A STUDY OF MYOSIN ISOFORM TRANSITIONS IN THE DEVELOPING MASSETER MUSCLE AND FOLLOWING OCCLUSAL PERTURBATION OF THE SPRAGUE-DAWLEY RAT

by

MICHAEL JAMES COUNSEL

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ABSTRACT

This study used immunohistochemical techniques to measure the relative percentage of fibres which stain positively for embryonic, neonatal, slow adult and fast adult myosin heavy chain (MHC) during the normal development of the masseter muscle in the Sprague-Dawley rat. In addition, the functional masticatory pattern of three-week-old developing rats was altered by weekly enameloplasty of the right maxillary molar teeth, testing the hypothesis that functional changes during development cause changes in MHC isoform transition. Third, growth related variations in the fibre size and distribution of fibre size were quantified during development and contrasted between experimental and control groups, to test the hypothesis that a functional change in the masseter muscle would be reflected in a gross structural alteration in that muscle after 3 weeks.

Sprague-Dawley rats of various ages (20 days gestation, 3 day, 3 week, 6 week, and 4 month) were used to establish the immunohistochemical and morphometric baseline data. The masseter muscle was dissected from the animal, oriented for cross-section in OCT and prepared for frozen sections. The enameloplasty was carried out on 3-week-old rats just after weaning, and 6-week-old rats served as their controls. In groups at 3 and 6 weeks of age and 4 months of age, the three muscle bellies of the masseter muscle were separated prior to freezing, and analyzed separately. Tissue sections were

incubated with monoclonal antibodies to one of four MHC antigens, while the omission of each of primary and secondary antibodies served as controls for the immunohistochemistry procedures. Positive control slides (longitudinal sections of myofibres showing intact A bands of myosin) were also run to corroborate positive MHC localization. Fibre diameter was measured using a computerized graphics tablet. Mean and standard error of the mean were computed for each parameter, and comparisons between groups were tested by appropriate analysis of variance, t-test, or Chi-square statistics.

As expected an increase in fibre diameter occurred from gestation to adulthood, reflecting the growth of myofibres during maturation. The proportion of fibre types present in the different age groups clearly showed significant changes. The proportion of neonatal and embryonic MHC was highest in the gestation group, and gradually decreased in the older groups of rats, until at 4 months of age, no embryonic and very little neonatal MHC was recorded. Conversely, the proportion of fibres that were positively labelled for fast MHC gradually increased from gestation to adulthood. In masseter muscle bellies from four month old rats, there was nearly 100% staining of fibres for the fast MHC isoform.

The comparison of control and experimental groups showed that both fibre diameter and myosin heavy chain isoform distribution were affected by the enameloplasty procedure. There was a significantly smaller fibre diameter in the 6-

week-old experimental (enameloplasty) group (p<0.01) compared to their age-matched control group, indicating that a functional change resulted in a significant alteration of gross muscle structure. A significant fibre size difference was also present between control and experimental fibres for each of the three muscle bellies of the masseter (the superficial belly, and the anterior and posterior deep bellies) (p<0.01). However, this change was particularly noted in the distribution of fibre diameter in the superficial masseter belly, suggesting that a muscle which is purely fast-twitch and changes toward a slower profile, will demonstrate the largest atrophic changes in response to reduced function.

In addition, there was a significant change in the proportions of fibres stained positive for fast, slow, neonatal, and embryonic MHC between the experimental group of rats and the age-matched control group. The latter 6-week-old control group did not have a significantly different profile of fibre types from the mature control adult rat at 4 months of age. The experimental group demonstated a significant increase in the proportion of fibres stained postively for slow, neonatal, and embryonic MHC. This change was consistently observed in all three muscle bellies, and clearly suggests that a change in the myosin isoform component of the contractile apparatus can occur as the result of a change in rat occlusal mechanics. However, the change toward slow MHC was most marked in the anterior deep masseter belly,

suggesting that it may be primarily involved in compensatory posturing of the mandible after enameloplasty has perturbed the normal occlusal relationship. The statistically significant reduction in mean fibre diameter and its distribution recorded in the 6-week-old experimental group confirms that a change in the function of the masseter muscle was produced by the occlusal adjustment.

These results indicate that short term functional changes affect both gross and molecular (isoform) structure of the masseter muscle, and suggest that such features may be used to monitor clinical changes induced by functional alteration of occlusion.

DEDICATION

For my parents, Mary and Jim

and

Julie

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1.0 INTRODUCTION

Nearly all of the information concerning skeletal muscle has been derived from studies of limb muscles. However, the muscles of mastication possess certain features that distinguish them from the well documented limb muscles since the muscles of mastication represent a distinct embryonic muscle cell lineage which may respond differently from limb muscles to neural, hormonal and functional stimuli. As myosin is important in the production of force and is developmentally regulated, the timing and mechanism of myosin isoform transformation during the maturation of craniofacial muscle fibres is likely to be an important indicator of the distinct features of craniofacial muscle. The factors which affect this maturation process must also be understood as their alteration may change normal development or function of muscle fibres.

Muscles of mastication are probably unique in terms of development, myosin isoform expression and response to various factors. Cervical and craniofacial muscles develop from the branchial arches, whereas limb muscles develop from somites. A distinct myosin isoform type IIM, or masticatory myosin, is expressed in the jaw muscles of some animals (Easton and Carlson, 1990). Further, muscles of mastication, according to Rubinstein et al. (1986), show a different response to thyroid hormone when compared to limb muscle.

During the development of skeletal muscles several myosin isoforms, which differ from the adult form of myosin, are

expressed. The transitions between isoforms are subject to neural input, endocrinological factors, especially thyroid hormone, and normal muscle function. Maturation of the immature fibres called myotubes, as they acquire more properties of adult muscle fibres, is best studied in animal models where the neural, hormonal and functional factors which influence development can be most clearly defined (Whalen et al., 1986).

Craniomandibular muscles have been characterized by their gross anatomy, their functional recruitment (as determined by electromyography) and their function, as defined biochemically by the presence and concentration of specific enzymes (Miller, 1991). Previously, enzyme histochemistry has provided a histological tool for visualizing the individual muscle fibres, the type of enzyme and its approximate concentration within the muscle fibre as outlined by Anderson et al., (1988). Depending upon the enzymes tested, the speed of contraction and the metabolic properties of a muscle can be predicted for individual muscle fibres and extrapolated to the function of the entire muscle by determining the proportion of the different muscle fibre types (Miller, 1991).

Immunohistochemistry utilizes antibodies to localize specific products in tissue sections. In the study of muscle fibre properties, antibodies to specific proteins within the muscle fibres can be identified. This technique is particularly useful in ascertaining the type of light and

heavy chains which compose the contractile protein myosin.

Recently, immunohistochemical methods have become increasingly common and, at least four different myosin types have been identified in rat muscle by using monoclonal antibodies (Schiaffino et al., 1988; Gorza, 1990). The present study was conducted to measure the relative percentage of fibres containing embryonic, neonatal, slow adult or fast adult myosin heavy chain present during the normal development of the masseter muscle in the Sprague-Dawley rat. Further to this, the functional masticatory pattern of the three week old developing rats was altered by weekly enameloplasty of the right maxillary molar teeth, since the altered functional demands on the right masseter after such a procedure may change the developing myosin isoform pattern, and also affect oxidative metabolism in the muscle. In a fast twitch muscle, increased oxidative metabolism would be demonstrated by an increased proportion of fast oxidative glycolytic fibres (FOG) and a decreased proportion of fast glycolytic fibres (FG), or by an increased proportion of fibres containing slow myosin. In a slow twitch muscle the same change would lead to an increase in the proportion of slow oxidative fibres (SO) and again possibly by some increase in slow myosin containing fibres.

While there may be changes in sarcomere number, as a result of a possible decrease in function, other structural changes (eg. fibre diameter) will enable comparison of

craniofacial muscles with previous reports on limb muscles in terms of muscle fibre response to changes in functional demands as well as their myosin isoform types and the timing of isoform appearance.

As the rat is used in maxillo-facial experiments to model orthodontic tooth movement procedures and temporomandibular joint mechanics, the structural correlates of developing masticatory muscle function act as an important baseline for further studies.

2.0 REVIEW OF LITERATURE

2.1.0 Skeletal muscle: overview

Skeletal muscle fibres are multinucleated, elongated cells which originate from smaller precursor cells called myoblasts that fuse before birth.

Myogenesis, or the process of muscle development, consists of stages where firstly myoblasts (committed cells which can differentiate into muscle fibres) fuse to become a primary myotube (a developing stage of the skeletal muscle where the central nucleus occupies most of the cell). These primary, or early myotubes in turn act as a scaffold for the alignment and formation of secondary myotubes and subsequently the mature cells themselves. During the early stages of embryogenesis a series of fibre generations thus occurs (Church, 1969; Kelly and Zachs, 1969). Successive (secondary) fibre generations form along the walls of and in alignment with the preceding (primary) generation of fibres.

Almost 80% of the fibre volume is composed of myofibrils, which are bundles containing thick and thin filaments in a repeating sarcomere pattern along the length of the fibril. The thick filament is composed primarily of the protein myosin and the thin filament contains actin and smaller amounts of the regulatory proteins tropomyosin and troponin. According to the sliding filament theory of contraction (Huxley and Huxley, 1964), the thick myosin filaments pull in the thin actin filaments by a cyclical attachment of cross bridges which

ultimately leads to a shortening of the sarcomere, shortening of the muscle and therefore the generation of force.

2.1.2. The muscle fibre: structural correlates of function

The movement produced by skeletal muscle is carried out by specialized cells called muscle fibres. A muscle fibre is in the form of a long cell of 10-100 microns in diameter extending between the proximal and distal muscle attachments onto either tendon or bone or skin. Each muscle contains hundreds to thousands of fibres (cells) but each fibre contains a few (30-40) myofibrils (Lehninger, 1975). The dimensions of the myofibril bundles vary slightly according to the type of muscle and the species from which it is obtained (Carlson and Wilkie, 1974). Myofibrils show the presence of a succession of transverse bands called I and A bands. When viewed under an electron microscope these bands can be subdivided into filaments of actin (I bands) and myosin (A bands) which are large polymerized protein molecules responsible for the contraction of muscle and constituting approximately 65% of total muscle protein. The actin filaments are attached at Z-discs and they extend from either side of the Z-disc towards the myosin rods. Myosin filaments aligned at the M-line interdigitate with the actin filaments. The unit of myofibril between the two Z-discs is called a sarcomere and sarcomere length varies such that actin filaments can overlap the myosin filaments. The interaction of actin and myosin is regulated by troponin, tropomyosin, calcium and other factors

causing the sarcomere to shorten and the muscle to contract and generate tension (Huxley and Huxley, 1964). In isometric contractions, where muscle does not shorten, the sarcomere length and myosin/actin overlap determines the amount of force which can be produced (Huxley, 1985).

As well as the abundant contractile proteins, myosin and actin, and the regulatory proteins, tropomyosin (Tm) and troponin (Tn), there are other regulatory proteins which mainly control the finely ordered structural alignment of the myofibrils. Among them, actinins are involved in the structural ordering of actin filaments, while M, C and I proteins are associated with myosin filaments. The scaffold or back bone proteins (connectin, desmin and Z-protein) are involved in maintaining the structural integrity of myofibrils. Connectin maintains longitudinal continuity of a myofibril while desmin (skeletin) transversely links neighbouring myofibrils at the Z-line levels. Z-protein forms the lattice structure in the Z-lines (Obinata et al., 1981). Dystrophin, a cytoskeletal protein just inside the fibre membrane, is important in maintaining cell integrity and stability and prevents muscular dystrophy (Koenig et al., 1988).

2.2.1 Fibre Types

Muscle fibres can be grouped by their similar functional properties into motor units, innervated by one motor neuron. For practical reasons, however, muscle fibre type

classifications have been based on similar staining reactions using histochemical methods (Dahl and Roald, 1991). Most histochemical identification methods for muscle fibre types have been based on myosin ATPase activity after preincubations at a variety of pH's, assessed alone or in combination with oxidative enzymes. Since Ranvier's description of red and white muscle fibres (Ranvier, 1873) the concept of muscle fibre types has been an important basis for discussion of muscle properties. Type I, or red fibres, are slowly contracting and fatigue resistant (slow oxidative) (Green, 1986). Type II fibres differ in many respects from type I fibres but generally type II fibres contract more rapidly than slow type I fibres and depend on glycolytic pathways for energy production. Type II, or white fibres, are subdivided into IIA which are fast twitch fatigue resistant fibres (fast oxidative glycolytic) and IIB which are fast twitch fatiguable (fast glycolytic) (Brooke and Kaiser, 1970a,b). In the adult craniofacial muscle of the rat there is very little, if any, type I myosin present. There is approximately 50% type IIA and 50% type IIB myosin present (Easton and Carlson, 1990). Interestingly, d'Albis et al., (1986) showed that in Wistar rats at three weeks of age no neonatal type myosins are observed in the EDL muscle while in contrast neonatal type myosins represent 50% of the total in the masseter of the three week old male rat. The neonatal type myosins persisted in significant amounts until about two months and were, on

average, found in higher concentration in males than in females. D'Albis et al. (1986) postulated that while the postnatal transitions of fast type myosins in the rat masseter have been shown to be influenced by thyroid hormone secretion, it appears that the myosin transitions in this muscle may be also regulated by additional factors such as innervation and sex hormones.

While muscle fibres can synthesize contractile proteins in the absence of motor neurons, once a muscle becomes innervated, the nerve supply and imposed activity influence the particular myosin isoform or regulatory protein form (Tm, Tn) which is subsequently synthesized (Rubinstein and Kelly, 1981). For instance, when the nerves to a fast twitch and slow twitch muscle are cut and redirected to reinnervate the opposite muscle type the former fast muscle switches to synthesize more slow myosin, while the formerly slow muscle changes, at least partially (and only partly) to more fast myosin synthesis (Weeds et al., 1974). Further, the fast to slow transition that occurs in cross-innervated fast muscles can be reproduced by a functional change in a muscle. For example, chronic stimulation of a fast motor neuron at a low continuous frequency induces the appearance of more slowly contracting, fatigue resistant fibres with a higher proportion of slow muscle fibres (Sreter et al., 1973). These transitions in function and isoforms occur within individual fibres (Rubinstein et al., 1978). Recent immunohistochemistry (see

below) has updated our understanding of distinctions between fast and slow fibres with developmental and physiological correlates (Hoh et al., 1988), however, the profile of fibre types with increasing age is not as clear as we might wish. For instance, it has been suggested that the proportions of type I and type II fibres in a muscle are genetically determined (Astrand and Rodahl, 1977; Komi et al., 1977) as evidenced by typical region-specific fibre type properties within a muscle of one species. While it has also been demonstrated that the nerve innervating a muscle fibre can influence its contractile characteristics and fibre type (Munsat et al., 1976), recent evidence indicates that expression of some proteins (Tnc, Tni) are not completely dependent on the nerve for isoform determination (Gauthier and Hobbs, 1982).

2.2.2 Myosin isoforms

Various mesodermal cell lineages are characterized by the replacement of one set of structures (molecules) by another during the course of development. The molecules involved in this transition are often related to each other in biochemical structure and sequence and are called isoforms (or isozymes) (Caplan et al., 1983).

In mouse muscle, analysis of the heavy myosin chains indicates that during fetal development there is an embryonic form of myosin. This changes at about the time of birth to a neonatal myosin heavy chain and later matures to the adult

form of the myosin heavy chain (Whalen et al., 1979). Transitions between isoforms which occur by replacement of one molecular form or another are subject to three general types of control - neural input, endocrinological factors (Whalen et al., 1986) and functional regulation. As development progresses, different myosins appear in different populations of muscle fibres. This gives rise to the mosaic pattern characteristic of most adult vertebrate muscles (Gauthier, 1990). As well one fibre may have more than one isoform at one time so there is a longitudinal heterogeneity in muscle development as well as a lateral heterogeneity of fibre types.

Maturation of immature myotubes from gestation and the neonatal periods as they acquire more properties of adult muscle fibres is best studied in animal models where the neural, hormonal and functional factors which influence maturation are most clear cut (Whalen et al., 1986). Four principal forms of isoforms have been identified in developing adult rat hind limb muscles (Whalen et al., 1981). However, they are not necessarily unique to any particular stage of development (Crow and Stockdale, 1986a,b).

Structurally, myosin molecule isoforms are very similar, and are comprised of two globular heads attached to an alpha helix rod-like tail (Slater and Lowey, 1967; Elliot et al., 1976) (see Figure 1).

The subunit structure of myosin consists of two heavy chains (MHC) (heavy meromyosin forming the two globular heads

and extending to the rod-like tail of the molecule) of molecular weight 200,000 and four molecules of light chains having a molecular weight of between 18,000 to 26,000 daltons (2 DTNB and 2 alkali myosin light chains) (Lowey and Risby, 1971).

Whilst myosins have a similar shape and subunit composition, the fine structure of the many diverse isoforms are different, consisting of various arrangements of one or other of the two types of core subfilament (Squire, 1973). There are also differences in regulatory mechanisms (eg. for light chain function and interactions between light and heavy chain and actin) (Wagner and Weeds, 1977) in filament formation (Whalen et al., 1981) and in enzymatic activity (Barany, 1967).

In early immunohistochemistry studies, which took advantage of the distinct antigenic sites on the different isoforms, it was believed that developing muscle fibres contain both fast and slow myosin isoforms irrespective of their fibre type. This concept arose following the observation that the two types of antibodies that would react only with fast or only with slow fibres in chickens would react with all embryonic muscle fibres in the chick embryo (Masaki and Yoshizaki, 1974). It had also been observed that the presumptive fast and slow embryonic muscles contained both fast and slow light chains (Rubinstein and Holtzer, 1979). However, it was subsequently demonstrated that embryonic

myosin was different from adult fast or slow myosin using peptide mapping techniques (Whalen et al., 1979). This latter technique suggested that three MHC isoforms appear sequentially during the development of the pectoralis major in the chick, which makes an excellent model for early developmental studies. One isoform (embryonic) is present from 10 days of embryonic development until hatching. A second isoform (neonatal) appears at hatching and completely replaces the embryonic MHC by 10-20 days and a third MHC isoform (adult slow or fast) appears at 40 days and completely replaces the neonatal MHC by eight weeks (Bandman et al., 1982). Monoclonal antibodies have been produced to pectoralis major muscle at different stages of development in chickens (Bader et al., 1982). The use of such monoclonal antibodies has allowed identification of MHC isoforms as they appear during development and maturation of the pectoralis major. This developmental sequence correlates with biochemical data of the enzyme histochemical studies and with the accumulation of different myosins during the development of this muscle (see Figure 2).

In contrast to avian systems there are no pure fast twitch or slow twitch muscles in mammals (Bandman, 1985). Rather all mammalian skeletal muscle fibres can be differentiated histochemically into type I and type II; type II fibres can be further subdivided into types IIA, IIB (and IIC) (Brooke and Kaiser, 1970a,b). This histochemical

differentiation is based on continued myosin ATPase activity following acidic or basic preincubation steps in the staining procedure. Subsequent experiments revealed that myosins from fast and slow muscles are immunologically distinct (Rubinstein et al., 1978; Gauthier and Lowey, 1979), allowing the preparation of antibodies against their unique sequences.

