hooks were also found on quadratojugals in stage 38 embryos following paralysis at stage 37 (Figure 10). A small amount of very densely staining cartilage matrix was found on these bones in the region corresponding to the outer edges of the cartilage pads seen in similar stage controls (Figure 7). The histological findings in paralyzed embryos examined at stage 37 and stage 38 indicate a process of either erosion and replacement or calcification of the secondary cartilage pads normally found on quadratojugals in embryos at these developmental stages.

In all bones examined, both from control embryos and from paralyzed embryos, the cells at the very tip of the bone (i.e. region of the germinal pool) appeared flattened and very densely packed. It was not possible to distinguish cytological differences in this region which, in stage 37 or stage 38 embryos, would permit separation of the chondrogenic precursor pool from the osteogenic precursor pool.

#### C. UDPG-DEHYDROGENASE

The specific activity of this enzyme in quadratojugal hooks of normally developing embryos, stage 35 through stage 38, and in hooks of stage 36 through stage 38 embryos which had been paralyzed 24 hours prior to examination, is shown in Figure 11. Each point represents the mean of four separate determinations, in each of which the sample was subjected to four assays in which different amounts of the enzyme extract were used. Statistical comparisons of these means are shown in Table 1.

UDPG-dehydrogenase was found to accumulate rapidly between stage 35 and stage 36 at which time a peak in activity was observed. No significant change in enzyme activity was found between developmental

Figure 11 Specific activity of UDPG-dehydrogenase in quadratojugal hooks of normally developing embryos (●—●) and in quadratojugal hooks of embryos which had been paralyzed 24 hours prior to the stage of assay (○). The activity in tibial epiphyseal cartilage (■) and in leg muscle (X) for stage 38 control embryos is also indicated. The standard error of the mean for each point is indicated by vertical bars.

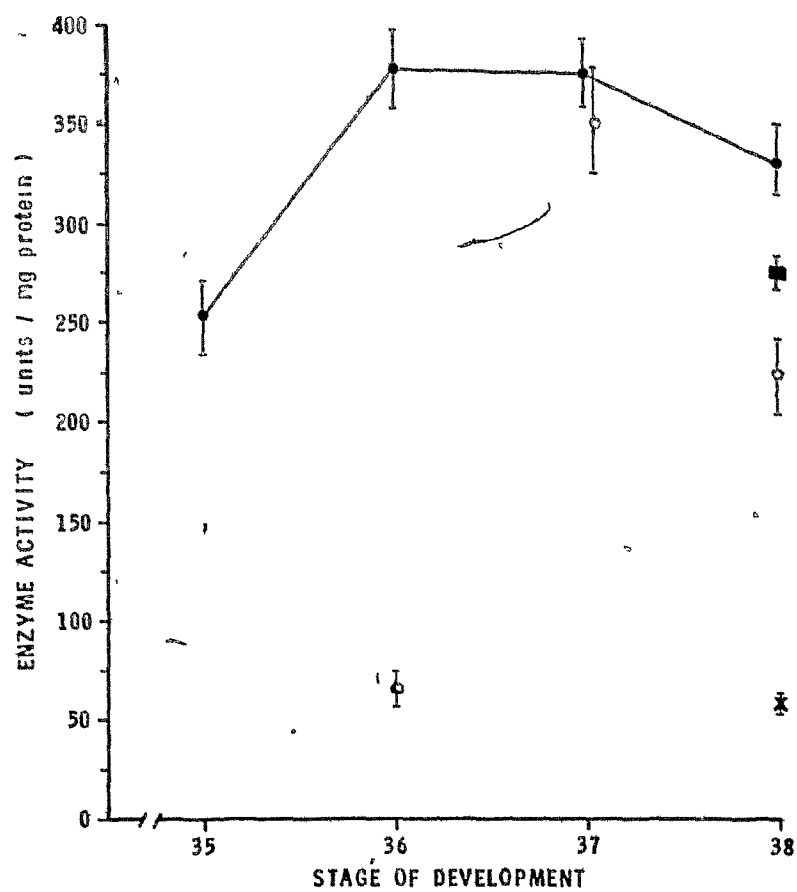


Figure 11

TABLE 1

## STATISTICAL COMPARISONS OF MEAN ACTIVITIES OF UDEG-DEHYDROGENASE IN

## QUADRATOJUGAL HOOKS OF CONTROL EMBRYOS AND OF PARALYZED EMBRYOS

STUDENT'S 't'							
	Stage 35 Controls	Stage 36 Controls	Stage 37 Controls	Stage 38 Controls	Stage 36 Paralyzed	Stage 37 Paralyzed	Stage 38 Paralyzed
Stage 35 Controls	253.07 <sup>1</sup> ±19.30	P≤0.01	P≤0.01	P≤0.05	P≤0.001	P≤0.05	NS
Stage 36 Controls		377.16 ±20.63	NS <sup>2</sup>	NS	P≤0.001	NS	P≤0.001
Stage 37 Controls			375.82 ±16.41	P≤0.1	P≤0.001	NS	P≤0.001
Stage 38 Controls				329.30 ±16.91	P≤0.001	NS	P≤0.001
Stage 36 Paralyzed					65.54 -9.36	P≤0.001	P≤0.001
Stage 37 Paralyzed						351.56 ±27.62	P≤0.001
Stage 38 Paralyzed							221.15 ±19.23

1 Mean enzyme activity (units/mg protein) ±SEM

2 No significant difference between means

stages 36 and 37, but a slight decline in activity was noted between developmental stages 37 and 38. While the significance of this decline is marginal (see Table 1), the real change in enzyme activity between stage 37 and stage 38 may have been partially masked as the result of slight variations ( $\pm 1/4$  stage) in the assessment of the developmental stage attained by embryos used to obtain stage 37 quadratojugal.

Severely reduced levels of enzyme activity as compared to similar stage controls, were found in quadratojugal hook tips of stage 36 paralyzed embryos (see Abbreviations and Conventions), enzyme levels in these hooks being comparable to those found in leg muscle of stage 38 control embryos (Figure 11). Muscle is representative of the non-cartilagenous embryonic tissues which have been shown to contain ubiquitous levels of this enzyme (Marzullo and Desiderio, 1972). Hooks in stage 37 paralyzed embryos showed no significant differences in level of enzyme activity as compared to similar stage controls (Table 1). A very significant reduction in level of enzyme activity was again observed in hooks from stage 38 paralyzed embryos (Figure 11, Table 1) as compared to similar stage controls. Indeed, activity levels did not differ significantly from those found in hooks of stage 35 control embryos (Table 1).

#### D. UDPG-4-EPIMERASE

The specific activity of this enzyme in quadratojugal hooks of normally developing stage 35 through stage 38 embryos and in hooks taken from stage 36 through stage 38 embryos which had been paralyzed 24 hours prior to examination, is shown in Figure 12. Each point represents the mean of three or four separate determinations, in each of which the sample was subjected to four assays in which different amounts of the extract

Figure 12 Specific activity of UDPG-4-epimerase in quadratojugal hooks of normally developing embryos (●—●) and in quadratojugal hooks of embryos which had been paralyzed 24 hours prior to the stage of assay (○). The standard error of the mean for each point is indicated by vertical bars.



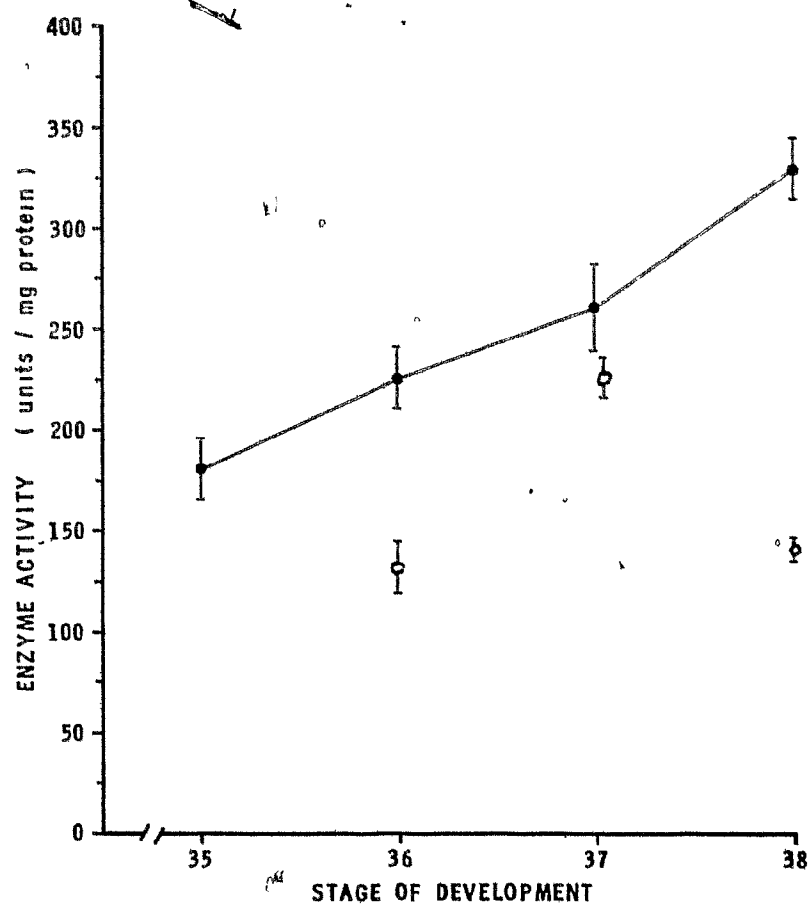


Figure 12

TABLE 2  
STATISTICAL COMPARISONS OF MEAN ACTIVITIES OF UDPG-4-EPIMPEASE IN  
QUADRATOJUGAL HOOKS OF CONTROL EMBRYOS AND OF PARALYZED EMBRYOS

STUDENT'S 't'							
	Stage 35 Controls	Stage 36 Controls	Stage 37 Controls	Stage 38 Controls	Stage 36 Paralyzed	Stage 37 Paralyzed	Stage 38 Paralyzed
Stage 35 Controls	179.61 <sup>1</sup> ±14.96	P≤0.09	P≤0.05	P≤0.01	P≤0.07	P≤0.09	P≤0.05
Stage 36 Controls		225.27 ±14.77	NS	P≤0.01	P≤0.01	NS	P≤0.05
Stage 37 Controls			260.42 ±21.48	P≤0.06	P≤0.01	NS	P≤0.001
Stage 38 Controls				327.69 ±14.55	P≤0.001	P≤0.05	P≤0.001
Stage 36 Paralyzed					131.54 ±12.89	P≤0.05	NS
Stage 37 Paralyzed						224.83 ±10.67	P≤0.05
Stage 38 Paralyzed							139.30 ± 6.18

1 Mean enzyme activity (units/mg protein) ±SEM

2 No significant difference between means

were used. Statistical comparisons of these means are shown in Table 2.