Mammalian fast and slow myosin isoforms have also been separated by native gel electrophoresis (Whalen et al., 1981; Fitzsimmons and Hoh, 1981; Lyons et al., 1983), as they have somewhat different molecular weights and mobilities.

In differentiated rat myotube cultures (grown from rats three days prior to birth), native gel electrophoresis reveals the presence of fetal myosin. In cultures obtained from rats two weeks post-natally, neonatal isoforms can be identified and in cultures obtained from three week old rats, adult myosin isoforms have appeared (Whalen et al., 1981), similar to the earlier reports for chicken muscle development. Since embryonic, neonatal and adult fast myosin heavy chain isoforms exist, and mammalian muscle also contains embryonic light chain (Whalen et al., 1978), it is probable that various combinations of light and heavy chain subunits give rise to the wide spectrum or range of myosin function present in fast muscle fibres. In developing rat slow muscle fibres, neonatal, slow and fast isoforms and adult slow myosin isoforms have been demonstrated by electrophoresis (Lyons et al., 1983; Butler-Browne and Whalen, 1984). Figure 3 summarizes myosin

transitions which occur during limb or somatic muscle development in the rat.

2.2.3 Neural impact on fibre type profile

Immunohistochemistry has revealed that many fibres express more than one isoform, a finding which suggests that transitions could occur during maturation of muscle. The results suggest that slow MHC's can be expressed in any muscle cell regardless of its current myosin content. It turns out that the signal to induce slow myosin and repress fast myosins has a large neural component. The activity of the slow type of motor neuron, which would induce slow fibre maturation in fetal muscle, however has not been demonstrated. A typical type II (fast) muscle can be transformed into a type I (slow) muscle by chronic stimulation of its nerve at a rate corresponding to the frequency of a slow muscle (10 Hz) (Salmons and Vrbova, 1969; Salmons and Sreter, 1976; Sreter et al., 1973), and it is the total number of stimulations rather than the rate in a short time that has the major influence on that transition. During this transformation of fibre type, the physiological, biochemical and ultrastructural properties of the muscle are altered in synchrony. For example, the speed of contraction, half time of relaxation, twitch and tetanus tensions, twitch potentiation and resistance to fatigue have been observed to change from that characteristic of a fast twitch muscle to that characteristic of a slow twitch muscle (Pette et al., 1973; Salmons and Henriksson, 1981).

Interestingly the transition from fast to slow is much more complete than one from slow to fast (Buller et al., 1960).

These altered properties are the result of changes of contractile protein isoforms and the consequent adjustment of their level of phosphorylation. Fast myosin light chains disappear with a corresponding increase in slow myosin light chains during transformation (Sreter et al., 1973; Rubinstein et al., 1978). Cross-innervating a fast twitch muscle with a nerve that originally supplied a slow muscle also causes the fast twitch muscle to change its physiological, biochemical and ultrastructural properties to those of a slow muscle (Sreter et al., 1975; Weeds et al., 1974). This again induces changes in myosin light chains (Sreter et al., 1975; Weeds et al., 1974), myosin heavy chains (Gauthier et al., 1983) and native myosin molecules (Hoh 1975). However, cross-innervation experiments in which a slow muscle was reinnervated with a fast motor neuron have yielded ambiguous results. Fast myosin can indeed be induced but slow myosin continues to be expressed (Gauthier et al., 1983). However, during normal development innervation acts as the major control of myosin isoform and fibre type expression (Kelly and Rubinstein, 1980).

In addition to the importance of the type of neural input to a fibre, muscle cells in most newborn mammals are polyinnervated (ie. innervated by branches from more than one motor neuron). During the first few weeks of life, muscle

cells become singly innervated by one neuron, and attain their adult characteristics from that single type (also of a fast or slow type) of neuron (Brown et al., 1976; Miyata and Yoshioka, 1980).

However, the denervation or removal of neural input to a newborn rat fast muscle only delays, but does not completely stop the appearance of adult fast myosin (Butler-Browne et al., 1982). Parallel results have also been obtained in the chicken (Matsuda, et al., 1983). Therefore innervation alone does not appear to be an absolute requirement for the myosin isoform or functional transitions characteristic of fast twitch fibres. By comparison innervation _is_ required for maturation of slow muscles. If the rat soleus is denervated at birth, slow myosin production progressively declines (Gambke et al., 1983). Furthermore it has been shown that when a mixed adult muscle (containing fast and slow fibres) is denervated, there is a selective maintenance of adult fast myosin (Carraro et al., 1982).

2.2.4 Non-neural impact on fibre type profile

Altering the pattern of functional activity, with no change in innervation of a muscle, can also lead to fibre type transformations. Endurance training can alter the overall fibre type composition of a muscle as revealed by myosin ATPase activity (Green et al., 1979) and immunohistochemical characteristics (Schantz et al., 1982). Green et al. (1979, 1983) observed a change from type IIB to type IIA (ie. toward

more oxidative) fibres in vastus lateralis in rats subjected to prolonged endurance training; that is, training tends to slow the functional character of fast muscles, and to increase their resistance to fatigue. However, unlike electrical stimulation and cross-innervation, endurance training did not seem to change significantly the contraction characteristics of the fast muscles although tension, fatigue-resistance and muscle hypertrophy increase towards values in the normal range of slow muscle (Salmons and Henriksson, 1981; Kennedy et al., 1986). It can be hypothesized that rigorous endurance programs would be necessary to increase the average level of muscle activity in order to effect a large change in myosin expression although the necessary degree of training may also be a function of the particular muscle, its normal fibre type proportion and of the individual in training.

Experiments with rats developing under euthyroid, hypothyroid and hyperthyroid conditions have shown that the thyroid hormones are able to affect myosin isoform transitions in fast muscle fibres. In normal healthy rats serum thyroid levels peak at about the same time as the transformation from neonatal to adult fast myosin takes place (Gambke et al., 1983). During normal fast muscle development, the switch in myosin composition is inhibited in hypothyroid animals and proceeds precociously in hyperthyroid animals, suggesting thyroid hormone directs the transition by activating adult fast myosin synthesis and inhibiting synthesis of neonatal

myosin. Indeed thyroid hormone is a mandatory requirement for normal muscle development of calcium handling and, therefore, contraction speeds (Everts and Van Hardeveld, 1987; Simonides and Van Hardeveld, 1989). However, thyroxine levels do not significantly alter the transition from neonatal to adult slow myosin, which is critically dependent on innervation (Gambke et al., 1983).

Other factors are also known to have effects on muscle development and function. For example, the serum level of growth hormone has been shown to be sensitive to thyroid hormone levels (Jacobsen, 1978; Seo et al., 1981; Daughaday 1979). Nemeth et al. (1989) found some degree of metabolic specialization in muscle fibre enzymes present at the time of birth in Sprague-Dawley rats. Further amplification of those metabolic differences in maturing muscle appeared to be regulated by multiple factors including the imposition of varied patterns of neural stimulation, differential use of available metabolic substrate and also response to hormonal stimulation. For instance, there was an increase in B-hydroxyacyl-CoA-dehydrogenase activity during adaptation of the neonatal rat to an aerobic extra-uterine environment and oxidative metabolism (Nemeth et al., 1989). Neonatal muscles also have slower velocities of shortening and relaxation (Close 1972) suggesting the newborn rat is adapted to making efficient use of restricted energy stores by the combination of mixed oxidative and glycolytic enzymes in muscles of the

limb. Since slow myosin has a low energy cost in the production of work (Crow and Kushmerik, 1982), the early expression of slow MHC in fetal and neonatal muscle (Narusawa et al., 1987) is consistent with this interpretation as is the finding by Whalen (1980) that ATPase activity of cultured rat muscle which mainly expresses embryonic myosin, is low and similar to that of adult slow muscle.

In addition to metabolic substrate and hormonal influence on MHC expression, it is clear that early patterns of MHC diversity precede innervation (Butler et al., 1982) and such findings are reproduced in vitro (Miller et al., 1985; Crow and Stockdale, 1986a,b). It seems that the commitment to specific patterns of muscle differentiation is intrinsically determined early in myogenesis, and that secondary fibre formation, which commences near the end of embryogenesis is the first stage at which dependence upon innervation is apparent (Crow and Stockdale, 1986a,b).

Unfortunately cell culture experiments designed to observe precisely which factors may be involved in regulating myosin isoform transitions, have revealed an inability to complete the normal sequence of myosin transitions seen in vivo. This implies that isoform alterations are not the sole architects of the myogenic program. Rather isoform changes are likely the typical net result of many external stimuli including innervation and activity which have yet to be elucidated.

2.3.1 Immunohistochemistry: Overview of immunological basis

Immunohistochemistry utilizes the properties of antibodies to localize specific products in tissue sections. In indirect immunohistochemistry, a tissue section is incubated with an unlabelled primary and then a labelled secondary antibody in sequence, then washed. The site of reaction of the antibody is identified, usually by a fluorescent, enzymatic or gold label on the secondary antibody (Ormerod and Imrie, 1989).

The target for an antibody is called an antigen and antibodies (which bind to the antigen), also called immunoglobulins, are produced by plasma cells. During an immune response, the antigen stimulates division of those plasma cells responsible for producing an antibody which reacts with that particular antigen (Ormerod and Imrie, 1989).

Structurally, an immunoglobulin is a dimer, with each half made up of two polypeptide chains, one heavy (containing approximately 440 amino acids) and the other light (containing approximately 220 amino acids) (Ormerod and Imrie, 1989). Figure 4 shows that the heavy chain has a constant region, which determines the class of the immunoglobulin, and a variable region. This latter region, together with the variable region on the light chain, forms the site which binds to the antigen. Immunoglobulins are sometimes split enzymatically producing fragments which are still reactive with the antigen. Pepsin digests the constant part of the

heavy chain leaving a dimeric fragment called F(ab')2. Papain creates two monomeric fragments, Fab, plus the constant region of the heavy chains, Fc. Fab fragments are sometimes used instead of the entire immunoglobulin (Ormerod and Imrie, 1989) for histochemical labelling.

Antibodies may be polyclonal or monoclonal. Polyclonal antibodies are "raised" (produced) by injecting an animal with the purified antigen in conjunction with a non-specific stimulant of the immune response (an adjuvant). The antigen consequently stimulates a variety of lymphocytes, each of which undergoes several divisions to produce a clone of antibody producing plasma cells (Ormerod and Imrie, 1989).

The site of an antigen which binds an antibody is called an epitope. An antigen may (and usually does) have several epitopes with different binding affinities to a variety of antibodies produced against the antigen. A solution of polyclonal antibody thus contains antibodies which bind to more than one epitope on an antigen. They also contain other immunoglobulins which were present prior to injection with the purified antigen. They may also contain antibodies reactive to impurities in the original preparation of antigen. This scenario is largely avoided with the use of monoclonal antibodies. These are made up by a cloned line of cells which produce a single molecular type of immunoglobulin to a single epitope. The cells are obtained by causing spleen cells, from an immunized animal, to fuse with a line of drug sensitive

myeloma cells (tumour plasma cells) which, by incubation in the presence of a drug, cause the fusion (Ormerod and Imrie, 1989). The only cells which grow following this procedure will be hybrids which result from a fusion of a normal spleen cell (which is drug resistant) and a myeloma cell (which brings immortality to the hybrid). The resulting cell lines (hybridomas) each produce indefinitely a single type of immunoglobulin (Ormerod and Imrie, 1989).

2.3.2 Application

In order to detect an antigen in a tissue section a direct or indirect method may be used. In the direct method a label is attached directly to the antibody. The advantage of this method is its speed as only one step is required. The disadvantage is that it potentially is less sensitive than the indirect method. More commonly, the primary antibody is left unlabelled and the label is attached to a different reagent which is then used to detect the primary antibody (Ormerod and Imrie, 1989). For example, if the primary antibody is a mouse immunoglobulin it may be detected by a labelled goat-antimouse immunoglobulin (see Figure 5).

The indirect labelling method is more sensitive than the direct method as more than one secondary antibody molecule can react with each first antibody. In order to visualize the antibody (which has attached to the antigen) four main methods can be used - fluorescent, gold with silver enhancement, enzymatic and occasionally a radioactive label followed by

autoradiography (Ormerod and Imrie, 1989). When enzyme labelling is used it is visualized by means of a reaction which gives an insoluble coloured product. Horseradish peroxidase (HRP) was the first such enzyme to be used. The substrate is hydrogen peroxide and the product oxidizes a chromogen. It can be used with DAB (3,3'-diaminobenzidine tetrachloride) which gives a brown precipitate at the site of reaction or with AEC (amino-ethyl carbazole) resulting in a brick red precipitate. The advantage of these stains is that they are permanent.

An enzymatic labelling system, using biotin and avidin, can be used. Avidin is a protein extracted from egg white and has four binding sites of high affinity for biotin, which is found in liver (Ormerod and Imrie, 1989). Instead of attaching an enzyme directly to avidin or streptavidin, the enzyme may be biotinylated, and unlabelled avidin used as a bridge. Potentially the larger complexes which are formed increase the labelling sensitivity (see Figure 6).

Therefore all four techniques described are modifications of the indirect method and essentially involve substitutions in the conjugation of the secondary antibody with a label (ie. an enzyme, a fluorescent molecule or a radioisotope). For each staining procedure a step in which the primary antibody is omitted is necessary. This ensures that any non-specific staining caused by the secondary reagents (used to detect the primary antibody) can be determined by comparison with the

original sections incubated with both the primary and secondary antibodies.

2.3.3 Alternative staining techniques

Histochemistry has provided a histological tool for visualizing the individual muscle fibre, the type of enzyme and its approximate concentration within a cell. Depending upon the enzymes tested, the speed of contraction and metabolic properties of a muscle can be predicted for individual muscle fibres and extrapolated to the function of the entire muscle by determining the proportion of muscle fibre types (Miller, 1991).

Different histochemical identification methods for muscle fibre types have been introduced. Most of these methods have been based on myosin ATPase activity after different kinds of preincubations, alone or in combination with oxidative enzymes. An important rationale for using these methods is the fact that the actomyosin ATPase activity can be used as an indicator of contractile speed (Barany, 1967). Consequently the ATPase reaction has become widely used to distinguish muscle fibre types. Some researchers make use of the susceptibility of the myofibrillar ATPase to different kinds of preincubation procedures (Brooke and Kaiser, 1970a,b) while other researchers rely on a combination of ATPase and the oxidative capacity as revealed by NADH-TR (nicotinamide-adenine dinucleotide tetrazolium reductase) or SDH (succinic acid dehydrogenase) activity (Peter et al., 1972).

Whilst several reports in the literature illustrate a lack of correspondence between different classification methods (Dahl and Roald, 1991), histochemical identification of insoluble, biological specimens can provide answers to the questions of identification, location and amount of the entity of interest (Horobin, 1989).

The functional properties of muscle fibres depend on a multitude of interacting factors like the actomyosin ATPase activity, the characteristics of the active state and the oxidative capacity of the muscle fibre. For instance, there is a correlation between contractile speed and myosin heavy chain content (Reiser et al., 1987), and between contractile speed and fatiguability (Nemeth et al., 1979; Anderson et al., 1988). Relatively recently (Nemeth and Pette, 1980, 1981) controversy has arisen about the lack of correlation between many different fibre type classifications. A variety of histochemical methods used in many physiological and histochemical studies of rat muscle have shown three main muscle fibre types. However, some studies have reported higher numbers of fibre types: Romanul, 1964 (eight fibre types); Gorza, 1990 (four fibre types). The advent of histochemical methods in the 1960's and 1970's was able to replace Ranvier's concept of red and white muscle fibres with a classification of fibre type that is functionally more meaningful. However, the lack of correspondence between the different enzymatic classification methods which investigators are currently

employing (Dahl and Roald, 1991), is mostly resolved by the use of immunohistochemical fibre typing, although there are different antibody preparations used in different laboratories, which again do not completely correspond.

Gel electrophoresis is a technique which can separate and identify the various proteins (eg. myosin) present in muscle. The term electrophoresis is usually applied to the movement of charged molecules in solution under the influence of an electric field (Grierson, 1990). A gel is the medium through which the molecules migrate with an electric field at moderate pH causing negatively charged molecules to migrate towards the anode. A variety of gel materials can be used and include agar, agarose and polyacrylamide (Grierson, 1990). A separation of the sample molecules is obtained as the smaller molecules move at a greater rate than the larger molecules. In the specific case of myosin, the protein isoforms which have a slightly different negative charge, move towards the anode at different rates. Thus myosin isoforms can be separated using gel electrophoresis and stained with a variety of blue or silver stains. However, the disadvantage of this technique is usually that each sample is a tissue average rather than a cell by cell study of isoform distribution as with immunohistochemistry, although some investigators study proteins from single muscle fibres (Rosser, 1991).

Immunoblotting is a separation technique which follows the same principles as outlined for electrophoresis. However,

the visualization of the bands is then made using specific antibodies to particular isoforms, in the case of myosin, much like immunohistochemistry. Both histochemistry and immuno- histochemistry enable the researcher to specifically examine individual fibres whereas electrophoresis and immunoblotting techniques allow only an averaging of what is present in the entire muscle sample (which has been previously homogenized as part of the preparation procedure used in these techniques). As the understanding of the relationship of myosin heterogeneity with function will ultimately require the isolation and characterization of myosin variants, the value of the aforementioned techniques and especially of immuno- histochemistry is readily apparent. Immunohistochemistry is especially valuable when the localization of different isoforms is of interest.

2.4.1 Muscle development

Exactly how different types of muscle fibres arise during development and become innervated in an orderly fashion has been the subject of investigation for many years. Until recently it was thought that each nascent muscle fibre differentiated according to signals from the motor neuron which captures its innervation during early development (Condon et al., 1989). This assumption was supported by two main lines of evidence. Firstly, adult fibre types can be significantly altered by innervating them with a foreign motor neuron (Buller et al., 1960) or by stimulating them with

activity patterns typically experienced by muscle fibres of a different type (Gorza et al., 1988). Secondly, neonatal fibres are poorly differentiated, at least on the basis of contractile properties (Buller et al., 1960; Close, 1964) or conventional histochemical methods for demonstration of contractile proteins and metabolic enzymes (Engel and Karpati, 1968).

There now appears to be four periods of striated muscle fibre formation during the life of the organism. These periods are the formation of a) myotomal fibres, b) primary fibres, c) secondary fibres and d) regenerating fibres (Stockdale et al., 1989) (see Figure 7).

The primary muscle precursor cells or myoblasts form in the myotomes (that portion of the embryonic somite that gives rise to striated muscle) of each somite (Stockdale et al., 1989), while the first fibres of the cervical and craniofacial muscles develop from the branchial arch muscle precursor (Easton and Carlson, 1990).

In the second period of myogenesis, primary fibres form in each skeletal muscle as it develops and before embryonic morphogenesis is completed. In the third period, secondary fibres then form parallel to and in contact with the primary fibres (Bennett, 1983). Secondary fibre formation commences toward the end of embryogenesis, continues during fetal development and is responsible for most of the increase in fibre number during this period (Stockdale et al., 1989).

Whilst primary fibre formation is independent of innervation, secondary fibre formation is influenced by neural stimulation (Crow and Stockdale, 1986a). During the fetal phase of growth, innervation becomes essential for maintaining a specific topographic pattern within each muscle and of modulating the myosin content of its fibres (Crow and Stockdale, 1986a,b).

The fourth period of fibre formation can be classified as regeneration of damaged muscle fibres. Fibre replacement itself is not dependent upon innervation and can occur at any stage after late fetal development, after the appearance of satellite cells which are "reserve myoblasts" or muscle precursor cells (Moss & Leblond, 1971) that appear at this time (Stockdale et al., 1989). Experimental results suggest that cellular elements, specifically the satellite cells, are the determinants of muscle allotype (a term used to describe different classes of skeletal muscle fibres with distinct intrinsic properties, eg. limb and jaw muscles) during regeneration.

In young, growing rat muscle, satellite cells have been shown to synthesize DNA and divide, and subsequently to contribute one of the daughter nuclei to the parent myofibre (Moss and Leblond, 1971). This process also occurs after muscle injury or disease, such as in muscular dystrophy and is better in young than in older animals and humans (Carlson and Faulkner, 1989; Zacharias and Anderson, 1991). It has been

accepted that in developing animals, muscle fibres increase in length by the addition of new sarcomeres and individual myofibres increase in diameter by the addition of myofibrils (Ontell and Dunn, 1978). Additional nuclei, to synthesize more proteins, are added to the growing fibre and the source of these new nuclei are satellite cells as discussed previously. Ontell (1979) has shown, using electron microscopy, that there are independent myofibres enclosed in their own basement membrane as well as clusters containing two or more myofilament containing cells. The largest, most developed fibre (primary fibre) is indistinguishable from an independent myofibre except that it is included in a cluster. Its average diameter is, however, larger than that of the independent fibre. Smaller satellite myofibres containing well developed myofibrils extend the full length of the primary fibre. Using the rat EDL muscle as a model, Ontell and Dunn (1978) showed that most of the muscle fibres in the adult rat are present at birth. Many are small, located in clusters, and cannot be identified with the light microscope. It was further concluded by Ontell and Dunn (1978) that the increase in the number of the independent filamented cells can be attributed to the breaking up of fibre clusters and the elongation of existing myotubes. A table in that paper showed the increase in mean diameter of filamented cells observed among two (6.5 ± 2.6 μm), five (7.4 ± 2.3 μm) and eight (9.5 ± 2 μm) day old extensor digitorum longus muscles.

In the two day old EDL muscle many of the myofibres were enclosed with other filamented cells (a satellite myofibre and/or myotube) in a common basement membrane forming a cluster. Clusters accounted for 40% of the total independent filamented cells and clusters in two day old muscles and for 2% in the five day old and less than 1% of the cells in the eight day old rat EDL (Ontell and Dunn, 1978). The decrease in the percentage of clusters occurred concomitantly with an increase in the number of independent cells, which increased by 90% by eight days.

Interestingly the largest fibres did not increase in diameter during the first week of postnatal development. Maximal fibre diameter in the two day old EDL being 15.9 μm and in the eight day old, 16.1 μm. In order to accommodate the myofibres, the muscle belly increased its girth and there was a decrease in the percentage of the area occupied by interstitial tissues, nerves and blood vessels (Ontell and Dunn, 1978). Much the same series of steps occurs in jaw mucles, although much less detail has been documented for the latter type of muscle.

Satellite cells of jaw and limb muscles are probably committed to express different subsets of myosin genes (Hoh et al., 1988, 1989). However, during fetal development the extracellular matrix is also likely to play a role in channelling the presumptive jaw and limb myoblasts into their respective allotypes. Specifically, the commitment of

myoblasts to distinct fibre type cell lineages is not intrinsically programmed (Butler et al., 1988). Fast and slow muscle fibres of the embryonic avian limb were unchanged (as determined by enzyme histochemistry and the proportion of fibre types present) in embryos in which the source of their precursor cells (the branchial somites) were replaced by thoracic somitic mesoderm from another embryo. It is known that fibre type profiles of these limb (somatic) muscles can emerge in the absence of nerves (Butler et al., 1982; Phillips and Bennett, 1984). During development the expression of one type of isoform or another is not only a manifestation of commitment of myoblasts, since a transition in MHC isoform expression can occur in culture in the absence of innervation or due to changes in innervation (Crow and Stockdale, 1984). Thus, it can be suggested that a non-neural factor may be responsible for the specification of different lineages of myoblasts destined to become various muscle fibres (Hoh et al., 1988). Stockdale et al. (1989) found by in vitro studies that satellite cells within different muscles of the chicken are committed to different fates, with some satellite cells committed to form fast fibres and others committed to form fast/slow fibres. Thus, the diversity of fibre types is an expression of different muscle specific proteins especially the expression of isoforms within the fast and slow classes of myosin heavy chain (Stockdale et al., 1989), as well as of the innervation or activity pattern as previously discussed.

The classification of myosin heavy chains into fast or slow groups is now based mainly on the reactivity of the MHC with monoclonal antibodies. The presence of these isoforms within fibres in vivo, that have been identified as fast or slow types by classical contraction velocity measurements and ATPase histochemical fibre typing, are good evidence for the assignment of distinct types of muscle fibres. In addition, electrophoretic mobility differences between the isoforms and the ATPase activity of the isolated isoform preparations also assists in the classification of fibre types (Stockdale et al., 1989).

The assignment of MHC isoforms to specific muscle fibre types is achieved either by immunohistochemistry or by microbiochemical analysis of dissected and histochemically identified single fibres (Termin et al., 1989). Gel electrophoresis of extracts from single fibres allows separation of MHC isoforms in rat skeletal muscle (Rosser et al., 1992) due to the differing electrophoretic mobility of these isoforms. Whilst the physiological and developmental significance of these different isoforms is unclear, the fact that biochemical differences do exist provides a reliable system for monitoring development at the molecular level (Crow and Stockdale, 1986a,b).

Comparative studies of different histochemical techniques have shown, however, that the different methods result in non-identical subgroups of Type II fibres (Dahl and Roald, 1991).

The functional properties of a muscle fibre rely on a variety of interacting factors such as ATPase activity and the characteristics of the active state and the oxidative capacity of the muscle fibre. Additionally, there seems to be a correlation between contractile speed and myosin heavy chain content of single rat plantaris muscle fibres (Reiser et al., 1987). Unfortunately, there is a lack of correspondence between muscle fibre types separated using different histochemical identification methods (Dahl and Roald, 1991). Consequently, immunohistochemical methods using labelled antibodies to detect homologous antigens have proved to be of great value in the study of biological systems (Seno et al., 1989) and currently a variety of immunohistochemical techniques exist allowing the researcher to detect precisely the sites of specific antigens in cells and tissues by light and electron microscopy.

In each of the four periods of myogenesis previously outlined, myoblasts are committed to distinct phases of developmental fate. However, the modulation of muscle specific proteins can and does occur at all periods of myogenesis within existing fibres. The regulatory mechanisms which underly this modulation of the MHC isoform include neural and endocrinological factors as well as changes in the activity in the muscle (Mahdavi et al., 1989; Bandman et al., 1989).

Recently Jellies (1990) examined the factors that

generate anatomically distinct muscles during embryogenesis and how a muscle comes to be innervated by the appropriate motor neurons during development. Myogenesis is believed to proceed in stages with the primary cells delineating the orientations of the muscle and acting as a scaffold to orient the secondary fibres (Crow, 1987; Jellies, 1990). A cellular scaffold is thus provided very early in embryogenesis and is used to pattern the assembly of large numbers of myoblasts. Such an early cellular framework may be necessary for the guidance of motor nerve axonal growth toward the appropriate region of fibres of a developing muscle (Jellies, 1990). By the time of birth in the Sprague-Dawley rat (22 days gestation) most of the essential muscle and neural elements of the limb muscles are present. However, maturation of intrafusal muscle fibres within muscle spindles and their neuromuscular connection occurs post-natally (Kucera et al., 1989).

Muscle spindles are small fusiform capsules within muscle which enclose three to eight intrafusal muscle fibres supplied by both afferent (Ia, IIa) and efferent (gamma motor) nerves in elaborate terminals. They function as both stretch receptors and in controlling muscle length and tone through a reflex via the spinal cord (Granit, 1975; Boyd, 1976). Intrafusal muscle fibres (which are centrally nucleated) differ from extrafusal muscle fibres which comprise the bulk of the muscle fascicles in every skeletal muscle regardless of

branchial or somatic origin. Two major types of intrafusal muscle fibres can be identified: nuclear chain fibres and nuclear bag fibres; bag I and bag II are further identified (Banker and Girvin, 1972; Kucera and Dorovini-Zis, 1979).

The distribution of spindles in individual muscles is variable. For instance, in a rodent such as the guinea pig, spindle density is more than four times greater in the soleus muscle than in the gastrocnemius (Maier et al., 1976). The muscles of mastication have very few spindles (Kubota and Masegi, 1972) although the anterior deep masseter of the rat possesses a major spindle cluster (Mascarello and Rowlerson, 1992; see Figure 12).

Muscle spindles are innervated with a single afferent nerve fibre (Fox et al., 1975). Unlike extrafusal muscle fibres in which innervation determines many physiological and histological properties, various types of intrafusal fibres have intrinsic immutable properties that differ despite the same innervation (Kucera, 1981), possibly because bag II fibres have a complex and variable myosin heavy chain content. It is interesting that spindles do not develop in rat limb muscles which have been deprived of innervation from birth (Zelena, 1957).

Cell culture experiments have shown that the fibroblast and myoblast must be in close proximity with each other for an external lamina to form around a developing myotube (Sanderson et al., 1986). Later in development (the third period of

myogenesis) connective tissue elements begin to separate individual myofibres, as a network of blood vessels and capillaries is established and each myofibre is finally surrounded by an external lamina (Mayne et al., 1989). Later stages of myogenesis can therefore be viewed as proceeding in an environment in which an external lamina separates the myogenic cells from the developing connective tissue and it is thought that growth factors, required for the development of skeletal muscle, may be concentrated in this developing external lamina (Folkman et al., 1988) consequently playing an important role in normal muscle development. For example, basic fibroblast growth factor, which promotes muscle cell division and also inhibits the terminal differentiation of muscle cells (Gospodarowicz et al., 1976) is found in extracellular matrix and binds to heparin sulphate proteoglycans in the external lamina. It is thought that increases in bFGF also play a regulatory role in muscle tissue repair and regeneration from disease states such as muscular dystrophy (Anderson et al., 1991 [animal]; Anderson et al., submitted [human]). The external lamina of mature jaw and limb muscles, by itself, plays little role in controlling normal myosin gene expression or in regulating the different fibre types of regenerating muscle cells (Hoh and Hughes, 1991). Cellular elements such as the satellite cells are much more important determinants of muscle allotype during regeneration (Hoh and Hughes, 1991).

In summary, muscle development is characterized by the synchronous fusion of primary myoblasts which produce a fixed and relatively small population of primary myotubes which extend from the somite as the limb bud grows, and elongate to form the length of the muscle. Secondary myotubes form after primary cells elongate, initially as small binucleate cells beneath the external lamina of the primary myotubes and overlapping their end plate region (Sheard et al., 1991). Subsequent elongation of the secondary myotubes is by fusion of myoblasts along the length of the developing myotubes (Harris et al., 1989). It has become accepted that the development of primary myotubes is autonomous of the nerve, while secondary myotube development occurs in the presence of innervation (Butler et al., 1982).

2.4.2 Influence of changes in innervation

As previously discussed for adult muscle, transformation of fibre types occurs upon innervation by foreign motor neurons (Buller et al., 1960; Pette and Vrbova, 1985), suggesting that motor neurons can largely determine fibre type. The observations that fibre types begin to differentiate concurrently with the arrival of axons in the muscle and with the formation of neuromuscular junctions support the hypothesis that the nervous system instructs the differentiation of each muscle fibre during development (Rubinstein and Kelly, 1981). It should be considered for completeness that vertebrate myosins are distinctly different

in smooth and striated muscle. In addition, distinct differences in the functional and developmental patterns are seen between skeletal and cardiac muscle.

Additionally, support for a role of the nervous system is obtained from experiments which show that denervation of newborn muscle impairs fibre differentiation as determined by myosin ATPase histochemistry (Engel and Karpati, 1968). However, whilst the differentiation of fast and slow contracting muscle fibres in the fetal rat occurs early in myogenesis, within a few days of the formation of fibres (Lyons et al., 1983; Narusawa et al., 1987), what exactly initiates this differentiation is equivocal. In a series of electron microscopic studies of the end plate region in young rat muscles, Duxon et al. (1986) observed that the primary myotubes were always densely innervated by multiple motor neurons whilst secondary myotubes were sparsely innervated. In those instances where an innervation site could be located on a secondary myotube, only one or two small nerve terminal profiles were present suggesting innervation by a single axon. These observations differ from a number of previous electrophysiological studies (Bennett and Pettigrew, 1974; Brown et al., 1976; Dennis et al., 1981).

Such earlier studies have reported that virtually 100% of embryonic myotubes are densely polyneuronally innervated. Sheard et al. (1991) have suggested that very young secondary myotubes, on average, have electrophysiological properties

quite different from those of primary or more mature secondary myotubes. It is further suggested that the initial development of young secondary myotubes has the potential to occur in the absence of direct neural input and that the biphasic nature of muscle development in the rat must be considered when analysing the extent and degree of polyneuronal innervation and synaptic arrangement within muscle. It is suggested further by Sheard et al. (1991) that young secondary myotubes are predominantly activated by end plate potentials which originate in adjoining primary myotubes and propagate electrically to the secondary myotube through the gap junctions which couple young secondary myotubes to the primary myotubes or by partial fusion of the nerve terminals themselves in cases where the terminal contacts a myoblast directly. This leads to the hypothesis that secondary myotubes do not require direct neural input for the initial cues for their development.

Polyneuronal innervation regresses to a mononeuronal configuration by approximately two weeks after the birth of the rat (Bennett and Pettigrew, 1974; Brown et al., 1976). Biochemically a sequential transition of myosin isoforms takes place with embryonic and neonatal forms occurring during fetal and postnatal development (Whalen et al., 1979, 1981; Butler-Browne et al., 1982).

Whilst the role of neural influence in establishing the phenotype during muscle development is somewhat equivocal, it

is established that the type of nerve which finally innervates a muscle fibre determines the myosin content in adult muscles (Salmons and Henriksson, 1981). Currently the use of immuno-histochemical methods to determine the types of myosins present during the rat postnatal muscle development, has suggested that developing muscle fibres will undergo a preprogrammed sequence of transitions in fast myosin unless they receive a signal which will stimulate the accumulation of slow myosin (Butler-Browne and Whalen, 1984).

In conjunction with myosin transitions, there occur changes in the functional properties of rat muscle in the first few weeks after birth. For instance, the isometric twitch contraction time of the EDL (fast-twitch in adult) and SOL (slow-twitch in adult) rat muscles at birth is about the same. Thereafter it decreases for both muscles but to a greater extent in the fast EDL muscle in proportion to its increased speed of shortening (Close, 1965). In the rat masseter muscle, most primary myotubes containing slow myosin heavy chain do not become slow fibres in the adult (Rowlerson et al., 1988). It is probable that the primary myotubes which do not evolve into adult slow fibres become fast fibres (rather than atrophying) (Mascarello and Rowlerson, 1992).

2.4.3 Masticatory muscles: Development and structure

By birth the masticatory muscles of the rat have reached a degree of maturity similar to that seen in limb muscles (Mascarello and Rowlerson, 1992) and the expression of slow

and neonatal MHC isoforms is similar to that which has been described for rat limb muscles (Harris et al., 1989; Condon et al., 1990; Mascarello and Rowlerson 1992).

There is an absence of extrafusal secondary myotubes in the spindle region of the developing rat masseter (Mascarello and Rowlerson, 1992). This finding supports the suggestion that the initial efferent contacts are withdrawn from presumptive bag II fibres since in the absence of such contacts, normal secondary myotube production should not occur (Duxon et al., 1989). This also explains why the spindles remain concentrated in certain areas of the muscle (eg. a major spindle cluster is present in the anterior deep masseter).

An increase in the concentration of spindle clusters is related to finely tuned proprioceptive and functional tasks as well as a short isometric twitch contraction time as associated with a fast twitch muscle. So it can be seen that the masseter muscle has an important proprioceptive or positioning role as well as a masticatory task to play (Zhang et al., 1992).

Adverse loading of the masticatory system can create muscle and joint strain and may lead to musculoskeletal pain (Seligman et al., 1988). Abnormal jaw and head posture may also perpetuate musculoskeletal dysfunction (McNeill, 1990). There is evidence to support the concept that increased loading of the temporomandibular joints which results from a

loss of posterior tooth support, subsequently produces craniomandibular dysfunction with associated muscle and joint strain (Christensen and Ziebert, 1986). Conversely other investigators have found no significant correlations between occlusion and craniomandibular dysfunction signs and symptoms (Roberts et al., 1987). Experimentally placed occlusal disturbances did not initiate signs or symptoms of craniomandibular dysfunction (Magnusson and Enbom, 1984). It does seem, however, that unconditioned reflexes originating in the temporomandibular joint (TMJ) do occur in pathological conditions involving the joint. Receptors in the TMJ, muscles, tendons and periodontal ligament conjointly serve to protect other sites in the stomatognathic system by sensing mandibular position (Storey, 1976).

2.4.4 Histochemistry

Histochemical studies have pointed out the striking differences in the properties of the masseter muscle depending on the species (d'Albis et al., 1986). The masseter of beef cattle, for example, which masticate slowly has only Type I fibres. The masseter muscle of rat and mouse, which eat rapidly, possess Type IIA and IIB fibres.

Miller and Farias (1988) compared the composition of the superficial temporalis, superficial middle masseter and anterior digastric muscles between adult male and female Rhesus monkeys (Macaca mulatta) and found no significant difference between the two sexes. However, while the

percentage of Type I and Type II fibres was similar, the female primates had significantly smaller cross sectional areas for both muscle fibre types in the muscles studied.

Detailed analysis of the effect of changing the pH of the preincubation step have indicated that the masseter muscle fibres have myofibrillar ATPase activity with a different pH lability than that of limb skeletal muscles (Maxwell et al., 1980). Interestingly, while the fibres of the temporalis muscle and limb muscles separate well into the different fibre types without alkaline preincubation, the masseter muscle fibres do not define as clearly without alkaline preincubation because the low activity fibres are not uniform in the intensity of their reaction. Indeed, some are only slightly less intense than the Type II fibres.

2.4.5 Changes in masticatory muscle histochemistry

Using the Rhesus monkey as model, a chronic edentulous condition (over a period of four years) was related to a significant decrease in cross sectional area of Type I fibres, although the IIA and IIB fibres did not change during this edentulous period (Maxwell et al., 1980). Biochemical assays of the superficial temporalis and masseter muscles showed a significant decrease in succinate oxidase confirming a loss of oxidative capacity within both these jaw closing muscles with a loss of dentition. Faulkner et al. (1972) have shown that reduced oxidative capacity in skeletal muscles can be correlated with relative inactivity. Maxwell et al. (1980)

concluded from electromyographic studies using the Rhesus monkey, that the temporalis and masseter muscles were less active both during the resting posture of the mandible and during mastication than in the dentate animal. It could not be differentiated, however, whether the low level recruitment, as in maintaining mandibular posture, or the high level recruitment required in high force activities such as mastication, contributed more significantly to the decrease in percentage of Type I fibres and a corresponding increase in Type IIB fibres. This latter finding correlates with studies in other skeletal muscles that have undergone experimental disuse (Booth and Kelso, 1973). Maxwell et al. (1981) analysed the effect of lengthening the masseter muscle on fibre composition and size by placing a bite opening appliance between the molar teeth of adult female Rhesus monkeys. Muscle fibre composition and fibre size were unchanged.