Increasing levels of enzyme activity were observed over the period of normal development from stage 35 to stage 38. While variations in staging of the embryos may have resulted in the 24 hour variations not being highly significant, the accumulation of enzyme in hooks over a 48 hour period (i.e. stage 35 to stage 37, or stage 36 to stage 38) is very significant (Table 2), with the level in stage 38 hooks being nearly double that in stage 35 hooks. The most rapid accumulation of enzyme activity appears to occur in hooks between stage 37 and stage 38 of development during which period a 25% increase in activity was observed (Table 2).

Quadratojugal hooks from stage 36 paralyzed embryos exhibited a 44% reduction in the level of UDPG-4-epimerase as compared to similar stage controls. This activity level in stage 36 paralyzed embryos also likely represents a small decrease as compared to that of stage 35 controls (Table 2). While hooks from stage 37 paralyzed embryos indicated a possible decrease in activity as compared to similar stage controls (Figure 12), statistical comparison of the two means did not verify this trend (Table 2). Quadratojugal hooks from stage 38 paralyzed embryos exhibited a very substantial and statistically significant decline in enzyme activity as compared to samples from both stage 38 and stage 37 control embryos (Table 2). Indeed, enzyme levels found in hooks from stage 38 paralyzed embryos did not differ significantly from those found in stage 36 paralyzed embryos (Table 2).

#### E. ESTIMATION OF URIDINE TRIPHOSPHATE

The concentrations of this nucleotide in quadratojugal hook tips of normally developing and paralyzed embryos, stage 35 to stage 38, are

Figure 13 Levels of UTP in quadratojugal hooks of normally developing embryos (●—●) and in quadratojugal hooks of embryos which had been paralyzed 24 hours prior to the stage of assay (○). The standard error of the mean for each point is indicated by vertical bars.

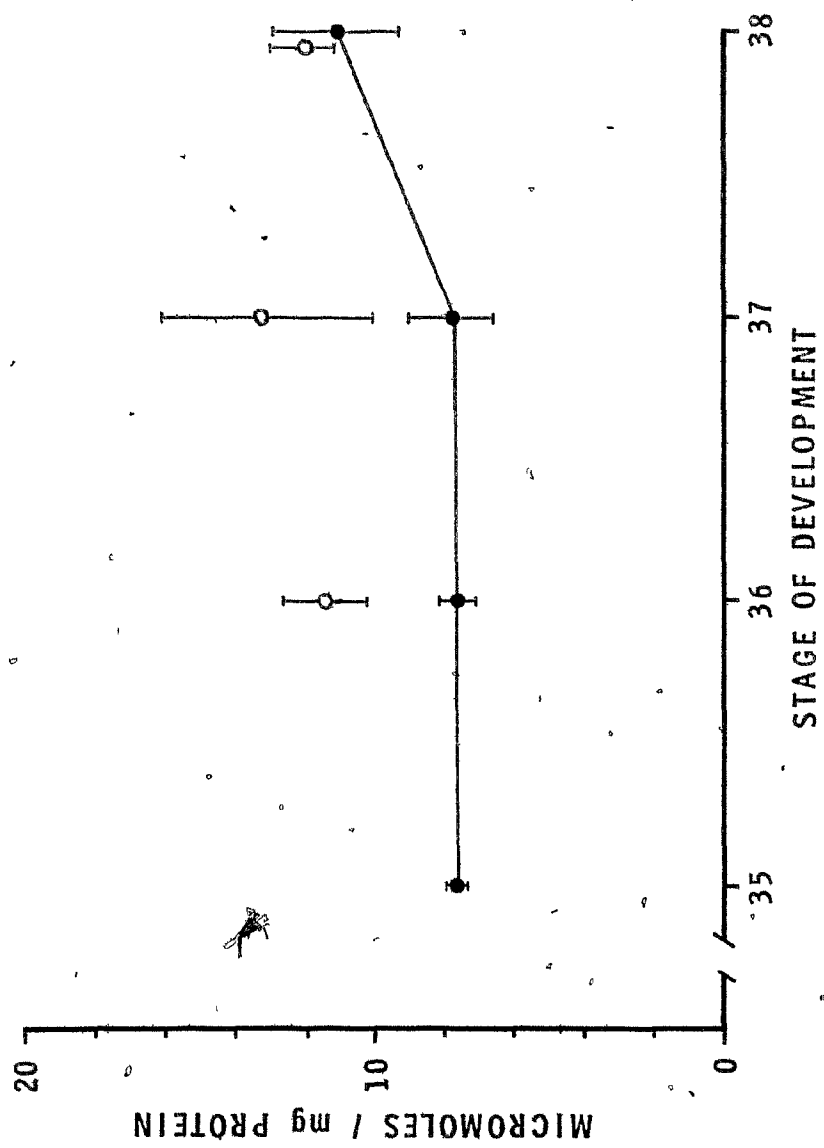


Figure 13

TABLE 3  
STATISTICAL COMPARISONS OF MEAN CONCENTRATIONS OF UTP IN

QUADRATOJUGAL HOOKS OF CONTROL EMBRYOS AND OF PARALYZED EMBRYOS

STUDENT'S 't'						
	Stage 35 Controls	Stage 36 Controls	Stage 37 Controls	Stage 38 Controls	Stage 36 Paralyzed	Stage 37 Paralyzed
Stage 35 Controls	7.80 <sup>1</sup> ±0.06	NS <sup>2</sup>	NS	NS	P≤0.09	NS
Stage 36 Controls		7.80 ±0.23	NS	NS	P≤0.1	NS <sup>1</sup>
Stage 37 Controls			7.63 ±1.17	NS	NS	P≤0.07
Stage 38 Controls				11.14 ±1.84	NS	NS
Stage 36 Paralyzed					11.44 ±1.2	NS
Stage 37 Paralyzed					NS	NS
Stage 38 Paralyzed					13.22 ±3.53	NS
						12.07 ±0.85

1 Mean UTP concentration (micromoles/mg Protein) ±SEM

2 No significant difference between means

shown in Figure 13. Each point represents the mean of three separate determinations, in each of which the sample was subjected to four assays in which different amounts of the extract were used. Statistical comparisons of these means are shown in Table 3.









Estimates obtained from the quadratojugal hooks of normally developing stage 35 to stage 38 embryos indicated the presence of constant levels of UTP over time (Figure 13). The apparent small increase in levels of UTP noted between stage 37 and stage 38 in control embryos was found not to be significant (Table 3).

Slight increases in UTP levels in hooks of stage 36 to stage 38 paralyzed embryos are indicated by Figure 13. However, these elevations proved to be of no significance when compared to levels in similar stage control embryos (Table 3). Thus, it is found that quadratojugal hooks from stage 35 to stage 38 embryos contained a constant quantity of UTP, irrespective of whether the embryos were paralyzed or developing normally.

#### F. CHROMATOGRAPHIC SEPARATION OF PHOSPHORYLATED AND UDP DERIVATIVES OF ACETYLHEXOSAMINES

The synthetic pathway leading to UDP-N-acetylgalactosamine and thence to chondroitin sulphate is outlined in Figure 1. The average relative amounts, of three separate determinations, of synthetic intermediates found in normally developing embryos, stage 35 through stage 38, and in stage 36 through stage 38 paralyzed embryos, are shown in Figure 14 and Table 4. A developed chromatogram is shown in Figure 15 and a typical spectrophotometric chromatogram scan is shown in Figure 16. Photographic records of developed chromatograms were difficult to obtain due to the low concentration of separating compounds and consequent weak staining of the chromatograms.

Figure 14 Histogram showing average relative proportions of the metabolic intermediates of chondroitin in cells of the quadratojugal hook at various developmental stages. Fifty to sixty hooks from similar stage embryos were pooled and homogenized. Following centrifugation of the homogenate the supernatant was lyophilized, then reconstituted and subjected to thin layer chromatography on silica gel G. See Abbreviations and Conventions for explanation of abbreviations employed.