Developmentally the rat proceeds from its earliest postnatal period of suckling to a period of suckling with mastication and finally to a stage in which mastication is the principle mode of mandibular movement during feeding (Maeda et al., 1987). Whilst the muscle fibre types are evident in the earliest weaning period, if similar aged animals are maintained on a fine grained diet to alter mastication for the four month period following the earliest weaning stage, the muscle fibres are significantly smaller and the oxidative capacity of the muscle significantly lower (Maeda et al.,

1987).

Individual muscle fibre types of the craniomandibular muscles appear to change under specific conditions but are resilient to subtle changes in function (Miller, 1991). Genetically the composition of fibre types appears to be determined well before muscles are used in mastication, and the craniomandibular muscle fibres are predetermined to modify contraction speed and velocity within a given range (Miller, 1991).

It appears that a most effective way of altering the composition of the craniomandibular muscles is the removal of the maxillary and mandibular teeth. Removing the dentition presumably decreases the maximum tension developed by these muscles, the time and duration of maximum activity and probably the overall level of muscle activity. The composition of these muscles shift with more Type IIB fibres emerging and fewer Type I fibres. The cross-sectional areas of the Type I fibres also become smaller (Miller, 1991). The mechanism for such changes probably lies in the pattern and activity of the motor neuron in relation to the muscle fibres in its motor unit. This proposal is based on the observation that direct electrical stimulation of a limb muscle, at a low frequency (eg. 10 Hz) changes the myosin contractile protein from that typical of Type II fibres to that of Type I (Salmons and Sreter, 1976), whereas stimulation with the same total number of pulses but at a higher frequency has the opposite effect.

Indeed, stimulation of the denervated tail skeletal muscles of the cat, at a frequency of 10 Hz, increases the concentration of mitochondrial enzymes and decreases the amount of glycogen while stimulation with the same number of pulses over time but at a higher frequency of 50 Hz has the opposite effect, decreasing the concentration of mitochondrial enzymes and increasing the concentration of glycolytic enzymes (Riley and Allin, 1973).

Studies which have recorded different patterns of motor neuron discharge also support this latter viewpoint. Specifically the discharge of the motor axon to its muscle fibres determines the type of muscle fibre as specified by contraction speed and fatigue resistance (Buchthal and Schmalbruch, 1970; Pette and Vrbova, 1985). Although most of these studies are on limb muscle, the results suggest that the type of myosin and myofibrillar ATPase synthesized, do not change to any large extent, except with changes in motor nerve output to its fibres. The activity of a motor neuron innervating its muscle fibres is the dominating factor in controlling the gene expression of the mammalian muscle fibre (Pette and Vrbova, 1985). Significantly, however, the muscle fibre can also be altered within the range of genetic expression for its contractile proteins so that craniomandibular muscles can exhibit similar fibre types to limb muscles as detected by some histochemical techniques while their biochemical properties are distinctly different

from that of limb muscles (Miller, 1991).

In conclusion, the majority of the scientific literature suggests that occlusion may well be a secondary contributing factor in the development of musculoskeletal dysfunction, and may also play a role after trauma, oral parafunctional habits, stress or dental iatrogenesis in determining the ultimate functional range of masticatory muscle (McNeill, 1990). None of these studies included an assessment of muscle fibre type proportions as a relatively objective means of assessing altered functional demands on activity of particular jaw muscles, nor did they take into account the interplay between injury, reinnervation and repatterning of muscle fibre type proportion. Therefore, it is important to assess the relationship between functional development and structural or MHC fibre type changes in development and then to apply these known criteria in the study of specific experimental pertubation of jaw muscle function.

2.5.1 The rat masseter muscle: Anatomy

The rat masseter muscle can be divided into three different parts for anatomical and functional reasons. The superficial masseter has a predominantly horizontal orientation of fibres and its function is to protract and elevate the mandible (Hiiemae and Ardran, 1968). As well its fibres and tendon are external to the remainder of the muscle and therefore superficial. The deep masseter is relatively long anteroposteriorly and has separate insertions anteriorly

into the maxilla and zygomatic arch, and posteriorly onto the lateral surfaces of the body and ramus of the jaw (Hiiemae and Houston, 1971). The deep anterior portion controls the incisors while the posterior portion provides the power needed in the chewing cycle (Becht, 1954).

The masseter is the largest of the jaw muscles in the rat. Superficially it is covered by fascia, thick above where it forms a direct continuation of the temporal fascia, and thin below where it attaches to the lower border of the mandible. The superficial masseter in the rat is a large unipennate muscle extending from an attachment on the angle and lower border of the mandible to a simple tendon on the maxilla (Hiiemae and Houston, 1971). The muscle originates from a thick tendon attached to a slight elevation on the lateral surface of the maxilla postero-inferior to the infraorbital fissure. The insertion of the superficial muscle belly is into the convex lower border of the mandible from the point at which the masseteric ridge reaches the lower border, backwards to the angle and into an internal aponeurosis originating from the masseteric ridge (Hiiemae and Houston, 1971). The deep masseter can be described as having three components: anterior deep masseter; anterior deep masseter (infraorbital); and deep masseter (this division is based on experimental work by Hiiemae (1971) which indicated that the various parts of the muscle have different activities). The deep masseter in the rat is a large trapezoid muscle extending

from the maxilla and zygomatic arch, to the lateral surfaces of the body and ramus of the mandible. The main part of the muscle forms a continuous sheet and its division into anterior deep and deep parts is partly arbitrary. The anterior deep (infraorbital) part of the muscle is, however, quite separate as it arises from the maxilla on the medial wall of the infraorbital foramen and does not come into intimate relation with the other parts until very close to its insertion (Hiiemae and Houston, 1971).

2.5.2 Function during mastication

In the rat all of the jaw muscles tend to elevate the mandible and, apart from the anterior and posterior temporalis, protract the mandible. The deep masseter (and anterior temporalis) elevate the mandible during the power stroke of mastication. The anterior deep masseter has the same function during the incisive stroke of ingestion and is assisted by the deep masseter. In addition to protracting the mandible, the superficial masseter acts as a regulator of jaw position during mastication and in the incisive stroke of ingestion (Hiiemae, 1971). Electromyography has been used by researchers (eg. Moller, 1966) to provide some idea of the activity of the craniofacial muscles. However, electromyography only provides a measure of the electrical activity of a muscle and gives no direct evidence of the type of contraction occurring, the direction in which it is effective or the force generated (Hiiemae, 1971). Therefore

Hiiemae (1971) constructed a biomechanical model which could explain the probable effects of contraction of the craniomandibular muscles in the rat.

2.5.3 Ingestive cycle

The movements of the lower jaw during mastication can be described as occurring in two cycles: an ingestive cycle in which the incisors occlude and a masticatory cycle (Hiiemae and Ardran, 1968). Both cycles have three strokes. As the rat shifts from the ingestive to masticatory stage, there is an extensive anteroposterior translation of the condyle in its fossa. The moment (force x perpendicular distance) of the superficial masseter reaches a maximum when the lower incisor approaches occlusion allowing this muscle to continue the incisive stroke against the resistance of the bolus of food.

The anterior deep masseter has a constant moment as the mandible approaches the completion of incisive stroke and passes through the recovery stroke, suggesting that this muscle can sustain an upward movement of the jaw throughout the incisive stroke. The deep masseter reaches its maximum moment at the beginning of the power stroke of mastication and its minimum at the completion of the power stroke. The moment then increases slightly as the jaw drops. In ingestion, the moment reaches a minimum with the jaw in the open position although it is still larger than at the completion of the power stroke. This relative increase in the moment as the mandible is moved to the commencement of the power stroke in

mastication suggests that the deep masseter produces the elevation of the mandible against the resistance of the food during mastication (Hiiemae, 1971).

The anatomy of the masticatory apparatus of the rat and, in particular, of the TMJ has led to the view that mandibular movement in the rat is propalinal (ie. in a forwards and backwards direction) (Hiiemae and Ardran, 1968). This viewpoint was confirmed by Hiiemae and Ardran (1968) by analyzing the feeding behaviour of "August" and "Wistar" rats using cinephotography and cinefluorography. It was shown that there is a fundamental separation of ingestive and masticatory function in the rat which can most probably be explained due to the morphology of the jaws and the disparity in the lengths of the mandibular and maxillary diastemas. To ingest the food, the rat brings the incisor teeth into occlusion by translating the mandible forward and the condyle into articulation with the anterior part of the fossa. During mastication the condyle is moved backwards to bring the molar teeth into occlusion and the condyle into articulation with the posterior part of the fossa. Once the mandible has been moved into the appropriate position for either ingestion, deglutition or mastication, there follows a cyclical pattern of horizontal and small transverse movements in addition to the fundamental vertical movement used to open and close the mouth.

In summary, the act of ingestion corresponds to the mandibular incisors moving upwards and forwards across the

palatal surface of the maxillary incisors, while the grinding action of mastication is a propalinal movement which carries the mandibular molars anteriorly across the maxillary with some small transverse movement. Thus the transversely ridged teeth can occlude effectively in grinding and in disengagement for the next chewing cycle. Whilst the rat can chew bilaterally, when it chews on one side only the mandible moves laterally as the mouth opens and medially as the teeth occlude so as to enable the food to be ground by a forwards and inwards movement of the lower molars across the uppers. In the process of bilateral chewing, there is an inwards movement of the body of the mandible (made possible due to the presence of a movable syndesmosis in the symphyseal region of the mandible). As both "halves" move forwards and medially the mandibular molar teeth are carried across the maxillary molar teeth with a transverse component of about 0.5 mm on each side (Hiiemae and Ardran, 1968).

3.0 Material and Methods

3.1 Purpose

The objective of this study was three-fold: first to measure the relative percentage of embryonic, neonatal, slow adult and fast adult myosin heavy chain present in the normal development of the masseter muscle in the Sprague-Dawley rat. This would establish baseline data for the normal developmental transition of MHC isoforms in an important craniofacial muscle. Second, alteration of the functional masticatory pattern of the three week old developing rats by weekly enameloplasty of the right maxillary molar teeth, would test the hypothesis that functional changes during development would cause rapid changes in MHC isoform transition. In turn, those changes might be applied to the understanding of masticatory muscle function. Third, growth related variations in the fibre size and distribution of fibre size would be quantified during development and contrasted between the experimental and control groups. This would test the hypothesis that a functional change in masseter muscle may be reflected in a gross structural alteration in that muscle. The structural correlates of a functional alteration, in the developing masseter muscle, will therefore be the subject of this investigation.

3.2 Experimental paradigm: overview

Sprague-Dawley rats of various ages (four month, three week, three day and 20 day gestation) were sacrificed by

initially using halothane anesthesia, an overdose of ketamine and xylazine (1.0:1.5) and cervical dislocation.

Under anesthesia, the right masseter muscle was dissected out after identification of the superficial and deep bellies. Tissue blocks were cut from the middle third of the superficial masseter, the anterior deep masseter and the posterior deep masseter. Blocks were embedded, frozen in isopentane cooled to -50 degrees Celsius. Serial sections were made and collected on gelatin subbed slides using a cryostat maintained at -40 degrees Celsius. Up to 15 slides per block were made with six sets of sections taken serially from each block of the superficial, deep anterior and deep posterior masseter. Slides were stored and sealed in slide boxes to prevent dehydration prior to the staining procedures.

3.3 Group Sizes and Ages

All rats were of the Sprague-Dawley strain, and six animals per group were used for statistical analysis of a number of parameters, consistent with the published literature (d'Albis et al., 1986; Bredman et al., 1992). Four-month-old males and one female (a timed pregnant rat used to supply the gestation group) comprised the adult group. Three-week-old male rats comprised the second group, sacrificed at the time of weaning. Three-day-old male rats comprised the third, neonatal group. The fourth group (20 days gestation) was obtained from a timed pregnant female rat. A group of four male rats, six weeks old, acted as the control group for the

enameloplasty experimental group. The experimental group of six male rats had three consecutive weekly occlusal equilibrations of the right maxillary molar teeth (using a high speed dental turbine and diamond burr) and were sacrificed at six weeks of age.

3.4 Dissection of rat muscle

Dissection procedures were practised in order to identify tendons and muscle fibre orientations. "Dissection Guides" by H.G.Q. Rowett was used as a reference for the dissection of the masseter muscle.

Once surgical anesthesia was obtained the skin on the right hand side of the rat, superficial to the masseter, was opened from lower incisor to ear. With blunt dissection, the underlying masseter muscle was exposed. The muscle controlling ear movement was cut away and the fascia, overlying the masseter muscle, was removed. This was done by spreading the beaks of scissors outward along the fascial plane, teasing the fascia from the underlying muscle in one piece. The superficial masseter was then identified and the anterior tendon held with tweezers and blunt dissected back to its insertion at the angle of the mandible. The superficial masseter was then cut from the animal by severing both attachments (Figure 9a&b). The muscle was positioned on dental baseplate wax. The middle one-third (approximately 2-3 mm in the adult animal and 1-2 mm in the three or six week old animals) was cut from the muscle belly at right angles to the

muscle fibres using a razor blade. For the three day old and gestation groups there was no identification of superficial or deep masseter since the muscle was so small; rather the muscle was excised en masse.

The tissue blocks were then placed into vinyl specimen moulds (cryomold biopsy moulds; 10 mm x 10 mm x 5 mm) which contained OCT compound (an embedding medium for frozen tissue specimens supplied by Tissue-Tek) in order to produce cross-sections of muscle fibres. The tissue moulds, containing the OCT and masseter tissue, were placed into isopentane cooled on dry ice to -50 degrees Celsius. The deep masseter was exposed after removal of the superficial masseter, cleaned of fascia and visually divided into fifths from anterior to posterior. The second fifth was collected as the anterior deep and the fourth fifth was collected as the posterior deep masseter, which were then each embedded in OCT and frozen in isopentane as previously described. In all cases, tissue blocks were oriented in cross-section with their anterior aspect facing upward out of the mould prior to freezing. The individually labelled moulds were then placed in hermetic resealable plastic bags and stored at -20 degrees Celsius until sectioned. Rats were then killed by severing the cervical spinal cord.

3.5 Preparation of slides and sectioning of tissue blocks

Glass slides were cleaned using distilled water and then subbed. To prepare the subbing solution 0.5 gm. of gelatin was

stirred into 50 ml of double distilled water, warmed on a hot plate to 65 degrees Celsius. Once the gelatin cooled it was mixed with 0.05 gm. of alum dissolved in 50 ml. in double distilled water. Once the two solutions were mixed together they were filtered. Each glass slide was dipped three times into this subbing solution and left to dry overnight at an angle under cover so as it ensure that no dust settled onto the drying slides.

The masseter muscle tissue sections were cut in eight μm thick sections using a cryostat machine (Model CTI, International Equipment Co.). The tissue blocks were removed from the freezer and mounted using OCT onto the cold (-40 degrees Celsius) cryostat chuck. Initial sections were made and checked using phase contrast microscopy for cross-sectional orientation. Re-orientation of the blocks was made if needed. The 8 μm thick tissue sections were placed in serial fashion on 15 slides until six sections had been placed onto each slide. The slides were labelled and stored in sealed slide boxes at -20 degrees Celsius until required for immunohistochemical staining.

3.6 Immunohistochemical staining

Freeze dried samples of tissue culture supernatant from four different characterized monoclonal antibodies were generously supplied by Dr. L.V.B. Nicholson (Newcastle-upon-Tyne, UK). The slow, fast, and neonatal MHC were generated using rabbit muscle myosin as the immunogen. The

embryonic/neonatal MHC was raised against neonatal rat myosin. They were reconstituted with distilled water and stored frozen in small aliquots until needed. The four antibodies bind with rat slow MHC (slow), fast MHC (fast), neonatal MHC (neo), and embryonic-neonatal MHC (RN). The myosin heavy chains were identified indirectly using a biotin-streptavidin system which combined the specificity of primary and secondary monoclonal antibodies with the high affinity binding of the small water soluble vitamin biotin to the bacterial protein streptavidin. By attaching biotin molecules to the secondary antibodies and linking horseradish peroxidase with the streptavidin, the biotin-streptavidin interaction was exploited to generate a visual signal that would identify the antigen-antibody interaction (Amersham Laboratories). After reacting the horseradish peroxidase (HRP) with a chromogen substrate, amino-ethyl carbazole (AEC) or 3,3'-diaminobenzidine tetrachloride (DAB), a brick red coloured precipitate or a brown coloured precipitate developed respectively.

Freeze-dried samples of monoclonal antibody to superfast IIM cat myosin were generously supplied by Dr. J. F. Y. Hoh (University of Sydney, Australia). They were reconstituted with distilled water and stored frozen in small aliquots until needed.

Four steps were involved in this histochemical staining procedure:

Step One:

The sections were outlined with a DAKO wax pen and slides were dried for one hour prior to applying the primary antibody. The appropriate primary antibody (slow, fast, neonatal or embryonic MHC) was diluted in a ratio of 1:5 with phosphate buffered saline (PBS) (0.01 M at pH 7.5 with 1% bovine serum albumin [BSA], 10% horse serum and 0.01% sodium azide). Horse serum and BSA were used to block non-specific binding of the antibodies. The primary antibody was flooded onto the sections. Slides were then incubated at 37 degrees Celsius for 60 minutes in a radiant heat oven and then slides were removed from the oven and rinsed in PBS for 10 minutes. It was important that during the staining procedures, the tissue sections on the slides were not allowed to dry out. To prevent drying the plastic containers in which the slides were stained contained a small amount of water to keep the environment at an appropriate humidity level.

Step Two:

A biotinylated secondary antibody (goat-anti-mouse) was diluted in PBS and 1% BSA in a ratio of 1:100. The tissue sections were then incubated with the biotinylated secondary antibody solution at 37 degrees Celsius for 60 minutes, and rinsed well in PBS for 10 minutes.

Step Three:

Streptavidin-HRP (Dimension Laboratories Inc., Mississauga, Ontario) mixed with PBS in a dilution of 1:200

was applied to the sections and incubated at 37 degrees Celsius for 30 minutes. The slides were removed from the oven and rinsed in PBS for 10 minutes.

Step Four:

Antibody location was visualized with the application of DAB or AEC. The use of DAB produced a brown coloured deposit upon reacting with peroxidase which was linked to the streptavidin. The colour was enhanced (blackened) by nickel chloride. The incubation time of the DAB substrate on the specimen was approximately five minutes at room temperature. AEC was used on a number of tissue sections as an alternate coloration for the enzyme substrate. Those slides treated with the AEC substrate showed antibody localized with a brick red coloured precipitate. Development of colour with this substrate was between 5 and 20 minutes at room temperature. After coloration sections were fixed in cold methanol and then rehydrated.

Tissue sections were incubated with monoclonal antibodies to one of four MHC antigens, while the omission of each of primary and secondary antibodies served as controls for the immunohistochemistry procedures. Positive control slides (longitudinal sections of myofibres showing intact A bands of myosin) were also run to corroborate positive MHC localization. The tissue sections were mounted with cover slips using an aqueous mounting solution (Aqua-Mount, Lerner Laboratories), and allowed to dry. The slides were then stored

in slide boxes at room temperature for future analysis.

3.7 Routine staining

One set of tissue sections from each animal were stained using an H&E (haematoxylin and eosin) staining method. Muscle fibres stain a deep pink colour, while nuclei stain a blue-purple colour. The procedure for routine H&E-phloxine is as follows. The slides were dried overnight to attach the sections well. The slides were then immersed twice in 100% ethanol for two minutes. This was followed by two minutes in 95% ethanol, and slides were then rinsed in tap water for five minutes. They were then immersed in Harris' haematoxylin for five minutes (which is appropriate for 8 μm thick sections), and washed again in tap water for five minutes. This was followed by three to ten quick dips in 1% acid alcohol, and again the slides were washed in tap water for five minutes. The slides were immersed in saturated lithium carbonate for two minutes, washed in tap water for five minutes, and placed in eosin Y&B-phloxine for two minutes (which is appropriate for 8 μm thick sections). After eosin staining, slides were dipped in tap water 10 times followed by 10 dips in 70% alcohol and 15 dips in 95% alcohol, immersed twice in 100% alcohol for two minutes and cleared in xylol in preparation for mounting with Permount.

3.8 Morphometric analysis of fibre diameter

Using a camera lucida (10X) attached to a Nikon alphaphot YS microscope, H&E stained tissue sections from each animal

and group were examined and the fibre diameters were measured. Minimum fibre diameter rather than the fibre area or volume was quantified, as previously reported (Finn et al., 1980), in order to minimize artifacts of sectioning plane. The results were recorded in an image analysis computer equipped with the SigmaScan program (Jandel Scientific, CA). Final magnification was 400 times for each of the following groups - adult, three week, three day, six week experimental, six week control, and 1000 times for the 20 day gestation group. At least 100 fibre diameters were measured per section with the operator visualizing an imaginary chord perpendicular to the largest diameter of the tissue section to systematically sample muscle fibres. Distance measurement was calibrated in millimeters, using an ocular micrometer which measured to 0.1 mm (Cambridge Instruments, USA). The data and calibration could be stored in the computer using the SigmaScan program, prior to importing into Lotus 1,2,3, then translated for statistical analysis into NWA Statpak or Harvard graphics for plotting.

3.9   Occlusal reduction (enameloplasty) procedure

The experimental group of rats underwent occlusal reduction of their right maxillary molar teeth at weekly intervals from three to six weeks of age. This occlusal adjustment (enameloplasty) was done using a long diamond burr attached to a high speed dental turbine after rats were anesthetized as previously described. A colleague, Dr. Walter Nider, assisted in this operation by steadying the head of the

rat and retracting the mandible and tongue. The surgical anesthesia obtained was adequate for the minor task which usually lasted less than five minutes per rat. After three weeks the experimental group was sacrificed as described previously. The weekly interval between occlusal adjustment was necessary because of the relatively quick eruption rate of the teeth in the rat. The rats from the experimental group were weighed at weekly intervals and rats from the control group were weighed at three weeks and six weeks of age (which corresponded with the time of their sacrifice).

3.10   Double blind study following occlusal adjustment: Control versus experimental rat.

In addition, a double blind assessment of the feeding pattern of a four-week-old control rat and a four-week-old experimental rat at one week post-enameloplasty was made in an attempt to observe any changes in the masticatory pattern of the rat which may have occurred following the occlusal adjustment. Both rats were observed over four days in the morning and afternoon for a period of approximately 20 minutes on each occasion. During these periods, the frequency and ability of rats to incise a strip of paper which was placed at the top of the cage, and their ability to incise and masticate food were observed and recorded. Food pellets were stored at the top of the cage requiring the rat to incise and break the pellets into pieces before they would fall to the bottom of the cage. Finally, each rat's ancillary oral habits

(specifically grooming) were observed and their frequency recorded. As well, a qualitative assessment of the rats' ability to masticate food was assessed.

3.11 Decalcified maxillary molar teeth: Sagittal cross-sections from the control and experimental groups.

The palates and maxillae were removed from the control and experimental rats. The maxillae plus molar teeth were decalcified in a solution of EDTA for 10-14 weeks. The molar teeth, from the right and left-hand side of the animal, were placed in blocks, sectioned sagittally and stained with H&E. The teeth were measured from the apical foramen to the mesial and distal aspects of the occlusal surface. This measurement was considered to be more reproducible than interocclusal space depth or the thickness of the dentin remaining on the adjusted occlusal surface. The morphometric analysis of crown height was carried out using an image analysis computer, the SigmaScan program and a camera lucida (10X magnification) attached to a Nikon alphaphot YS microscope (using an ocular lens at 2.5X magnification).

3.12 Double blind study between the control and experimental groups following immunohistochemical staining.

A double blind study was undertaken once the tissue sections of the six week control and experimental groups were stained with the four monoclonal antibodies. All slides were coded by another independent student investigator (Marianne Krahn) from Dr. Anderson's laboratory, and only decoded after

all the staining results for all muscle sections were tabulated. Without knowledge of the antibodies used or which group (control or experimental) was being viewed, the stained tissue sections were examined using an Olympus microscope (VH2-RFCA). A selected stained tissue section was then assessed in comparison with both positive and negative control slides from that staining run. The number of positively stained muscle fibres (more than 50% coloured by DAB or AEC substrate deposition), and the total number of fibres in the field of view were counted. This binary grading system was used in preference to a comprehensive densitometric assessment of the staining intensity within a population of cells, due to its simplicity. A manual counter was used to count the number of fibres and then the proportion of positively stained fibres was expressed (the number of positively stained fibres divided by the total number of fibres). The other groups (gestation, three day, three week and adult) were assessed in a similar fashion, however, the operator had knowledge of which group was being examined. This was largely due to the power of observation. The large fibre diameters and peripherally located nuclei exhibited by the adult group, were in stark contrast to the small fibre diameters and centrally located nuclei of the gestation group, for instance, and easily biased a coded assessment of these groups.

3.13 Statistical tests

The present design compared immunohistochemical staining

proportions and fibre size data between the different groups of rats. Statistical assessment was made using the NWA Statpak computer program (Northwest Analytical, Inc.) after consultation with a statistician from the University of Manitoba. The mean and standard error of the mean (SEM) of each group were determined for each parameter. A one-way ANOVA determined the presence of an effect of age or treatment. Duncan's multiple range test was used post hoc to test differences between pairs of groups where appropriate. In addition, the data from the control and experimental groups of rats was analysed by two-way ANOVA which enabled comparison which took advantage of the paired data available for each subject. Chi square statistics were used to test for differences in the distribution or proportion of MHC (fast, slow, neonatal and embryonic), and also of the distribution of fibre diameter between the different groups. The mean weight (±SEM) for each group of rats was also determined, as was the molar tooth length for control and experimental groups. In all cases finding a probability of $p \leq 0.05$ was used to indicate a significant difference between samples or groups.

4.0 RESULTS

4.1 Demographics

Six groups of Sprague-Dawley rats were tested using the protocol described above. One group consisted of six rats of 20 days gestation (gest). A second group consisted of six male rats, three days of age (3 day). A third group consisted of six male rats, three weeks of age (3 week). The fourth group consisted of six male rats, four months of age (and a seventh adult female rat which was the mother of the gestation group) (Adult). A fifth group consisted of four male rats, six weeks of age (6 week control). The sixth group consisted of six male rats which underwent enameloplasty at weekly intervals from three to six weeks of age (6 week experimental). A seventh group of two male rats (one of which underwent occlusal adjustment of the right maxillary molar teeth at three weeks of age) was observed at four weeks of age to assess any observable differences in the feeding pattern following the enameloplasty procedure.

4.2 Body Weight

The average body weight ($\pm$SEM) is shown in Table 1. There was a significant increase in body weight at each increment of age ($p < 0.01$). There was no difference in body weight between the six week control and six week experimental groups ($p > 0.31$).

4.3 Fibre diameter data

The fibre diameter for each group of rats is reported in Table 2. As expected, there was an increase in the mean fibre diameter reflecting the age dependent growth of muscle cells. There was also a significantly smaller mean fibre diameter in the six week experimental group ($p < 0.01$) than in the six week control group: this difference was also present for each of the superficial, anterior deep and posterior deep bellies of the muscle ($p < 0.01$). However, there was no difference between the muscle bellies in this reduction in fibre diameter.

The distributions of fibre diameter are shown in Figures 8a-i. The graphs indicate the significant shift ($p < 0.01$) to larger diameters in the fibre populations of the masseter muscle at increasingly older ages. In addition, the six week experimental group fibre distribution is significantly left shifted ($p < 0.01$, see Figure 8d) compared to the six week control distribution. The left shift was particularly noted in the population of fibres within the superficial masseter muscle. These findings indicate that there was a muscle fibre population change in response to a perturbation of the occlusion and masticatory activity in the six week experimental group.

4.4 Effectiveness of chromogen substrates

The use of diaminobenzidine tetrachloride (DAB) and amino-ethyl carbazole (AEC) chromogens worked equally well in

the MHC immunohistochemistry. The DAB substrated produced a brown coloured deposit upon reacting with peroxidase and the muscle fibres which reacted were clearly and discreetly stained. The AEC produced a brick red coloured precipitate at the same site of the enzyme (HRP) and was used interchangeably with DAB. For photography, AEC did produce better contrast. Both these stains were preferred to fluoroscein-linked secondary antibodies, as the fluorescent label is labile under mercury vapour lamp illumination and therefore proportions of positively stained fibres were difficult to count. Furthermore, such staining is not permanent, and the transience would prohibit future observation.

4.5 Histological observations of immunohistochemical staining

The immunohistochemistry revealed different staining patterns for each group of rats and each monoclonal antibody. In all cases, there was little or no coloration of fibres when primary or secondary antibodies were omitted (Figure 10b). In the gestation group (Figure 11), the embryonic antibody revealed discreet areas of darkly stained cells. The neonatal antibody revealed a similar dark coloration in each cell but the staining distribution was more evenly spread throughout the muscle. The slow antibody was prominent in muscle spindle fibres (detected by H&E staining, see Figure 13), but was seen in a scattered fashion throughout the extrafusal fibres in the masseter.

The three day and three week old group of rats exhibited

a progressive decrease in the relative proportional area stained with the neonatal and embryonic MHC antibodies.

In the adult group, the fast antibody (Figure 10a) stained nearly all fibres in the masseter bellies although the colour of the AEC stain was often yellow brown rather than brick red, possibly due to a different pH of the tissue, or to inadequate chromogen substrate penetration or application. In these cases the muscle cytoskeleton between fibrils was more deeply stained and reddish in colour. In this group the neonatal and embryonic antibodies stained little or no extrafusal muscle cells, although the neonatal and embryonic antibodies did stain spindle fibres. The slow antibody exhibited a similar pattern to the neonatal and embryonic antibodies with large areas of clear unstained muscle cells, However, there were a few single cells which stained for slow MHC.

The six week control group (Figure 13) exhibited a similar staining pattern to the adult group while the six week experimental group (Figure 14) revealed discreet areas of neonatal and slow MHC stained cells. The embryonic MHC antibody, on occasion, stained groups of cells in a similar discreet fashion although this was not as consistent an observation as was revealed for the slow and neonatal MHC antibodies in the experimental group.

The H&E staining (Figure 15) allowed the observer to quantify changes in fibre diameter as well as observe a

progressive decrease in the percentage of area occupied by interstitial tissue, nerves and blood vessels from gestation to three weeks of age. The changes reflect the increase in the myofibre size with age and at low magnification the increase in muscle girth was also noted between groups of increasing ages. It was clear from microscopic examination of the tissue sections stained with the IIM antibody that superfast myosin is not present in the Sprague-Dawley rat masseter muscle. Tissue sections from the three bellies of the experimental and control groups were stained with IIM antibody and showed no difference in staining patterns when compared to the control (no primary antibody) slides.

4.6 Fibre type distributions

Table 3 shows the proportions of different fibre types present in each group. There was a significant change (p < 0.01) in the proportion of MHC between the gestation age group and the three day age group, due to a decrease in positively stained embryonic, neonatal and slow fibres and an increase in positively stained fast fibres. A significant difference (p < 0.01) also existed between the three day age group and the three week age group and between the three week and the six week control groups with a further increase in fast fibres at the expense of neonatal and slow fibres. There was no significant change between fibre type proportions of the six week control group and the adult age group (p > 0.18) indicating that the fibre type proportions are relatively

mature at six weeks of age in the Sprague-Dawley rat. However, there was a significant difference in the proportion of fibre types between the six week control and experimental groups (p < 0.01), in that there was a persistence of embryonic and neonatal fibres and a reappearance of slow fibres in the experimental enameloplasty group. By comparsion the superficial masseter muscle from the left-hand (non-enameloplasty) side of one six-week-old experimental rat, did not show persistence of embryonic or neonatal fibres or reappearance of slow fibres which were observed in the right masseter muscle (enameloplasty side).

4.7 Observations of behaviour

After decoding all the observations made in double blind fashion, it was determined that the control rat had no difficulty in incising the rat chow biscuits, ingesting the food, masticating it and swallowing it. Mastication was coincident with the control rat lifting its head from its lowered position near a large chunk of the food and chewing the food with a propalinal movement of the mandible. For each bolus of food, which was passed from forelimbs to mouth, there was approximately 10-20 chewing cycles prior to swallowing. This habit proceeded without significant change over the four day observation period. The experimental rat was less inclined to incise the rat chow biscuits from the roof of the cage. However, whilst the incising and breaking up of a chow biscuit by this rat was less frequent than control rat, on the

occasions it was done there seemed to be little difficulty in incising the biscuits. The experimental rat seemed to observe a "pecking order" and on two occasions was observed to engage in a tussle with the control rat for food particles. The experimental rat was also observed to forage for food on the floor of the cage whilst the control rat was not observed to follow this behaviour. The control rat was also more inclined to incise at a strip of paper which was held at the top of the cage. Both rats groomed each other at various times. The experimental rat was observed to engage in ancillary oral behaviour, especially self-grooming, on a greater number of occasions than the control rat. The control rat more frequently interspersed grooming of the body fur with feeding. Licking and combing of the fur, using the tongue and incisor teeth, followed an upward movement of the head across the fur. No definitive difference in oral function could be deduced from this observational study (see Table 4), and no difference in the relative overall health of the two rats was noted.

4.8 Occlusal reduction (enameloplasty) procedure

There was a significant difference in the maxillary molar crown height of the experimental versus control group of teeth (Table 5). The difference indicated that the height of the crown (measured from the apical foramen) was much smaller in the enameloplasty group at the time of sacrifice (Figure 16).

4.9 Summary of results

The average body weight of the rats from three weeks to

adulthood increased as expected. There was no difference in body weight between the experimental and control groups. A concomitant increase in the fibre diameter was observed and recorded from gestation to adulthood reflecting the maturation of each age group of animals. However, there was a significantly smaller fibre diameter in the six week old experimental group ($p < 0.01$). A significant fibre size difference existed between the superficial, anterior deep and posterior deep bellies of the masseter muscle in both experimental and control groups ($p < 0.01$). In the experimental group the superficial muscle fibre diameter was smaller than the deep posterior muscle fibre diameter which, in turn, was smaller than the deep anterior muscle fibre diameter.

The proportion of fibre types present in the different age groups clearly showed significant changes. The proportion of neonatal and embryonic MHC, for instance, was highest in the gestation group and gradually decreased in the older groups of rats until at four months of age no embryonic and very little neonatal MHC were recorded. Conversely in this adult age group there was positive labelling of fast MHC in 100% of the fibres which reflected the normal increase observed from gestation through to adulthood in fast MHC and the decline of embryonic and neonatal MHC in the same time period. Slow MHC similarly progressively declined from gestation through until the adult age. There was a significant change in the proportions of fast, slow, neonatal and

embryonic MHC present between the control group of rats (which approximated the adult proportions) and the six week experimental group of rats. The experimental group demonstrated an increase in fibres stained positively for slow, neonatal and embryonic MHC. This adjustment was found in each of the three muscle bellies studied and clearly suggests a change in structural myosin components of the muscle as a result of a change in rat occlusal mechanics. The significant difference in the proportion of fibre types between the six week control group and the six week experimental group reflected a persistence of embryonic and neonatal fibre types and the reappearance of slow fibre types. The statistically significant reduction in mean fibre diameter and its distribution recorded in the six week experimental group compared to the six week control group of rats also indicates a change in the function of the masseter muscle produced by the occlusal adjustment procedure.

5.0 DISCUSSION

This study quantified the relative proportion of embryonic, neonatal, slow adult and fast adult myosin heavy chain during the normal development and growth of the masseter myofibre population in the Sprague-Dawley rat. Subsequently, the impact of an induced change in maturation of mastication, produced by enameloplasty between three and six weeks of age, on myofibre growth and MHC expression was assessed. The results of characterizing normal development supported previously reported findings and the general trend of myosin transition agreed with the literature. For example, d'Albis et al. (1986) reported that in the adult Wistar rat masseter muscle, there is between 0 and 30% of neonatal MHC and that the development transitions of myosin in the rat masseter, when compared with the skeletal muscles of the same animal, showed a delay in the appearance of the adult MHC as well as in the disappearance of the neonatal MHC in the masseter muscle.

A similar maturation of MHC profiles occurred in all three of the muscle bellies studied, although the anterior deep belly had more slow MHC compared to the other two bellies. The fibre diameter of the masseter muscle was observed to increase from gestation to four months of age as expected and in agreement with the literature on age-related changes (eg. Ontell and Dunn, 1978; Anderson et al., 1988). The observed decrease in the percentage of area occupied by

interstitial tissue, nerves and blood vessels from gestation to three weeks of age, reflected the increase in the myofibre size as well as the development of secondary myofibres in the enlarged muscle girth after the gestation period.

The experimental group of rats, which underwent occlusal reduction of their right maxillary molar teeth, revealed a significantly different fibre type profile to the control group of age-matched rats (see Table 3). The delayed fibre growth accompanied a change in distribution of muscle fibre diameter in all three muscle bellies. The persistence of embryonic and neonatal MHC's may reflect a change in the type of activity of the mandible during mastication, and the observation supports the hypothesis that function is an important factor in the progression of MHC's to their more mature fast adult state. The reappearance of slow MHC's, in the experimental group in addition, suggests that there was likely an increase in oxidative metabolism of fast oxidative glycolytic fibres and an accompanying decreased proportion of fast glycolytic fibres, since this is a typical sequel of a functional change which requires a relative increase in the duration of muscle work by those fibres. The persistence of staining for embryonic and neonatal MHC also suggests that the expression of those MHC's, in particular, are maintained by different types of activity (including suckling and unilateral mastication) while they are disallowed by maturation which is typified by bilateral mastication as the primary mode of

mandibular movement during feeding. This information is new to the literature, since developmental changes are primarily not perturbed after three weeks of age when mononeuronal innervation of fibres and adult usage and sexual development patterns have been established. It is also possible that there was an increased use of the tongue and buccinator on the side of occlusal reduction in order to position the food between the molar teeth. Such an adjustment in masticatory behaviour may have prevented noticeable change in the feeding patterns as observed, or as noted by the lack of change in weight gain in the experimental group. Unfortunately, tongue and buccinator fibre diameter or MHC expression were not examined to test the idea that an operational compensation by other muscles was involved during the response to enameloplasty. As well, the strength of observing precisely the same cells over several serial sections, as applied to immunohistochemistry (Anderson et al., 1988) was not fully utilized in this study.