Legend		stage 35 controls
		stage 36 controls
		stage 37 controls
		stage 38 controls
		stage 38 controls tibial epiphyseal cartilage
		stage 36 paralyzed
		stage 37 paralyzed
		stage 38 paralyzed



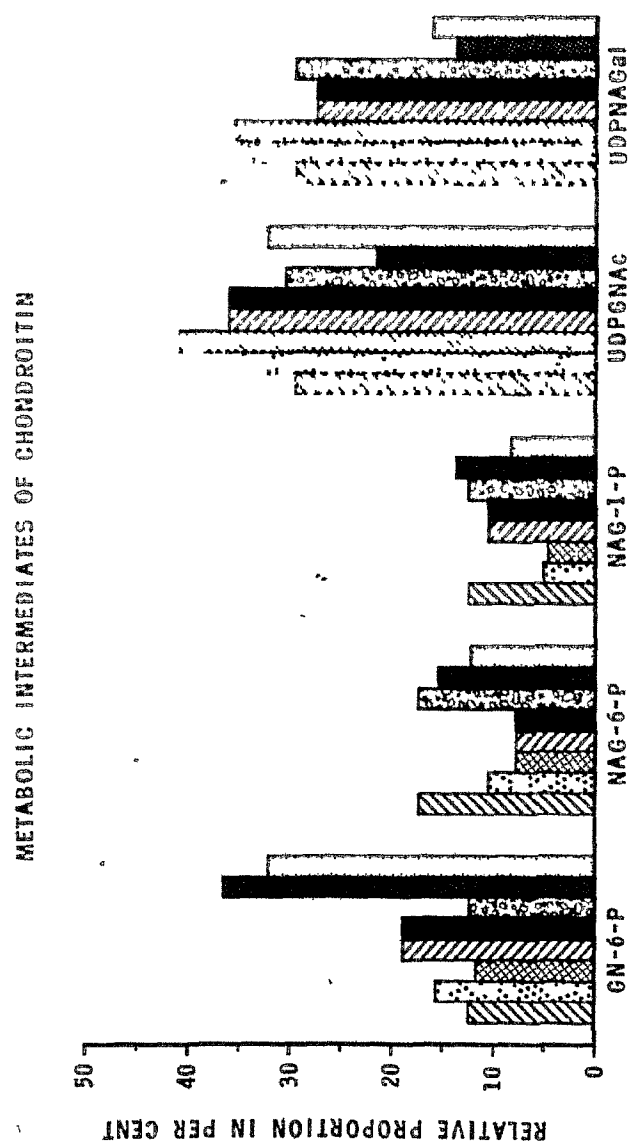


Figure 14

TABLE 4

## AVERAGE RELATIVE PROPORTIONS OF PHOSPHORYLATED AND URIDINE DIPHOSPHATE

## DERIVATIVES OF ACETYLHEXOSAMINES IN HOMOGENATES OF QUADRATOJUGAL HOOK TIPS

INTERMEDIATE	STAGE OF DEVELOPMENT							
	35	36	37	38	Cartilage <sup>1</sup>	36P <sup>2</sup>	37P	38P
Glucosamine-6-Phosphate %	12.2	15.7	11.7	13.8	13.8	12.2 (-22%) <sup>3</sup>	36.4 (+211%)	32 (-70%)
N-Acetylglucosamine-6-Phosphate %	17.1	10.5	7.8	7.8	7.8	17.1 (+63%)	15.2 (+95%)	12 (+54%)
N-Acetylglucosamine-1-Phosphate %	12.1	4.9	4.7	10.2	10.2	12.2 (+149%)	13.6 (+190%)	8 (-10%)
UDP-N-Acetyl-Glucosamine %	29.3	34.5	40.6	35.9	35.9	29.3 (-15%)	21.2 (-48%)	32 (-11%)
UDP-N-Acetyl-Galactosamine %	29.3	34.5	35.2	27.3	27.3	29.3 (-15%)	13.6 (-61%)	16 (-41%)

1 Stage 38 tibial epiphyseal cartilage

2 Paralyzed embryo (See Abbreviations and Conventions)

3 Change from similar stage controls, in parentheses

Figure 15 A developed silica gel G thin layer chromatogram showing separation of the phosphorylated and UDP precursors of chondroitin.

- Legend
1. Glucosamine-6-phosphate
  2. N-acetylglucosamine-1-phosphate
  3. N-acetylglucosamine-6-phosphate
  4. UDP-N-acetylgalactosamine
  5. UDP-N-acetylglucosamine
  6. Glucose

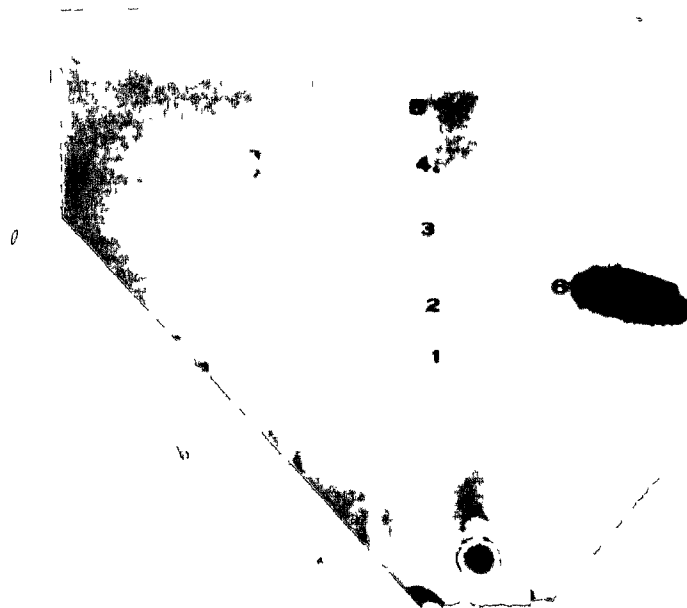


Figure 15

Figure 16 Typical spectrophotometric scan of a developed silica gel G thin layer chromatogram. Note that peaks not readily visible in the chromatogram are clearly indicated by the scan (cf. fig. 15).

- Legend
1. Glucosamine-6-phosphate
  2. N-acetylglucosamine-1-phosphate
  3. N-acetylglucosamine-6-phosphate
  4. UDP-N-acetylgalactosamine
  5. UDP-N-acetylglucosamine

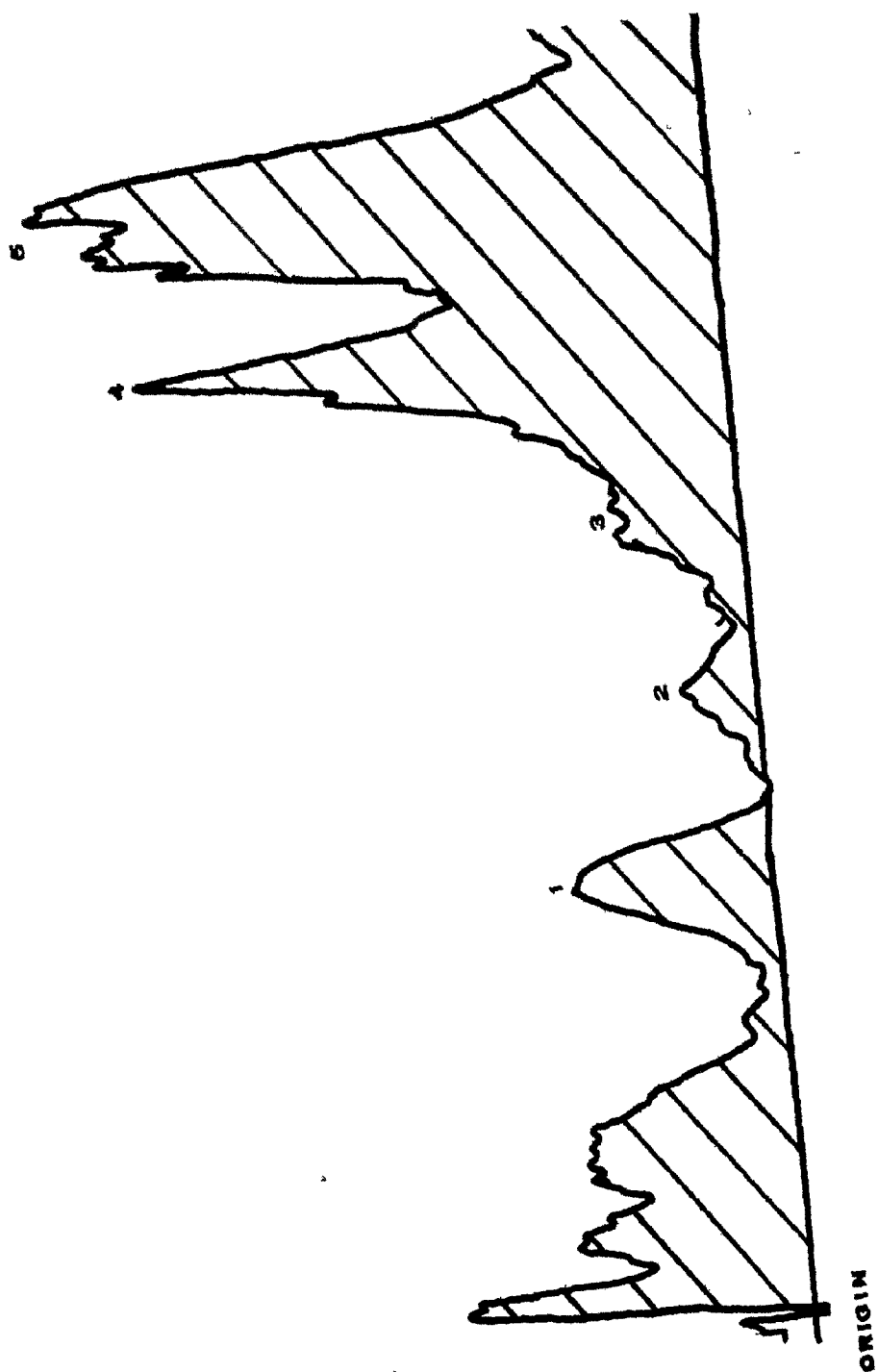


Figure 16

The initial broad peak, extending from the origin, in all chromatogram scans (Figure 16) can be seen in Figure 15 to be a darkly staining area across the entire base of the plate and does not represent a true separation peak. The cause of this band of dense stain is not known. The peaks representing the compounds of interest migrate beyond this dense band however, and were thus readily indentifiable. Solutions of UDPGNac were found to migrate to approximately the same Rf value as that measured from Marzullo and Lash (1967). Since standards for the remaining compounds in the synthetic pathway under consideration were not readily available commercially, these compounds were located by comparison of Rf values to those measured in Marzullo and Lash (1967) and standardized throughout the series of chromatograms by their Rglucose values.

Chromatograms of quadratojugal hooks from stage 35 through stage 38 control embryos revealed a relative preponderance of the terminal intermediates UDPGNac and UDPNAGal. The GN-6-P proportion was variable, increasing from 12.2% at stage 35 to 15.7% at stage 36 (a 28% change), decreasing to 11.7% at stage 37 (a 25% change) and again increasing to 18.8% at stage 38 (a 38% change) (Figure 14). NAG-6-P fractions declined by 64% between stages 35 and 37 (from 17.1% to 7.8%) and then remained constant while NAG-1-P fractions declined by 60% between stages 35 and 37 (from 12.2% to 4.7%) (Figure 14). A 117% recovery in the amount of NAG-1-P occurred between stages 37 and 38, to a proportion of 10.2% (Figure 14). Although variations in the relative amounts of these three hexosamines were seen to occur over the developmental period examined, it is to be noted that at any given developmental stage the total proportion of these intermediates never exceeded 44% of the total of the substrates examined.

The relative proportions of UDPGNac and UDPNAGal varied between 29.3% and 40.6% of the total of the substrates examined (Figure 14). Each of these two intermediates exhibited an 18% increase in relative amount between stage 35 and stage 36 (from 29.3% to 34.5%). Although the proportion of UDPGNac increased a further 13% between stages 36 and 37 (to 40.6%), the level of UDPNAGal remained constant during this period. Both intermediates showed relative declines between stage 37 and stage 38, UDPGNac declining 12% to a proportion of 35.9% and UDPNAGal falling 22% to a proportion of 27.3% (Figure 14). Similar chromatogram patterns were observed with quadratojugal hooks from stage 38 controls embryos and with cartilage obtained from the distal end of the tibia of these same embryos (Figure 14).

Quadratojugal hooks in paralyzed embryos were found to contain substantially different proportions of substrates in the UDP-Nacetyl-galactosamine synthetic pathway as compared to similar stage controls (Table 4, Figure 14). The relative proportion of all five substrates recorded in hook tips from stage 36 paralyzed embryos was identical to that found in stage 35 controls. Stage 37 paralyzed samples exhibited, as compared to similar stage controls, relative increases of 211% in GN-6-P, 95% in NAG-6-P, 190% in NAG-1-P, and relative reductions of 48% in UDPGNac and 61% in UDPNAGal (Figure 14, Table 4). Hooks from stage 38 paralyzed embryos exhibited, as compared to similar stage controls, relative increases in GN-6-P and in NAG-6-P of 70% and 54% respectively, and relative declines of 18% in NAG-1-P, 11% in UDPGNac and 41% in UDPNAGal (Table 4).

#### G. AUTORADIOGRAPHY

Measurable amounts of labelled precursor were incorporated into the



secondary cartilage pads of chick embryos quadratojugals following a four hour pulse in vitro. The pulse label was not chased with unlabelled precursor as these experiments were designed to study the rate of precursor uptake rather than the time course of incorporation.

#### I) TRITIATED GLUCOSAMINE

The mean grain counts per unit area of a 10 x 10 micrometer eyepiece are shown in Table 5. Areas of secondary cartilage showing no indications of hypertrophy were considered to be actively developing. This non-hypertrophic cartilage was considered separately from the mature hypertrophic cartilage when both occurred simultaneously (i.e. stage 37 and stage 38 controls) as the non-hypertrophic areas were found to incorporate label mainly over the extracellular matrix while the uptake in hypertrophic cartilage was mainly intracellular. Statistical comparisons of the mean grain counts are shown in Table 7.

No detectable incorporation of tritiated glucosamine occurred in quadratojugal hooks of stage 35 control embryos. In quadratojugals from stage 36 control embryos no histological evidence of cartilage was seen but incorporation of tritiated glucosamine was observed in two regions, one anterior to the bony hook and one posterior to the bony hook (Figure 17). The label was noted to be mainly extracellular. The anterior region incorporated this precursor in significantly greater levels than did the posterior region (Table 5, Table 7). In stage 37 control quadratojugals, cartilage pads were evident and higher levels of precursor incorporation than in stage 36 control samples were detected in all regions (Table 5, Figure 18). Labelling over areas of non-hypertrophic cartilage were greater in the anterior pad than in the posterior pad. The highest levels of incorporation at this developmental stage were observed

TABLE 5  
AVERAGE NUMBER OF GRAINS TRITIATED GLUCOSAMINE INCORPORATED PER UNIT AREA  
MICROMETER EYEPIECE OF SECONDARY CARTILAGE IN QUADRATOJUGALS INCUBATED IN VITRO<sup>1</sup>

TREATMENT	VALUES ARE MEANS $\pm$ SEM			
	ANTERIOR PAD		POSTERIOR PAD	
	NON-HYPERTROPHIC	HYPERTROPHIC	NON-HYPERTROPHIC	HYPERTROPHIC
Stage 36 Control (10) <sup>2</sup>	18 $\pm$ 1	NC <sup>3</sup>	9 $\pm$ 1.8	NC
Stage 37 Control (10)	23 $\pm$ 1.3	33 $\pm$ 1.3	12 $\pm$ 0.7	NC
Stage 38 Control (10)	19 $\pm$ 1.6	25 $\pm$ 2.0	15 $\pm$ 1.0	18 $\pm$ 1.9
Stage 36 Paralyzed (10)	NC	NC	NC	NC
Stage 37 Paralyzed (10)	NC	NC	NC	NC
Stage 38 Paralyzed (10)	NC	19 $\pm$ 0.8	NC	4 $\pm$ 0.7

1 See Methods and Materials for incubation conditions and counting protocol

2 Number of quadratojugals labelled, in parentheses

3 No cartilage present

Figure 17 Quadratojugal, from a stage 36 control embryo, exposed in vitro to [ $^3\text{H}$ ]-glucosamine. Note the mainly extracellular label both anterior and posterior to the bony hook (B).

AB. X 80.

Figure 18 Quadratojugal, from a stage 37 control embryo, exposed in vitro to [ $^3\text{H}$ ]-glucosamine. Note the dense labelling over cartilage anterior to the bony hook (B) and slight labelling along the posterior surface of the bony hook. Cartilage matrix is obscured by the labelling. Anterior below.

AB. X 100.



Figure 17

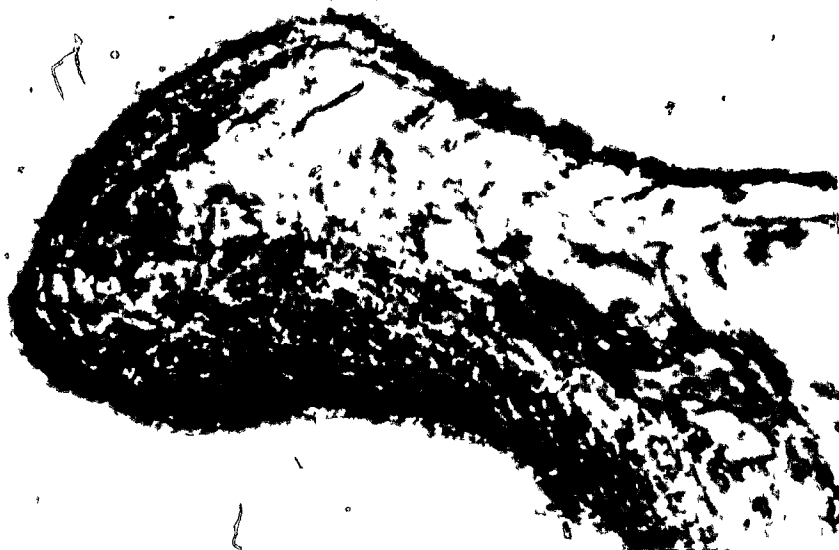


Figure 18

over the hypertrophic cartilage present in the anterior pad. Although only a marginally significant decrease in the labelling over areas of non-hypertrophic cartilage in the anterior pad was observed between developmental stages 37 and 38 (Table 7), the hypertrophic cartilage in the anterior pad of quadratojugals from stage 38 control embryos (Figure 19) incorporated significantly less tritiated glucosamine than did similar cartilage in stage 37 control quadratojugals (Table 5, Table 7). Non-hypertrophic cartilage in the posterior pad of stage 38 control quadratojugals incorporated significantly more tritiated glucosamine than did similar areas of stage 37 controls (Table 5, Table 7). It was also noted, in quadratojugals from stage 38 control embryos, that the non-hypertrophic cartilage in the posterior pad incorporated significantly less tritiated glucosamine than did similar cartilage in the anterior pad (Table 5). No significant difference in labelling was detectable between hypertrophic and non-hypertrophic areas of the posterior pad in stage 38 control quadratojugals (Table 7).

Quadratojugals from stage 36 and stage 37 paralyzed embryos contained no cartilage and no incorporation of tritiated glucosamine could be detected in the areas anterior to and posterior to the bony hook. Some hypertrophic cartilage was present in the anterior and posterior pads of quadratojugals from stage 38 paralyzed embryos and incorporation of this precursor was observed in these regions (Figure 20). However, this incorporation was significantly less than that observed in similar areas of stage 38 control quadratojugals (Table 5). It is noted that the label found in stage 38 paralyzed quadratojugals, unlike that observed over hypertrophic cartilage of stage 37 and stage 38 control bones, was almost exclusively located over the extracellular matrix (Figure 18, Figure 19, Figure 20).

Figure 19 Quadratojugal, from a stage 38 control embryo, exposed in vitro to [ $^3\text{H}$ ]-glucosamine. Note that labelling over hypertrophic cartilage (HC) is mainly intracellular while labelling over non-hypertrophic cartilage (C) is mainly extracellular. AB. X 60.