Previous reports (Maxwell et al., 1980; Faulkner et al., 1972) have shown that a decrease in masticatory activity in monkeys is correlated with a decrease in oxidative capacity of the jaw closing muscles. Electromyographic studies, using edentulous Rhesus monkeys, have shown that the masseter muscles are less active during the resting posture of the mandible and during mastication. It could not be determined, however, whether the low level recruitment in maintaining mandibular posture or the high level recruitment required in

high force activities such as mastication contributed more significantly to the decrease in percentage of Type I (slow) fibres and a corresponding increase in Type IIB (fast glycolytic) fibres in those studies.

Other work in the literature (d'Albis et al., 1986) has reported an atypical presence of neonatal forms of myosin in the murine adult masseter muscle suggesting that myosin expression in this muscle is subject to unusual regulations. A very large variation in fibre type proportions of slow or neonatal fibres, for example, from one mammal to another is somewhat uncommon and quite possibly reflects a degree of muscle adaption to specific functional requirements in each species. For instance, the masseter of the guinea pig is made up of only Type IIA (fast oxidative glycolytic) fibres. The masseter of the rabbit consists of Type I, IIA and IIB fibres while the corresponding muscle of rat and mouse consists of Type I and Type IIA fibres (d'Albis et al., 1986) and according to Easton and Carlson (1990) the superficial masseter muscle of the Sprague-Dawley rat predominently consists of Type IIA and Type IIB fibres. Predatory animals, such as the cat, have a high percentage of IIM (superfast) fibres while the masseter of beef cattle, which masticate slowly, has only slow Type I fibres. However, the present study did not find very large between-animal variations in MHC profiles except in the six week experimental group.

The myosin composition of the masseter in these different

animals is thought to be due to the different patterns of functional use in the different animals. For instance, it has been noted that in addition to small amounts of slow myosin, rabbit masseter contains three fast isoforms while the same muscle of the dog, a predator, contains only one fast myosin which may be different from the three species present in rabbit (Shelton et al., 1985). Evolutionary constraints may also play some role in these differences. As well, the known technical difficulties (lack of consistency) in every enzyme histochemical study and different pH labilities between different animals and among different muscles, even within one animal, should also be remembered when interpreting reported species differences which have been analyzed without the use of immunohistochemical methods.

Masticatory patterns and function, in general, therefore can be seen to influence myosin isoform transition in the masseter muscle. That muscle is of branchiomeric origin and is innervated by the mandibular branch of the trigeminal nerve, a cranial nerve, rather than a skeletal motor nerve. While this study did not undertake a comprehensive examination of the contralateral masseter muscle, previous literature (d'Albis et al., 1986) did not observe any difference in the content of neonatal myosins between paired contralateral masseter muscles. In the present study the masseter bellies, contralateral to the occlusal adjustment side, did not show persistence of the neonatal, embryonic or slow fibres observed

ipsilaterally suggesting that the adjustment in function and fibre structure was largely unilateral and ipsilateral to the change in occlusion. This study did not use a sham occlusal adjustment group to control for possible manipulative trauma. Such an addition would have more clearly shown the specific impact of the adjustment procedure itself.

The timed course of maturation of the postnatal myosin pattern of rat masseter muscle demonstrates long delays both in the appearance of adult type myosin and in the disappearance of neonatal type myosins (d'Albis et al., 1986) when compared with skeletal muscles such as the EDL and the sternomastoid muscles. The transitions observed in the latter two muscles are more rapid and complete than those described for other leg muscles (Whalen et al., 1981). A more comprehensive analysis of the contralateral masseter muscle in this study would have enabled comparison with the extensively studied ipsilateral masseter muscle in terms of the correlation between the degree of occlusal change and its effect on MHC proportion or fibre diameter. Nor is it clear how long such a change would have persisted. Specifically, if the experimental group had been sacrificed at 10 weeks or 15 weeks of age (as opposed to 6 weeks of age) the muscle fibre composition may have adapted to reflect its genetic pattern and the neonatal and slow MHC may not have persisted that long. Such an investigation should be blinded so the investigator would not have a bias when analyzing the MHC

composition. However, in a 10-15 week old rat, mastication is the primary mode of mandibular movement during feeding and perhaps the change that this study revealed in the developing rat would not manifest in a completely mature animal.

This study did not undertake a large scale blinded behavioural study. An observational analysis, such as this, could have been supplemented with electromyographic analysis indicating activity levels in the ipsilateral and contralateral masseter muscles. Although no overt pain responses were noted, the possibility of inflammatory cell indicators of pulpitis cannot be overlooked. However, large or moderate increases in neutrophil and lymphocyte numbers were not observed in the tooth sections from the enameloplasty group compared to sections from the control group.

Altering the diet early in the maturation of a mammal shifts the muscle fibre composition so that smaller muscle fibres and a decreased proportion of Type I muscle fibres remain. Therefore a comparison of the delay in MHC maturation and morphometric development of muscle fibres following the administration of hard or soft foodstuffs or of a liquid only diet during the maturation of the animal would have allowed study of changes following an imposed pattern of feeding and correlated with different ages.

As it is known that fibres types begin to differentiate concurrently with the arrival of axons in the muscle and with the formation of neuromuscular junctions, the unilateral

denervation of the masseteric nerve in a developing rat would have allowed analysis as to whether such denervation delays or accelerates the maturation of MHC isoform in the masseter muscle. As it takes about 1 1/2 weeks for the nerve to regenerate (Carlson, personal communication) such analysis would be restricted to a relatively short time span unless the nerve was prevented from reaching the muscle. A more sophisticated approach would be the cross-innervation of the masseter muscle by a skeletal muscle nerve although this would be technically unfeasible unless a limb was sacrificed. As jaw closing muscles arise from myoblasts of the presomites in the paraxial mesoderm (Noden, 1983) and limb and trunk muscles are derived from somites, it would be interesting to observe the degree to which the cross-innervation by the opposite type of motor nerve influences the expression of myosin isoform type.

The results of the present study reinforce the concept developed in previous reports that the muscles of mastication and, in this case the masseter muscle specifically, are probably unique in terms of development, myosin isoform expression and response to various factors. That neonatal myosin persists in rat extrinsic ocular muscles which are also innervated by cranial nerves (Wieczorek et al., 1985) suggests that innervation of branchiomeric muscle by cranial nerves presents a distinct set of limits on the regulation of myosin isoform expression in those muscles.

Mascarello and Rowlerson (1992) noted the slower or less

complete replacement of neonatal myosin with adult myosin in rat masticatory and extra-ocular muscles using immunohistochemical methods. That report concluded that the slower process of neonatal myosin replacement in some of the fibres could not be attributed to a late onset of development.

The finding in the present study, of a decrease in fibre diameter following what was effectively the unilateral removal of the posterior dentition is in agreement with previous literature on disuse (Maeda et al., 1987; Miller, 1991). The occlusal adjustment procedure presumably led to an immediate decrease in the maximum tension developed by the right masseter muscle. The decrease in tension could have occurred concurrently with a decrease in the duration of maximum activity (with an increase in the work done by the contralateral occlusion) and consequently the persistence of the more immature MHC's. It is also possible that the mandible was repositioned in a compensatory move to re-enable force generation during mastication and that the repositioning, itself, caused increased demands on some fibres which responded by expressing slow MHC. The statistically significant difference between the control and experimental group, in terms of their MHC profile and fibre diameter, also could be due to the lack of function which delays the replacement of neonatal isoforms with the adult isoform. Perhaps the maturing muscle " attempts" to make more efficient use of the available metabolic substrate by maintaining the

neonatal MHC isoform and restricting the morphometric growth of the muscle fibre. As neonatal muscles have slower velocities of shortening and relaxation than adult muscles (Close, 1972) and since neonatal and slow myosins have lower energy costs in the production of work (Crow and Kushmerik, 1982), the maintenance of both slow and neonatal MHCs can be understood. Miller (1991) reported that following the removal of the dentition in the Rhesus monkey the composition of the masseter muscle shifts with more Type IIB fibres emerging and fewer Type I fibres. The cross-sectional area of the Type I fibres also becomes smaller. The mechanism for such change probably lies in the pattern and activity of the motor neuron in relation to its muscle fibres within its motor unit. Pette and Vrbova (1985), for instance, suggest that the activity of a motor neuron, innervating its muscle fibres, is the dominating factor in controlling the gene expression of the mammalian muscle fibre. This proposal is based on observations that direct electrical stimulation, at least of a limb muscle at low frequency, alters the myosin contractile protein from Type II to Type I (Salmons and Sreter, 1976).

Alternatively a decrease in tension may have produced an increase in the duration of maximum activity and hence increased contraction times, increased work and the persistence of slow MHC, a process which is analogous to endurance training. However, the decrease in fibre diameter of the experimental groups masseter muscle argues against this

second alternative. Although the diameter of different fibre types was not separated, the increased use of slow fibres would have produced a less clear cut change in fibre diameter towards a small dimension.

So whether the decrease in fibre diameter is due to disuse and posturing of the mandible or to a lack of activity due to unilateral mastication with the contralateral muscle and dentition, the results in this study are consistent with previous reports in the literature. Bredman et al. (1992) described the various MHC isoforms in rabbit masseter muscle during postnatal development. This study's finding of cardiac alpha MHC in the rabbit masseter muscle fibres, supports the hypothesis that individual fibres may, by varying their MHC content, adapt their contraction speed to the conditions in the muscle during development. As cardiac alpha MHC is present in both cranial and masticatory muscles of adult rabbit, it is probable that embryonic neural crest cells play a role in inducing the expression of specific MHC's in skeletal muscle. This study shows that immunohistochemistry may be particularly useful in the quantitative and dynamic assessment of muscle function following dental procedures such as orthognathic surgery and growth modification orthopaedic-orthodontic techniques, presuming that baseline assessment is also made prior to treatment. Changing the function of the craniomandibular muscles can result in significant and early alterations in the muscle fibre profile primarily related to

the consequent activity of the muscle. The application of immunohistochemistry to define myosin contractile molecules has refined the enzyme histochemical techniques which were recently popular. Immunohistochemistry has the potential to become an important diagnostic tool in assessing craniomandibular muscle function in the adolescent and adult in respect to orofacial dysfunction and diagnosis.

A relatively objective means of assessing altered functional demands on the activity of a major masticatory muscle is thus outlined, which takes into account the morphometric and immunohistochemical profile of a normal and a perturbed system. Specifically, this study assessed the relationship between functional development and structural and MHC fibre type changes which occur in the masseter muscle following perturbation of the stomatognathic system of the growing rat.
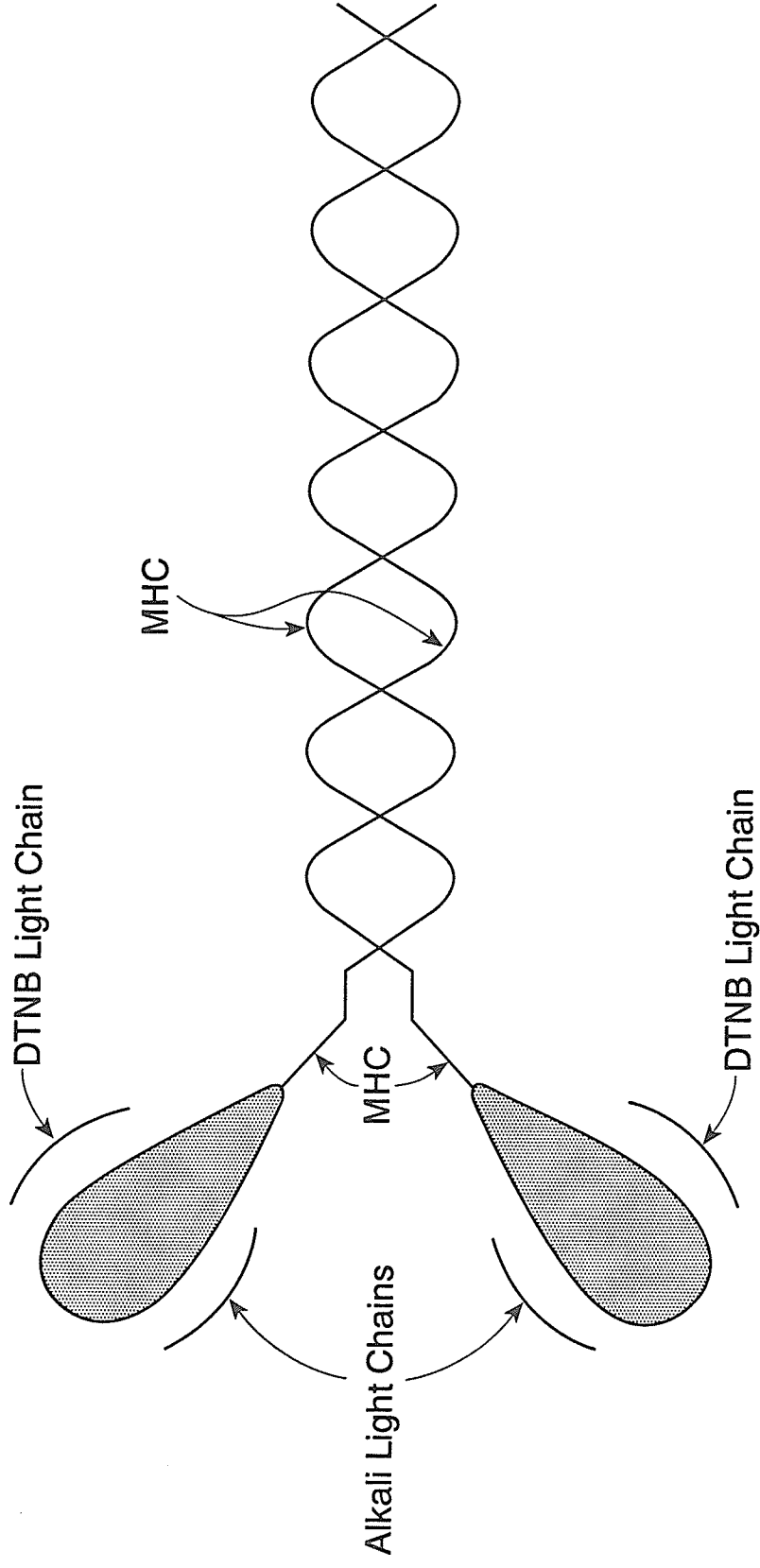
Figure 1

Schematic diagram of a myosin molecule consisting of two heavy chain subunits, two alkali light chains, and two DTNB light chains.
(After Bandman, 1985)
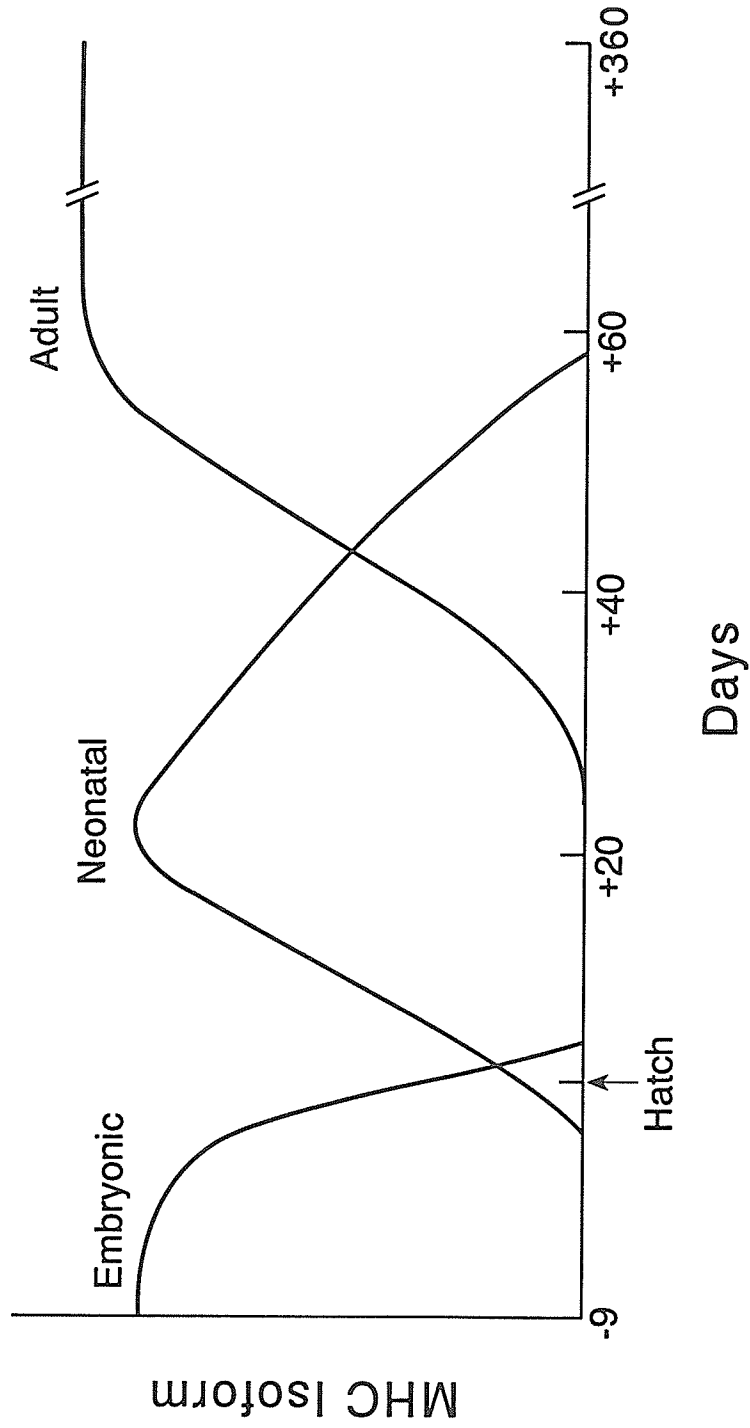
Figure 2
Diagram showing the expression of different myosin heavy
chains during development of the pectoralis major.
(After Bandman, 1985)

Slow   Slow   Slow

Emb→Neo→Fast
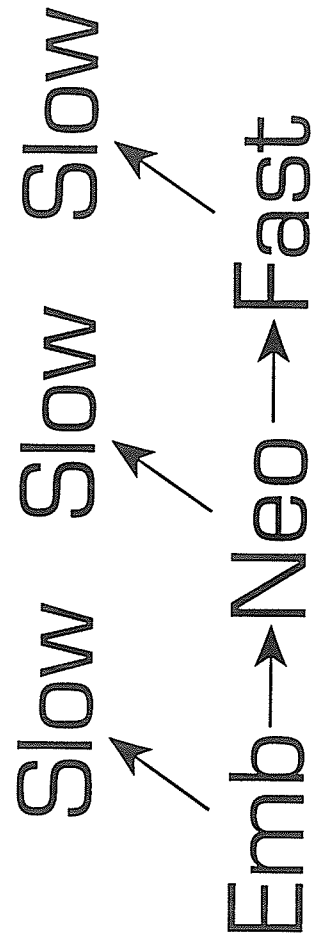
Figure 3

Diagrammatic summary of myosin transitions that occur during muscle development in the rat.