Figure 20 Quadratojugal, from a stage 38 paralysed embryo, exposed in vitro to [ $^3\text{H}$ ]-glucosamine. Note that only low levels of extracellular labelling are seen anterior to the bone (P) present where cartilage is normally found (cf. fig. 19). AB. X 100.



Figure 19



Figure 20

II) [ $^{35}\text{S}$ ]-SULPHATE

Mean grain counts per unit area are shown in Table 6. Discrimination was again made between the hypertrophic and non-hypertrophic cartilage in the quadratojugal secondary cartilage pads as uptake of this precursor resulted in label located mainly over the extracellular matrix in non-hypertrophic cartilage while labelling over areas of hypertrophic cartilage was equally dense intra and extracellularly. Statistical comparisons of the mean grain counts are shown in Table 7.

No detectable incorporation of [ $^{35}\text{S}$ ]-sulphate occurred in quadratojugal hooks from stage 35 control embryos. In stage 36 control embryos, although no histological evidence of cartilage was observed in the quadratojugal, incorporation of [ $^{35}\text{S}$ ]-sulphate was seen in the region anterior to the bony hook (Figure 21). In stage 37 control quadratojugals (Figure 22) cartilage pads were evident and the hypertrophic cartilage present in the anterior pad incorporated significantly more precursor than did the non-hypertrophic cartilage areas (Table 6, Table 7). No significant difference in incorporation of [ $^{35}\text{S}$ ]-sulphate was noted between anterior pad and posterior pad non-hypertrophic cartilage at this developmental stage (Table 7). In stage 38 control quadratojugals (Figure 23), both hypertrophic and non-hypertrophic cartilage in the anterior pad, incorporated significantly more of this precursor than did similar regions in stage 37 control quadratojugals (Table 6, Table 7). No significant change in the labelling over areas of non-hypertrophic cartilage in the posterior pad was observed between developmental stages 37 and 38 (Table 7). It was also noted in stage 38 control quadratojugals that the hypertrophic cartilage of the anterior pad incorporated significantly more [ $^{35}\text{S}$ ]-sulphate than did similar cartilage in the



TABLE 6

AVERAGE NUMBER OF GRAINS [ $^{35}\text{S}$ ]-SULPHATE INCORPORATED PER UNIT AREA  
MICROMETER EYEPIECE OF SECONDARY CARTILAGE IN QUADRATOJUGALS INCUBATED IN VITRO<sup>1</sup>

VALUES ARE MEANS  $\pm$  SEM

TREATMENT	ANTERIOR PAD		POSTERIOR PAD	
	NON-HYPERTROPHIC	HYPERTROPHIC	NON-HYPERTROPHIC	HYPERTROPHIC
Stage 36 Controls (10) <sup>2</sup>	1 $\pm$ 0.2	NC <sup>3</sup>	NC	NC
Stage 37 Controls (10)	2 $\pm$ 0.2	3 $\pm$ 0.6	1 $\pm$ 0.2	NC
Stage 38 Controls (10)	3 $\pm$ 0.5	10 $\pm$ 0.7	3 $\pm$ 0.7	7 $\pm$ 0.5
Stage 36 Paralyzed (10)	NC	NC	NC	NC
Stage 37 Paralyzed (10)	NC	NC	NC	NC
Stage 38 Paralyzed (10)	NC	3 $\pm$ 0.3	NC	2 $\pm$ 0.3

1 See Methods and Materials for incubation conditions and counting protocol

2 Number of quadratojugals labelled, in parentheses

3 No cartilage present

TABLE 7  
STATISTICAL COMPARISONS OF AVERAGE NUMBER OF GRAINS [3H]-GLUCOSAMINE AND [3S]-SULPHATE INCORPORATED  
PER UNIT AREA MICROMETER EYEPIECE OF SECONDARY CAPTILAE IN QUADRANTS INCUBATED IN VITRO

	STUDENT'S 't'											
	ANTERIOR						POSTERIOR					
	NON-HYPERTROPHIC			HYPERTROPHIC			NON-HYPERTROPHIC			HYPERTROPHIC		
	Stage 36	Stage 37	Stage 38	Stage 37	Stage 38	Stage 38	Stage 36	Stage 37	Stage 38	Stage 36	Stage 37	Stage 38
	Control	Control	Control	Control	Control	Paralyzed	Control	Control	Control	Control	Control	Paralyzed
Stage 36 Control	P<0.01	NS <sup>1</sup>		P<0.01	P<0.01	P<0.01	P<0.01	P<0.01	NS	NS	P<0.01	
Stage 37 Control	P<0.1		P<0.1	NS	P<0.01	P<0.01	P<0.01	P<0.01	P<0.01	P<0.1	P<0.001	
Stage 38 Control	P<0.01	P<0.01		P<0.05	P<0.01	P<0.01	P<0.01	P<0.01	P<0.05	NS	P<0.01	
Stage 37 Control	P<0.01	P<0.05	NS	P<0.01	P<0.01	P<0.001	P<0.01	P<0.001	P<0.01	P<0.01	P<0.001	
Stage 38 Control	P<0.01	P<0.001	P<0.01	P<0.01		P<0.01	P<0.01	P<0.01	P<0.01	P<0.05	P<0.01	
Stage 38 Paralyzed	P<0.01	P<0.01	NS	NS	P<0.01		NS	P<0.01	P<0.01	P<0.01	P<0.01	
Stage 36 Control								NS	NS	P<0.05	NS	
Stage 37 Control	NS	NS	P<0.05	P<0.01	P<0.01	P<0.05			P<0.01	P<0.01	P<0.01	
Stage 38 Control	P<0.05	NS	NS	NS	P<0.01	NS		NS		NS	P<0.01	
Stage 38 Paralyzed	P<0.01	P<0.001	P<0.01	P<0.01	P<0.01	P<0.01		P<0.01	P<0.01			
Stage 38 Paralyzed	NS	NS	P<0.01	P<0.05	P<0.01	P<0.01						

[3S]-SULPHATE

1 No significant difference between means

[3H]-GLUCOSAMINE

Figure 21 Quadratojugal, from a stage 36 control embryo, exposed in vitro to [ $^{35}\text{S}$ ]-sulphate. Note the very slight extracellular labelling anterior to the bony hook (D). Anterior below. AB. X 100.

Figure 22 Quadratojugal, from a stage 37 control embryo, exposed in vitro to [ $^{35}\text{S}$ ]-sulphate. Note the low levels of extracellular labelling over non-hypertrophic cartilage (C) anterior to the bony hook (D). A slight amount of extracellular labelling is present posterior to the bony hook. Anterior below. AB. X 100.



Figure 21



Figure 22

Figure 23 Quadratojugal, from a stage 38 control embryo, exposed in vitro to [ $^{35}\text{S}$ ]-sulphate. Note the low levels of mainly extracellular labelling over areas of non-hypertrophic cartilage (C). Labelling over areas of hypertrophic cartilage (HC) is more dense and is evenly distributed intra and extracellularly. AB. X 60.

Figure 24 Quadratojugal, from a stage 38 paralyzed embryo, exposed in vitro to [ $^{35}\text{S}$ ]-sulphate. A slight amount of extracellular labelling is present over the hypertrophic cartilage (HC) remaining anterior to the bony hook (B). Anterior below. AB. X 100.



Figure 23



Figure 24

posterior pad (Table 6). No significant difference in labelling was detected between areas of non-hypertrophic cartilage in stage 38 control quadratojugal hooks (Table 6)..

Quadratojugals from stage 36 and stage 37 paralyzed embryos contained no cartilage and no incorporation of [ $^{35}\text{S}$ ]-sulphate could be detected in the areas anterior to and posterior to the bony hook. The hypertrophic cartilage remaining in both anterior and posterior pads in quadratojugals from stage 38 paralyzed embryos incorporated this precursor (Figure 24), but at levels significantly less than that observed in similar areas of stage 38 control quadratojugals (Table 6, Table 7). It is noted that, as was found with incorporation of tritiated glucosamine, incorporation of [ $^{35}\text{S}$ ]-sulphate into quadratojugals from stage 38 paralyzed embryos was restricted almost exclusively to the extracellular matrix (Figure 24).

## DISCUSSION

It has been demonstrated that the differentiation of secondary cartilage on the quadratojugal of embryo chicks is associated with an increased activity of two cartilage specific enzymes, as well as an increase in the rate of incorporation and secretion of [ $^3\text{H}$ ]-glucosamine and [ $^{35}\text{S}$ ]-sulphate. Paralysis of the embryo inhibits differentiation of the secondary cartilage, suppresses activity of the two cartilage specific enzymes and lowers the rate of incorporation and secretion of the radio-labelled precursors. These observations have been supported by comparisons of the relative proportions of the hexosamine intermediates in the synthetic pathway of chondroitin sulphate.


The pattern of secondary cartilage development on the quadratojugal hook and the effects of embryo paralysis on the development of this cartilage have been examined in detail histologically (Murray, 1957, 1963; Murray and Smiles, 1965; Hall, 1972a). Histological examination of representative quadratojugals employed in this investigation yielded results in accordance with those recorded by others and served to relate the biochemical findings to the stage of morphological development of the secondary cartilage.

The activity of cartilage specific enzymes has been examined in a variety of systems undergoing differentiation and maturation of cartilage. Medoff (1967) noted linear increases in the activity of UDPG-dehydrogenase and in the activity of UDPCNAc-4-epimerase in both developing whole chick limbs and in cultures of chick limb bud aggregates. The rate of increase in activity of UDPG-dehydrogenase was more rapid than that of UDPCNAc-4-epimerase in both systems studied. Marzullo and Desiderio (1972) observed



rapid, linear increases in the activities of UDPG-dehydrogenase and UDPG-4-epimerase in embryo chick sterna over the period of histological maturation of this tissue. Johnson and Hunt (1974) have observed increased levels of activity of UDPG-dehydrogenase and UDPG-4-epimerase during the post partum attempt of achondroplastic mice to recover from the abnormally low rate of embryonic cartilage synthesis. It is to be noted that the increased activity of these cartilage specific enzymes, observed during embryonic cartilage development, have been shown to be the result of higher concentrations of the enzymes and not to be the result of the loss of an inhibitor or the acquisition of an activator (Medoff, 1967; Marzullo and Desiderio, 1972).

The essentially linear increase in activity of UDPG-4-epimerase found, in this investigation, to accompany the development of secondary cartilage on chick quadratojugal bones, and the detection of an increased activity of this enzyme prior to histological differentiation of the cartilage, correlates well with the findings in other chondrogenic systems. However, the developmental changes in activity of UDPG-dehydrogenase observed in this study are at variance with those noted in several other systems. Medoff (1967) observed a slight decline in the activity of this enzyme in cultures of chick limb bud aggregates between 6 and 7 days of culture, but this was no doubt due to adverse in vitro conditions resulting from the prolonged cultivation period. Similar declines in the activity of UDPG-dehydrogenase have not been found to accompany maturation in other cartilage systems. It is to be noted, however, that studies prior to the present have been conducted primarily on tissues which have passed the stage of initial, post-induction, rapid proliferation. Histological examination of developing chick quadratojugals



reveals that between stage 35 and stage 36 of development there is a large proliferation of cells in the presumptive secondary cartilage regions. Murray and Smiles (1965) and Hall (1967a, 1967b, 1968a, 1969, 1972a) have shown that induction of secondary cartilage on chick quadratojugals occurs between these two developmental stages. Thus, the rapid rise in activity of UDPG-dehydrogenase observed in this study is seen to accompany both cartilage induction and the proliferation of prechondroblasts.

Tomida et al. (1974, 1975) and Moscatelli and Rubin (1975) have shown the proliferation of chick embryo fibroblasts and rat fibroblasts in vitro to be associated with an increased rate of hyaluronic acid synthesis. Toole and Gross (1971) and Toole (1972) have shown that the proliferation and migration of chondrocytes in culture is accompanied by hyaluronidate synthesis. Hall (1970c) has shown that injections of cortisone acetate inhibit secondary cartilage formation on chick embryo quadratojugal, pterygoid and surangular bones. This inhibition, however, did not appear to affect the proliferation of germinal cells or young chondroblasts and, while these cells failed to synthesize sulphated glycosaminoglycans, they did produce small amounts of non-sulphated glycosaminoglycan. Hyaluronic acid is a non-sulphated glycosaminoglycan. Since the repeating disaccharide unit of hyaluronic acid is composed of glucuronic acid and N-acetylglucosamine, the rapid rise in UDPG-dehydrogenase activity observed in quadratojugal hooks between developmental stages 35 and 36 may be the result of hyaluronic acid synthesis in the proliferating prechondroblasts. Oohira et al. (1974) have shown that young, mitotic chondrocytes have a low rate of chondroitin sulphate synthesis and that this rate increases to a maximum in chondrocytes that

have begun to hypertrophy. The levelling and subsequent decline in activity of UDPG-dehydrogenase observed in this study between developmental stages 36 and 38 is associated with a reduced rate of cellular proliferation, observed histologically, in the chondrogenic tissue and may reflect a switch to predominantly sulphated glycosaminoglycan synthesis. The synthesis of these compounds can be detected histologically in quadratojugal hooks shortly after stage 36 of development has been passed and by developmental stage 37 the chondrocytes are separated by moderate amounts of secondary cartilage matrix which can be stained with techniques specific for sulphated glycosaminoglycans (Hall, 1968c). Further, the levels of [ $^{35}\text{S}$ ]-sulphate incorporation noted in this investigation, suggest that while little sulphated glycosaminoglycan is synthesized by the chondrogenic tissue of stage 36 quadratojugals, synthesis of these compounds increases significantly between stages 36 and 38 of development.

The activities of UDPG-dehydrogenase and UDPG-4-epimerase observed, in this investigation, following paralysis of the embryo, do not display a single, generalized pattern. Reductions in the activity of both enzymes were observed following paralysis of the embryo at developmental stages 35 and 37 while paralysis at stage 36 of development had no effect on the activity of these enzymes. That paralysis of the embryo inhibited secondary cartilage formation on the quadratojugal hook is evidenced by the histological observations. Inhibition of cartilage in this system as a result of paralysis and the attendant lack of mechanical stimulation is a well documented phenomenon (Murray, 1963; Murray and Smiles, 1965; Hall, 1970a, 1972a). However, conflicting results on the activity of UDPG-dehydrogenase and of UDPG-4-epimerase in inhibited cartilage have been reported. Marzullo and Lick (1970) found no decrease

in UDPG-4-epimerase activity following inhibition, with bromodeoxyuridine, of chondrogenesis in chick embryo sternal chondrocyte cultures. Levitt and Dorfman (1973) observed only slight reductions in the activities of UDPG-dehydrogenase and UDPGNac-4-epimerase following bromodeoxyuridine inhibition of chondrogenesis in stage 23-24 embryonic chick limb bud cell cultures. Marzullo (1972) reported that bromodeoxyuridine inhibition of chondrogenesis, in cultures of 15 day embryo chick sternal cells, was associated with significant reductions in the activities UDPG-dehydrogenase and UDPG-4-epimerase, to the low levels found in a variety of tissues, i.e. presumptive chondrogenic tissues of the early embryo and non-chondrogenic tissues at all stages of development (Marzullo and Desiderio, 1972). The activity of UDPG-dehydrogenase, observed in the present study, in quadratojugal hooks in stage 36 paralyzed embryos is the same as that found in leg muscle in stage 38 control embryos. The activity observed in stage 38 paralyzed embryos is significantly less than that recorded in similar stage controls but is not significantly different from that recorded in stage 35 control embryos. The activities of UDPG-4-epimerase observed in quadratojugal hooks of stage 36 paralyzed and stage 38 paralyzed embryos are similar and significantly less than the activities found in stages 36 to 38 control embryos. It is proposed that paralysis of the embryo prior to the induction of secondary cartilage on quadratojugal hooks (i.e. paralysis at stage 35 of development) inhibits the formation of what Marzullo (1972) has called the cartilage specific excess of these two enzymes. Inhibition of continued differentiation of the secondary cartilage (i.e. paralysis at stage 37 of development) similarly inhibits UDPG-4-epimerase. However, the apparent cartilage specific excess of UDPG-dehydrogenase is only partially suppressed following

paralysis of the embryo at stage 37 of development. It may be that UDPG-dehydrogenase accumulates in the cells following inhibition of chondrogenesis and hence the activity of this enzyme estimated in vitro is not a true reflection of its rate of synthesis. It is also possible that the paralysis of stage 37 embryos inhibits the chondroitin polymerization enzymes and that the decreased activity of UDPG-dehydrogenase observed in quadratojugal hooks from stage 38 paralyzed embryos is the result of an increased concentration of UDP-xylose, a compound known to inhibit UDPG-dehydrogenase (Garney and Phelps, 1972; Baldwin et al., 1973). Whether the decreased activity of UDPG-dehydrogenase observed in this investigation following paralysis of the embryo at stage 37 is the result of a decreased rate of synthesis of the enzyme or the result of the acquisition of an inhibitor cannot be determined from the available results.

The failure to demonstrate a significant decrease in the activity of either UDPG-dehydrogenase or UDPG-4-epimerase in quadratojugal hooks from stage 37 paralyzed embryos is somewhat puzzling. Also, contrary to the findings of Murray and Smiles (1965), no cartilage could be found on histological examination of these bones. However, these histological observations do indicate that prior to the paralysis of stage 36 embryos some cells had already been induced to form secondary cartilage and these cells continued to produce cartilage but the matrix of this cartilage had begun to calcify at the time of examination. These observations are compatible with those reported for the same system by Hall (1972a), and stem from the fact that while the Canadian strain of chickens acquire secondary cartilage at the same stage of development as do the Australian strain, subsequent calcification of this cartilage occurs much more rapidly

in the Canadian strain (Hall, 1976). Lash (1968) has suggested that the increased activity of cartilage specific enzymes in chondrogenic tissues is the result of stabilization of the metabolic pathway by an appropriate stimulation. Ellison and Lash (1971), Solursh and Meier (1974), and Koshier and Lash (1975) have all shown that the presence of proteoglycans in the cell's immediate vicinity is an appropriate stimulation for the stabilization of the synthesis of cartilage. Thus, the synthesis of cartilage by cells already induced in quadratojugals at the time of paralysis could be expected to stabilize, for at least a short period of time, the chondrogenic metabolic pathway in the chondroblasts. In light of this thesis, no significant difference in the activity of either UDPG-dehydrogenase or UDPG-4-epimerase would be expected between quadratojugal hooks from stage 36 control embryos and those from stage 37 paralyzed embryos. A similar relationship in the activity of these enzymes is not expected between quadratojugal hooks from stage 37 control embryos and those from stage 38 paralyzed embryos if the pattern of enzyme activity, particularly that of UDPG-4-epimerase, in normally developing embryos is examined carefully. The increase in activity of this enzyme is seen to occur in two waves, one between developmental stages 35 and 36 and one between developmental stages 37 and 38. This developmental pattern of enzyme activity suggests that chondrogenic induction of germinal cells in quadratojugal hooks also occurs in two waves. The significant reduction in the activities of UDPG-dehydrogenase and of UDPG-4-epimerase found in quadratojugal hooks in stage 38 paralyzed embryos is the result of inhibition of the induction of chondrogenesis normally occurring at or shortly after developmental stage 37.