(After Butler-Browne and Whalen, 1984)
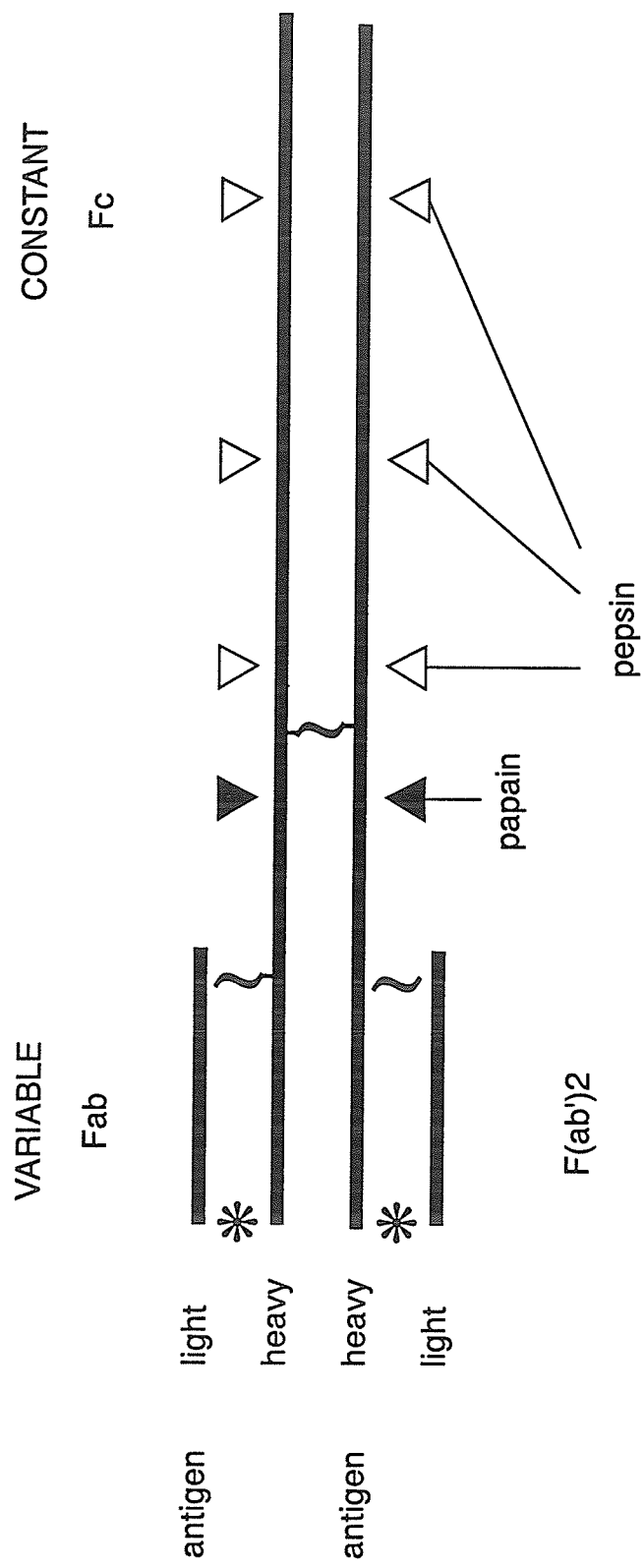
Figure 4

A simplified representation of an Ig molecule showing the antigen binding site and the sites of cleavage of papain and pepsin. ⟩ represents a disulphide bond.

(After Ormerod and Imrie, 1989)

2 (goat-anti mouse Ig)

1 (mouse Ig)

b

~ IgG

a
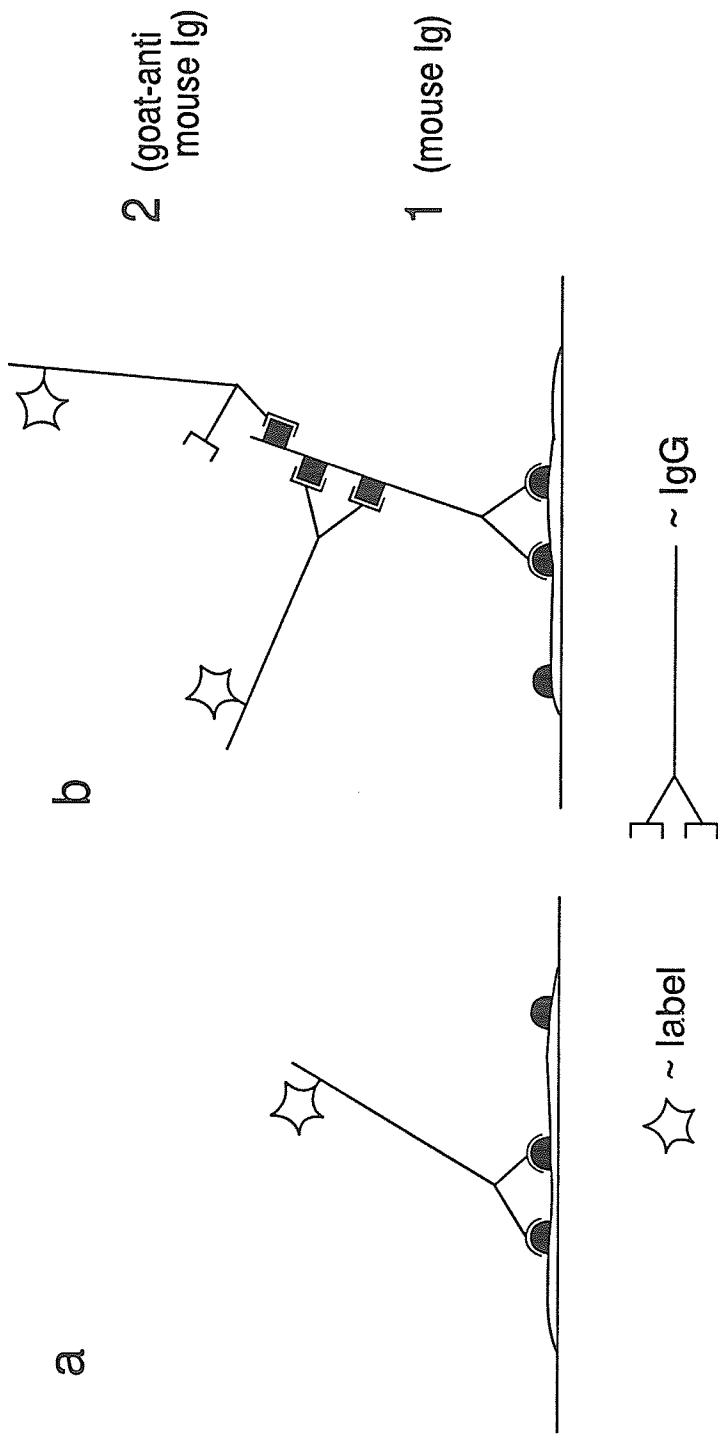
~ label

Figure 5

The (a) direct and (b) indirect methods of visualizing the reaction site of an antibody on a tissue section. 1, primary antibody; 2, labelled second antibody

(After Ormerod and Imrie, 1989)
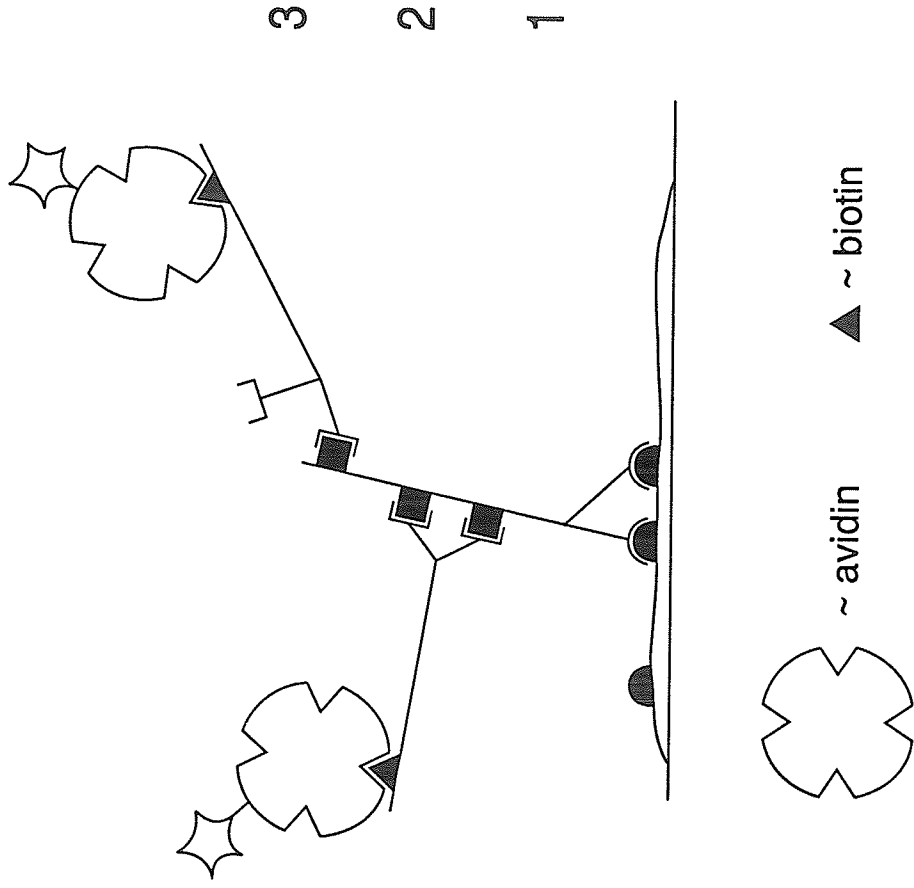
Figure 6

The biotin-avidin method. 1, primary antibody; 2, biotinylated second antibody; 3. labelled avidin

(After Ormerod and Irmie, 1989)

~ avidin   ▲ ~ biotin

Figure 7

PERIODS OF MYOGENESIS

Fertilization

| Somite Structures | Limb Bud Structures | Fetal Structures | Adult Structures |

Embryonic Myoblasts

Fetal Myoblasts

Satellite Myoblasts
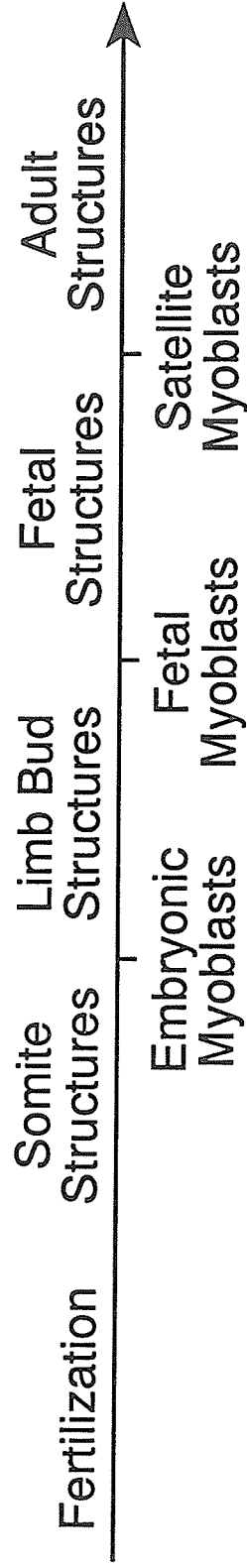
There are four periods of myogenesis in vertebrates

(After Stockdale et al., 1989)

Figure 8: Distribution of fibre diameter (mm) for different age groups.

a) gestation,

b) 3-day-old,

c) 3-week-old,

d) 6-week-old masseter muscle (3 bellies pooled): control versus experimental,

e) 6-week-old masseter muscle, superficial belly: control versus experimental,

f) 6-week-old masseter muscle, anterior deep belly: control versus experimental,

g) 6-week-old masseter muscle, posterior deep belly: control versus experimental,

h) adult, 3 masseter muscle bellies pooled, and

i) adult, 3 masseter muscle bellies separately plotted: anterior deep, posterior deep, and superficial.

Fibre distribution
gestation

frequency (%)

fiber diameter (mm)
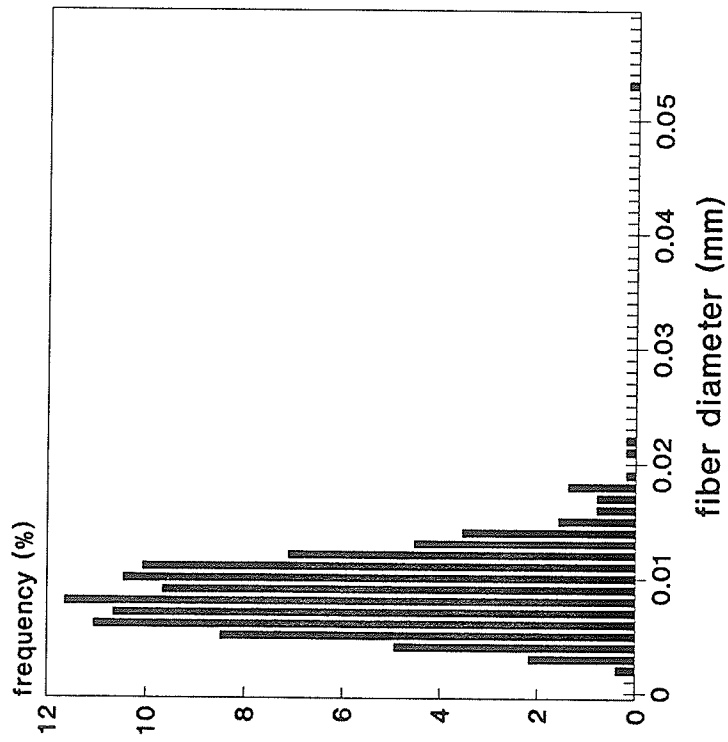
gestation

8a

Fibre distribution
3 day old

frequency (%)

fiber diameter (mm)
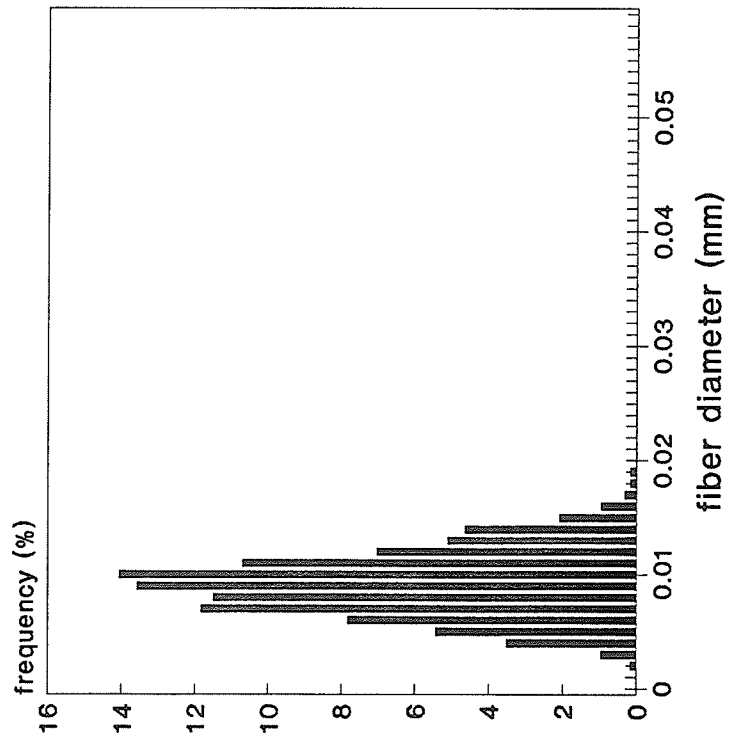
3-day-old

8b

Fibre distribution
3-week-old

frequency (%)

fiber diameter (mm)
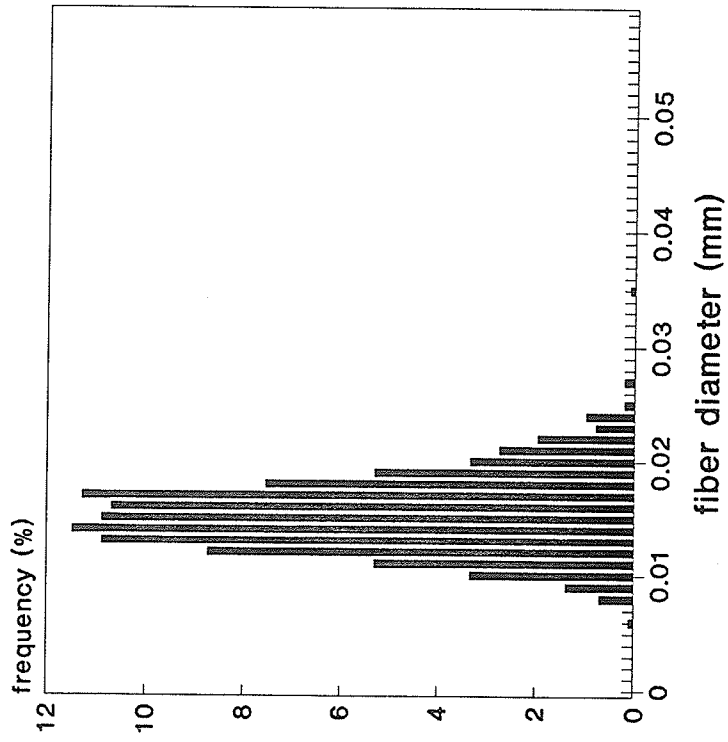
3-week-old

8c

Fibre distribution
6-week-old

frequency (%)

12

10

8

6

4

2

0

fiber diameter (mm)

0.01   0.02   0.03   0.04   0.05

control   experimental   8d
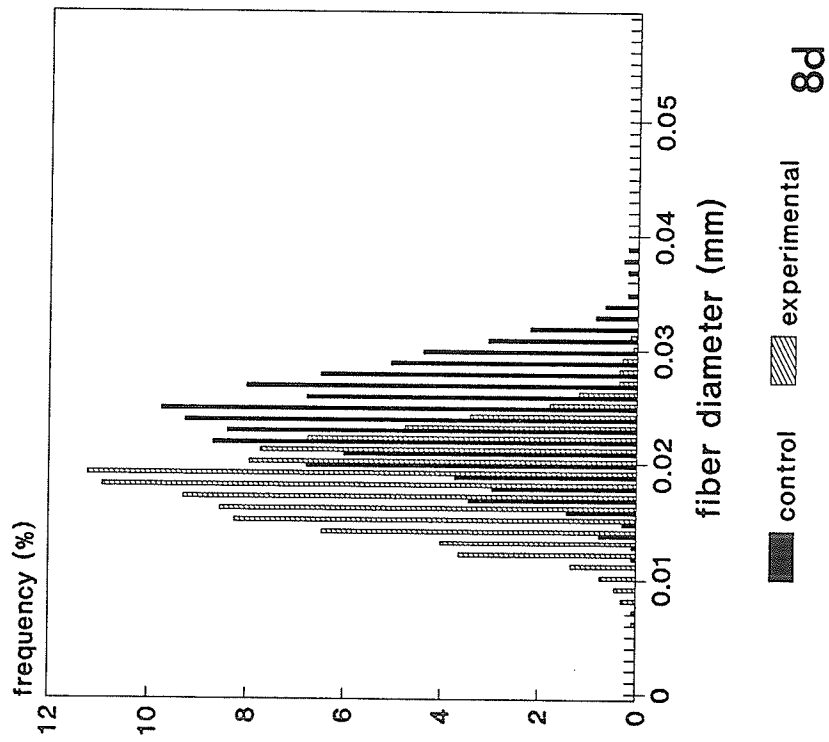
Fibre distribution
6-wk superficial

frequency (%)

fiber diameter (mm)
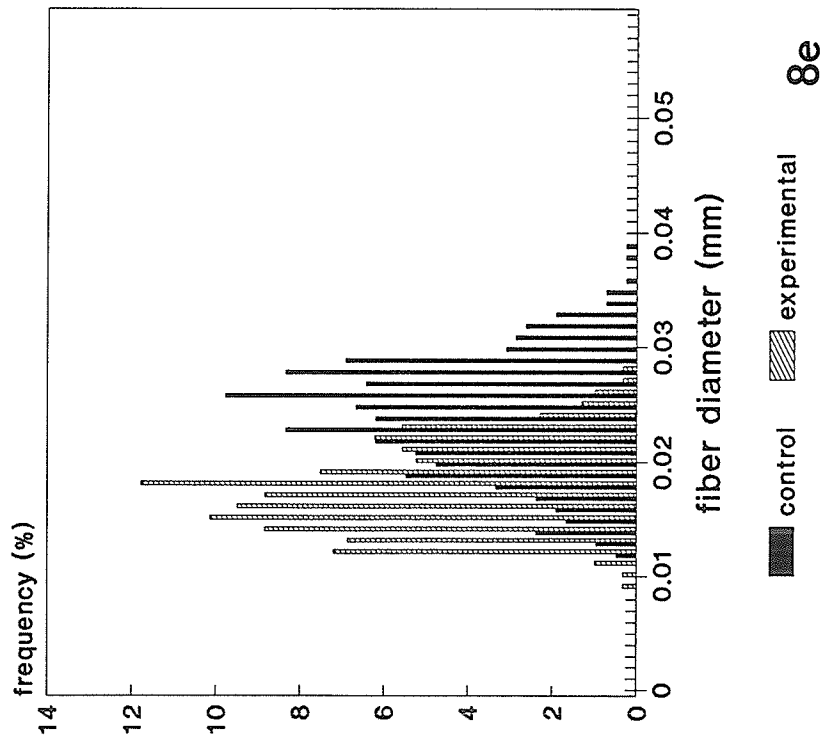
■ control ▨ experimental