The level of activity of both UDPG-dehydrogenase and UDPG-4-epimerase observed in this study, in quadratojugal hooks from stage 36 and stage 38 paralyzed embryos suggest that an event important to the formation of secondary cartilage on these bones occurs prior to stage 35 of development. That induction of chondrogenesis in germinal cells around the hook of the quadratojugal occurs in embryo chicks between stage 35 and stage 36 of development has been discussed above. However, the results of this investigation indicate that a cartilage specific excess of at least two cartilage specific enzymes is present in quadratojugal hooks in stage 35 chick embryos. Therefore, it is postulated that the induction of chondrogenesis in germinal cells around the hook of the quadratojugal in embryo chicks between stage 35 and stage 36 of development is not the result of a single event, but rather represents an event permitting the expression of a bias acquired by these cells as the result of an induction at an earlier stage of development. It has been shown that movement in embryo chicks commences at 3 to 4 days of incubation and that these movements increase sharply in frequency at 7 days and again at 9 to 10 days of incubation (Hamburger et al., 1965; Oppenheim, 1972; Foelix and Oppenheim, 1973). It may be that cells around the hook of the quadratojugal acquire a chondrogenic bias in response to these mechanical stimuli which, having reached a critical threshold, are known to induce secondary chondrogenesis between stages 35 and 36 of development.

Care was taken to ensure that assay media employed during this investigation contained a constant amount of essential substrates and that these substrates were present in excess. With these conditions, it is possible that all differences in the activities of UDPG-dehydrogenase and of UDPG-4-epimerase observed in this study, in quadratojugal hooks from both normally developing and paralyzed embryos, reflect changes in the

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Vmax of a constant amount of enzyme. Alternately, the possible presence of inhibitors, which would alter the Km values of the enzymes, has been mentioned above. However, the results reported herein have been obtained from an embryonic system in which the biosynthetic pathway is being turned on, a series of events known to produce patterns of enzyme activities resembling those seen in inducible enzyme systems. This fact, combined with the findings of activator-inhibitor studies in similar systems (Medoff, 1967; Marzullo and Desiderio, 1972), suggests that the in vitro changes in the activities of UDPG-dehydrogenase and UDPG-4-epimerase in quadratojugal hooks observed in this investigation are true reflections of changes in the in vivo concentration of these enzymes.

The supply of UTP available to the chondrocyte has been suggested as a potential source of regulation of the synthesis of glycosaminoglycans. Lash (1968) found that the chondrogenic tissues of the early chick embryo accumulated label from [<sup>3</sup>H]-glucosamine in the UDP-N-acetylhexosamines whereas non-chondrogenic tissues accumulated label in the earlier steps of the pathway, suggesting that the conversion of NAG-1-P to UDPGNAC could be rate limiting during chondrogenesis. This step is catalyzed by the enzyme UDPGNAC-pyrophosphorylase and requires the presence of UTP. Marzullo and Lash (1970) found that even though considerable amounts of this pyrophosphorylase were present, this metabolic step was blocked in cultures of chick embryo sternal chondrocytes following the inhibition of chondrogenesis by added chick serum. Winterburn and Phelps (1970, 1971) found that UTP reduced the inhibition of L-glutamine-D-fructose-6-phosphate amidotransferase (the first step in the biosynthesis of UDP-N-acetylhexosamines) caused by UDPGNAC. These authors suggest that the regulation of glycosaminoglycan synthesis by UDPGNAC is not due to simple feedback mechanisms but rather also involves an alteration in the binding constant



of this intermediate evoked by radiations such as UTP. The concentrations of UTP, estimated in this study, available to chondroblasts around the hook of the quadratojugal do not vary as secondary cartilage is differentiated and appear little affected by conditions which inhibit this secondary cartilage. It would appear that UTP is not involved in the control of secondary cartilage differentiation of the chick quadratojugal.

Estimates of the relative proportions of the intermediates in the biosyntheses of UDP-N-acetylhexosamines were conducted in order to obtain an indication of the rate of all enzymatic reactions in this pathway. These estimates indicate that the two reactions converting NAG-6-P to UDPGNac occur fairly rapidly in quadratojugals during normal differentiation of secondary cartilage. While a slight accumulation of GN-6-P is noted in these hooks, the predominate intermediates observed at all developmental stages examined were UDPGNac and UDPNAGal. The slight increase in accumulation of UDPGNac from stage 35 to stage 37 followed by a decline in relative proportion between stage 37 and stage 38 is consistent with the increased activity of UDPG-4-epimerase noted between stage 37 and stage 38. The predominance of UDPGNac and UDPNAGal suggests that these intermediates are being produced faster than they are required. This finding is hard to understand, especially in light of the fact that UDPGNac has been shown to be a regulator of this synthetic pathway via feedback inhibition of the enzyme leading to the synthesis of GN-6-P (Kornfeld, 1967). It may be that UDPGNac does not possess this inhibitory capacity in embryonic systems, thereby maximizing the polymerization of glycosaminoglycan. This theory would place the control of cartilage differentiation on the xylosyltransferase step or some subsequent step. That xylosyltransferase may regulate the synthesis of chondroitin sulphate

via selective dissociation and reassociation with the main complex of polymerizing enzymes has been suggested by Stoolmiller et al. (1972), Schwartz (1974) and Speciale et al. (1974). Conversely, UDPGNac may be directly stimulating the rate of chondroitin sulphate synthesis. Vessey et al. (1973) have shown that UDPGNac binds to the glucuronyltransferase producing an allosteric change that increases the enzyme's affinity for glucuronic acid, thereby assuring a high rate of glucuronic acid incorporation even at low glucuronic acid concentrations.

The inhibition of secondary cartilage on chick quadratojugals results in relative declines in the proportions of UDPGNac and UDPNAGal and in relative increases in the proportions of the earlier intermediates, in particular increased proportions of GN-6-P. The predominate increase in the proportion of GN-6-P would suggest that paralysis of the embryo suppresses the velocity of the phosphoglucosamine transacetylase step. This enzyme requires the presence of Acetyl CoA. The partial suppression of this transacetylase following paralysis is not entirely unexplainable. Hall (1969, 1970a, 1970b) has shown that low oxygen tensions favor formation of secondary cartilage in cultured quadratojugals whereas high oxygen tensions inhibit this chondrogenesis. It may be that paralysis of the embryo, by altering the oxygen tension in the cells' immediate environment, or by direct action on the cell membrane, enhances general oxidative metabolic pathways in the chondroblasts and germinal cells, thereby restricting the quantities of Acetyl CoA available to the glycosaminoglycan synthetic pathway.

The rise in proportion of intermediates prior to UDPGNac (i.e. GN-6-P, NAG-6-P, and NAG-1-P) following inhibition of secondary cartilage formation is compatible with the study of Lasb (1968) which demonstrated an accumulation of these intermediates in the non-chondrogenic tissues of the

early chick embryo. The decline in proportions of UDPGNac and UDPNAGal following inhibition of the secondary cartilage does not correlate with the observations of de la Haba and Holtzer (1965), who found an accumulation of UDPGNac following inhibition of chondrogenesis with puromycin, or with the observations of Levitt and Dorfman (1973) that no change in the UDP-N-acetylhexosamine pool followed inhibition of chondrogenesis with bromodeoxyuridine. However, this variation in observations may be the result of variation in the mechanism of cartilage inhibition. The authors mentioned above suggest that both puromycin and bromodeoxyuridine exert their effect via inhibition of the proteoglycan core protein. The only investigation known to be relative to the current findings suggests that the inhibition of chondrogenesis in chick quadratojugals is not mediated by the inhibition of general protein synthesis (Hall, 1968c). The pattern of change in the proportions of UDPGNac noted in quadratojugals hooks from stage 36 to stage 38 paralyzed embryos does, however, correlate with the pattern of UDPG-4-epimerase activity observed in these hooks; that is the proportion of UDPGNac is lowest in stage 37 paralyzed hooks while the activity of UDPG-4-epimerase is highest in stage 37 paralyzed hooks.

Handley and Phelps (1972) observed that the normal equilibrium of the epimerization enzyme in chondrocytes favored UDPGNac and concluded that this step was not rate limiting during glycosaminoglycan synthesis. This investigation has shown that while relative proportions of UDPGNac exceed those of UDPNAGal in quadratojugal hooks both from embryos paralyzed after the induction of secondary chondrogenesis and from control embryos after the appearance of secondary cartilage, these two intermediates are present in equal proportions prior to the induction and/or histological differentiation of secondary cartilage (i.e. stage 35 and stage 36). It may be

that the epimerization step is rate limiting during the initiation of secondary chondrogenesis as this process appears to be accompanied by a shift in the normal equilibrium parameters of the epimerization enzyme. Paralysis of the embryo not only reduces the activity of the epimerase enzyme but also alters the equilibrium of the enzyme so that the UDPGNac: UDPNAGal ratio exceeds that in similar stage controls.

Labelled precursor incorporation studies were conducted in order to examine the rate of chondroitin sulphate synthesis as well as to correlate this synthesis with the histological differentiation of secondary cartilage. Lash (1968) has shown that [ $^3\text{H}$ ]-glucosamine is metabolized by chondrogenic tissue to UDPNAGal, and thus may be used to detect glycosaminoglycan synthesis in this tissue. Minor and Lash (1972) have shown that, in chick embryos more than 5 days old, [ $^{35}\text{S}$ ]-sulphate labelling is restricted to the chondrogenic tissues. Medoff (1967) and DeLuca et al. (1973) have shown that 3'-phosphoadenosine-5'-phosphosulphate (PAPS), the active sulphate intermediate in the synthesis of sulphated glycosaminoglycans, can be labelled with exogenously supplied [ $^{35}\text{S}$ ]-sulphate, while Adams and Meaney (1961), Richmond et al. (1973) and Gordon and Lash (1974) have shown that this label can be transferred from PAPS to chondroitin sulphate. The results of Hardingham and Muir (1972) indicate that a 4 hour incubation is a sufficient time in which to detect the synthesis and export to the extracellular matrix of [ $^{35}\text{S}$ ]-labelled chondroitin sulphate.

Although no histological evidence of secondary cartilage synthesis was observed in quadratojugals from stage 36 control embryos, significant amounts of [ $^3\text{H}$ ]-glucosamine labelling was noted in those regions where the secondary cartilage pads are found later in development. The predominately extracellular location of this label suggests that the cells in these regions were actively synthesizing and secreting glycosaminoglycan. These

observations support the thesis, suggested earlier on the basis of the developmental pattern of UDPG-dehydrogenase activity, that germinal cells in the embryo chick quadratojugal hook may respond to the induction of secondary cartilage with an initial burst of hyaluronic acid synthesis.

The normal developmental pattern of incorporation of both [ $^3\text{H}$ ]-glucosamine and [ $^{35}\text{S}$ ]-sulphate support the histological observation that development of secondary cartilage on the anterior aspect of the quadratojugal hook preceeds that on the posterior surface of the hook. Further, these incorporation studies show that the rate of sulphated glycosaminoglycan synthesis, most likely that of chondroitin sulphate, is higher in hypertrophic cartilage than in non-hypertrophic cartilage. That actively differentiating secondary cartilage rapidly exports synthesized matrix materials as indicated by the predominately extracellular location of these labelled precursors in non-hypertrophic cartilage. The predominately intracellular location of [ $^3\text{H}$ ]-glucosamine label in hypertrophic cartilage is suggestive of a decreased rate of glycosaminoglycan export from hypertrophic chondrocytes. This may be the result of a reduced activity of the PAPS synthetic enzymes as indicated by the comparatively equal intracellular and extracellular location of [ $^{35}\text{S}$ ]-sulphate labelling.

From the virtual total lack of incorporation, of either [ $^3\text{H}$ ]-glucosamine or [ $^{35}\text{S}$ ]-sulphate, by cells in the hook of quadratojugals from paralyzed embryos, it is concluded that the failure of secondary cartilage differentiation in these hooks is the result of inhibition of the primary step(s) in the synthetic pathway of chondroitin sulphate. Consideration of the low levels of incorporation, of both [ $^3\text{H}$ ]-glucosamine,

and [ $^{35}\text{S}$ ]-sulphate, over the remaining hypertrophic cartilage in quadratojugal hooks from stage 38 paralyzed embryos must take into account the fact that the cells in this tissue were, in all probability, already induced at the time of paralysis. Of significance is the predominately extracellular location of labelling in this cartilage, as compared to the predominately intracellular label in quadratojugal hooks from similar stage control embryos. Fitton-Jackson (1970) and Hardingham et al. (1972) have shown that 11 to 12 day embryo chick tibial chondrocytes responds to enzymatic depletion of the matrix with an increased rate of glycosaminoglycan synthesis. It may be that hypertrophic chondrocytes in quadratojugal hooks of paralyzed embryos respond to depletion of the secondary cartilage matrix (i.e. failure to form normal amounts of matrix) with an increased rate of synthesis and/or export of chondroitin sulphate.

The results of these current investigations demonstrate the instability, (i.e. susceptibility to control by environmental factors) of cartilage specific enzymes in the chondroblasts of the secondary cartilage which develops on the quadratojugal bone of embryo chicks. Also, these results suggest levels at which the control of expression of this cartilage, by germinal cells around the hook of the bone, may act. From the normal developmental pattern of activity of the epimerase enzyme, and the alterations in the relative proportion of UDPGNAC observed both during normal development of secondary cartilage and following inhibition of secondary cartilage formation, it is suggested that control of secondary chondrogenesis on the embryo chick quadratojugal is exerted at the level of the epimerase enzyme reaction. However, the results do not confirm that this reaction is a single point of control. It remains possible that the changes in the biosynthetic pathway of chondroitin sulphate observed

in this investigation represent feedback alterations due to control mechanisms active at the level of the glycosaminoglycan polymerization enzymes.

Two proposals advanced herein are of particular significance:

1) that the induction of secondary cartilage on the quadratojugal of embryo chicks, the differentiation of which cannot be totally blocked after developmental stage 35, represents the expression of a bias acquired by the germinal cells at an earlier stage of development: a bias that may be induced by subthreshold levels of the same mechanical stimuli known to induce secondary chondrogenesis between stage 35 and stage 36 of development, and 2) that germinal cells may respond to the induction of secondary cartilage with an initial burst of non-sulphated glycosaminoglycan synthesis.

Further investigation of these proposals, as well as investigation of the activity of the glycosaminoglycan polymerization enzymes during differentiation of secondary cartilage, would be expected to further elucidate the factors controlling the evocation of this tissue. The significance of both the current and proposed investigations, not only to an understanding of the factors controlling the evocation of secondary cartilage in embryo chicks, but also to an elucidation of factors controlling chondrogenesis and cartilage maintenance in general, are noted.

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## APPENDIX I

## RELATION BETWEEN HAMBURGER-HAMILTON DEVELOPMENTAL

STAGES<sup>1</sup> AND DAYS OF INCUBATION<sup>2</sup>

DEVELOPMENTAL STAGE	LENGTH OF INCUBATION
35	9 DAYS
36	10 DAYS
37	11 DAYS
38	12 DAYS

1 Degree of development of nictitating membrane utilized to determine stage

2 Exact days plus 3 hours in a forced-draft incubator at  $37 \pm 1^{\circ}\text{C}$  and  $57 \pm 2\%$  relative humidity

## APPENDIX II

## ALCIAN BLUE - CHLORANTINE RED STAIN

(Adapted from Lison, 1954)

Chlorantine Red: 0.5% aqueous solution.

Alcian Blue: 1% aqueous solution/1% acetic acid (1:1). Mix, filter and add 10 to 20 mg thynol.

stain in Mayer's Haemalum .....	20 minutes
blue in tap water .....	as required
Alcian Blue .....	5-10 minutes
distilled water .....	30 seconds
phosphomolybdic acid (1% aqueous) .....	10 minutes
distilled water .....	30 seconds
Chlorantine Red .....	1-2 minutes
distilled water .....	very brief
100% alcohol (2 changes) .....	5 minutes each
xylene (2 changes) .....	5 minutes each

Bone matrix stains red, Cartilage matrix stains blue-green.

## APPENDIX III

## UDPG-DEHYDROGENASE ASSAY

(Pontis and Leloir, 1962)

Principle:  $\text{UDPG} + 2\text{NAD} + \text{H}_2\text{O} \longrightarrow \text{UDP-glucuronic acid} + 2\text{NADH} + 2\text{H}^+$

Measure NADH formation spectrophotometrically at 340 nm.

Reagents: Glycine buffer, 1M, pH 8.7; NAD, 0.05M; UDPG, 0.01M.

Procedure: Mix 10 microliters UDPG, 10 microliters NAD, and 50 microliters glycine buffer. Add enzyme extract and water to 0.5 ml. Read O.D. at 340 nm at 1 minute intervals until no further reaction detected.

Calculations: One unit of enzyme activity gives absorbancy increase of 0.001 per minute.

## APPENDIX IV

## UDPG-4-EPIIMERASE ASSAY

(Pontis and Leloir, 1962)

## Principle:



Measure NADH formation spectrophotometrically at 340 nm.

## Reagents:

Glycine buffer, 1M, pH 9.0; UDPGal, 7mM; NAD, 0.05M;  
UDPG-dehydrogenase.

## Procedure:

Mix 5 microliters UDPGal, 10 microliters NAD, 50 microliters glycine buffer, and 200 units UDPG-dehydrogenase. Wait for constant absorbance at 340 nm, then add enzyme extract to 0.5 ml. Read O.D. at 340 nm at 1 minute intervals for 4 minutes.

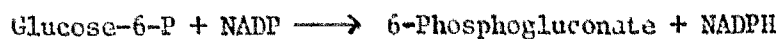
Calculations: One unit of enzyme activity gives absorbancy increase of 0.001 per minute.



## APPENDIX-V

## ESTIMATION OF UTP CONCENTRATION

## Principle:



## Reagents:

Tris buffer, 0.5M, pH 7.8;  $\text{MgCl}_2$ , 1M; NADP, 0.05 M;  
ADP, 0.02M; Glucose, 1M; Nucleoside diphosphokinase;  
Hexokinase; Glucose-6-P dehydrogenase.

## Procedure:

Mix 100 microliters Tris buffer, 10 microliters  $\text{MgCl}_2$ ,  
10 microliters NADP, 10 microliters glucose, 10 units  
nucleoside diphosphokinase, 10 units Hexokinase, 10 units  
glucose-6-P dehydrogenase. Add water and extract and  
immediately read O.D. at 340 nm ( $E_1$ ). Add 10 microliters  
ADP (final volume 0.5 ml) and read O.D. at 340 nm at  
1 minute intervals until a constant absorbancy is  
reached ( $E_2$ ).

Calculations:  $\Delta E$  (i.e.  $E_2 - E_1$ ) is a measure of NADPH production and

6.22 is the extinction coefficient of NADPH at 340 nm.

Therefore,  $\frac{\Delta E}{6.22} = \text{micromoles UTP in extract.}$

## APPENDIX VI,

BENZIDINE REAGENT<sup>1</sup>

Dissolve 1 g benzidine in 40 ml glacial acetic acid, warming, if necessary. Dissolve 30 g trichloroacetic acid in 40 ml water. These two solutions are then mixed to give a monophasic solution which is stable in the refrigerator although it slowly darkens in colour. The benzidine stock solution is diluted with acetone (1:9, V/V) immediately before use.

Most sugars react on heating to 100-110°C to yield brown spots on a light yellow background.

1. From: Chromatographic Techniques, Edited by Ivor Smith, pg 168.

W. Heinemann Medical Books Ltd., London, 1958.