8e

Fibre distribution
6-wk anterior

frequency (%)

fiber diameter (mm)

control   experimental

8f
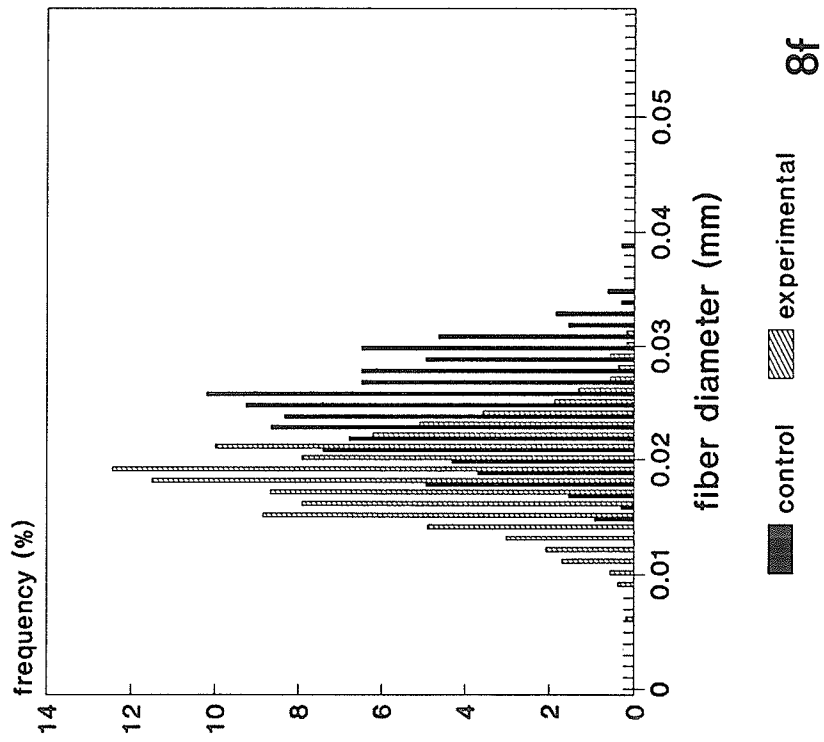
Fibre distribution
6-wk posterior

frequency (%)

fiber diameter (mm)

control ▦ experimental

8g

Fibre distribution
adult

frequency (%)

8
6
4
2
0

fiber diameter (mm)

0.01   0.02   0.03   0.04   0.05

adult

8h

Fibre distribution
adult

frequency (%)

10

8

6

4

2

0

0   0.01   0.02   0.03   0.04   0.05

fiber diameter (mm)

anterior   posterior   superficial

8i
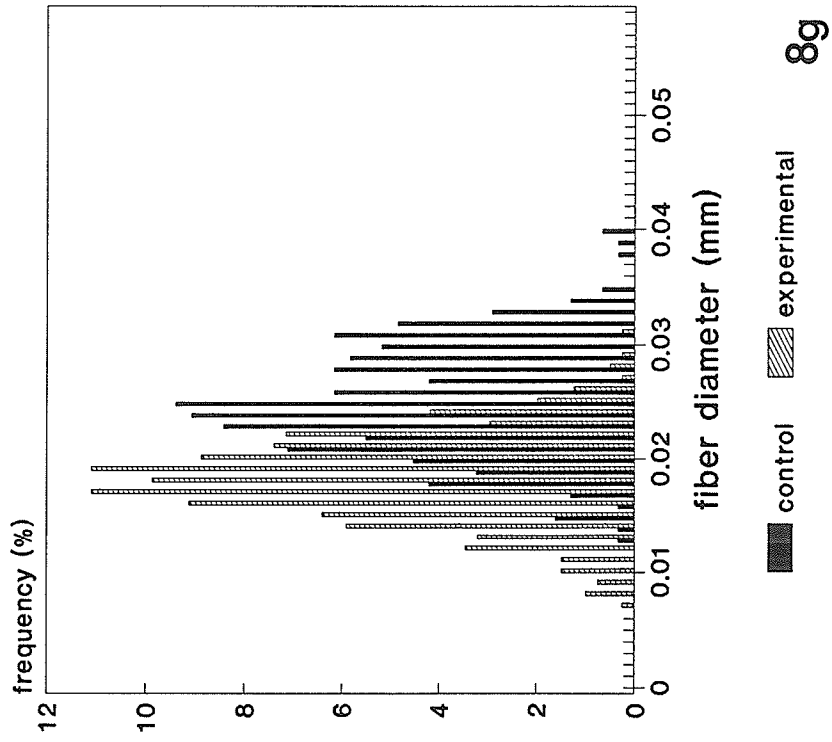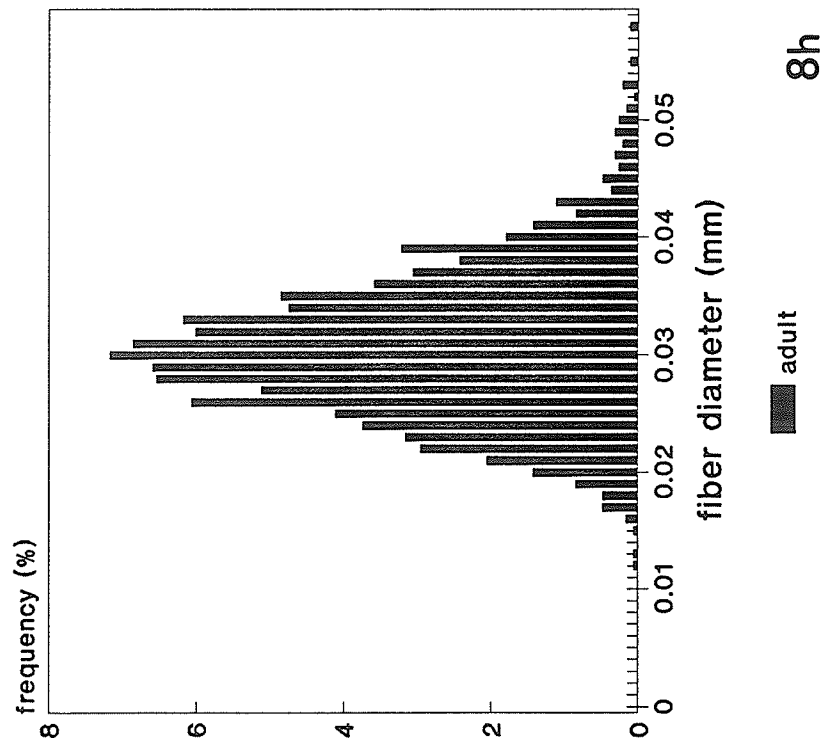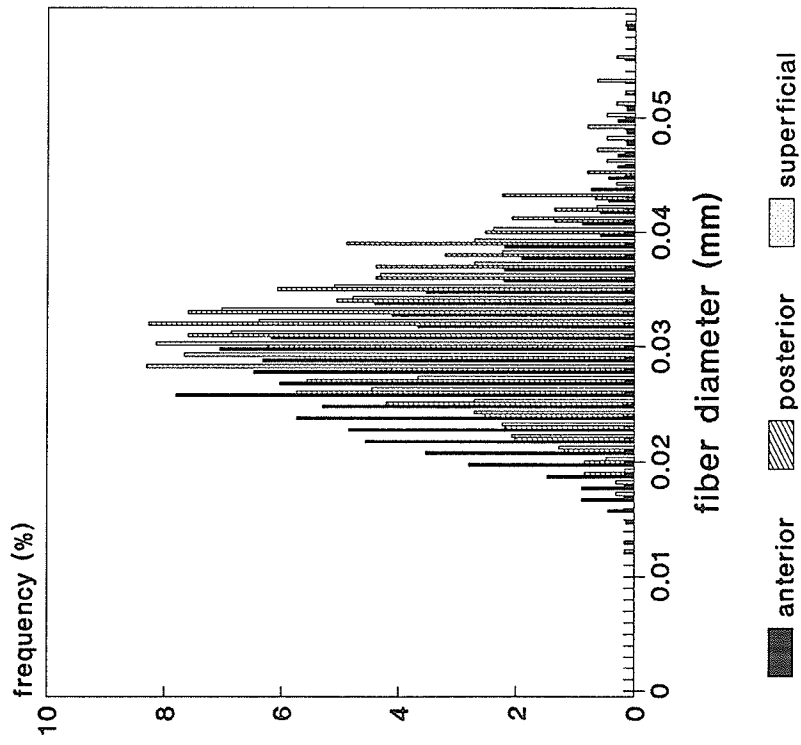
Figure 9: Dissection of masseter muscle bellies. The anterior of the mandible is to the left of the figure. (X7.5)

a) The superficial muscle belly is shown between the anterior tendon and the posterior angle of the mandible. The facial nerve lies along the superficial masseter.

b) The deep muscle belly is shown overlying the wooden applicator sticks.
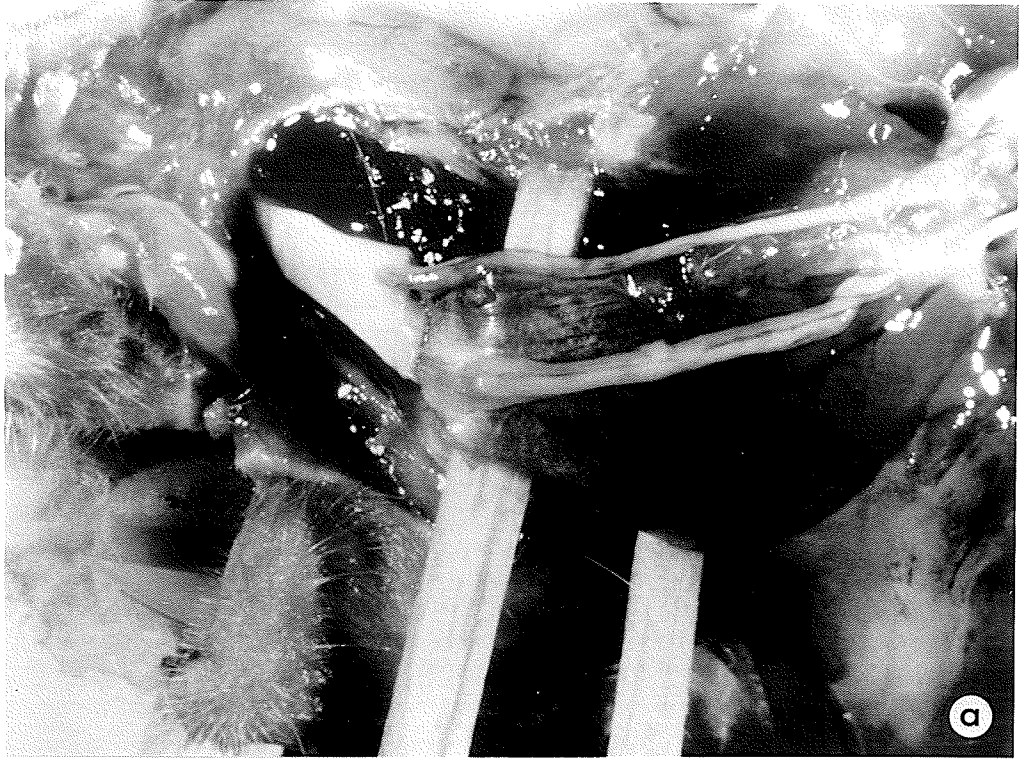
Figure 10: Micrographs of adult masseter muscle. (X120)

a) The section has been stained with anti-fast MHC, and shows uniform staining of all myofibres in the muscle.

b) During incubation of this section, the primary antibody was omitted. The myofibres show little or no staining in this negative control.
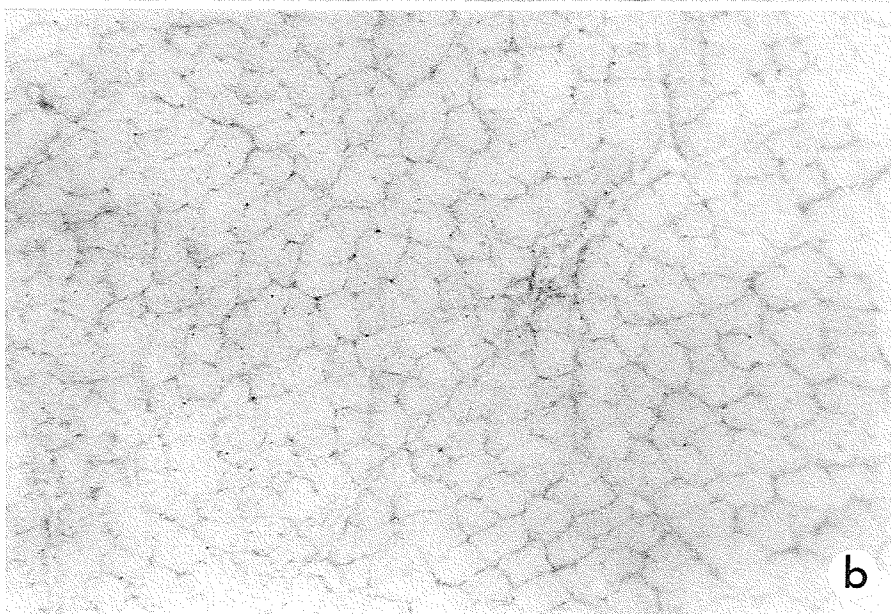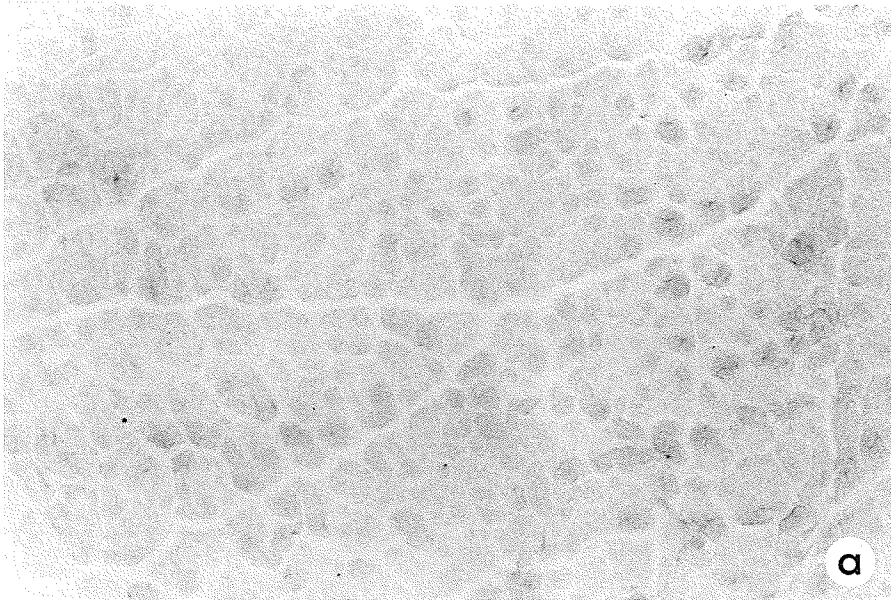
Figure 11: Micrographs of the masseter muscle from one of the rats at 20 days of gestation.

a) This section was stained with anti-fast MHC, and shows clumps of positively stained myofibres within the fascicles in this muscle belly. Each fibre demonstrates stain in a ring pattern, surrounding the central nucleus of the immature myofibre (myotube) present at this stage of development. (X120)

b) This section was stained with anti-slow MHC, and shows most fibres stain positively for this isoform. (X120)

c) This section was stained with anti-neonatal MHC. Occasional fibres stain positively for neonatal MHC, although the majority of fibres do not. (X120)

d) This section was stained with anti-embryonic MHC using the AEC chromogen. Many myotubes stain positively for the embryonic MHC isoform. The spaces between the fibre fascicles contain connective tissue at this stage of development. (X240)
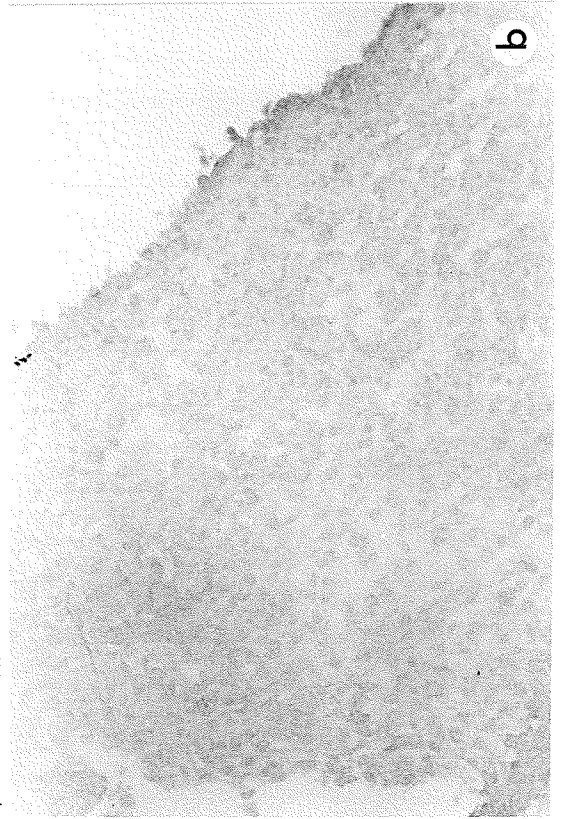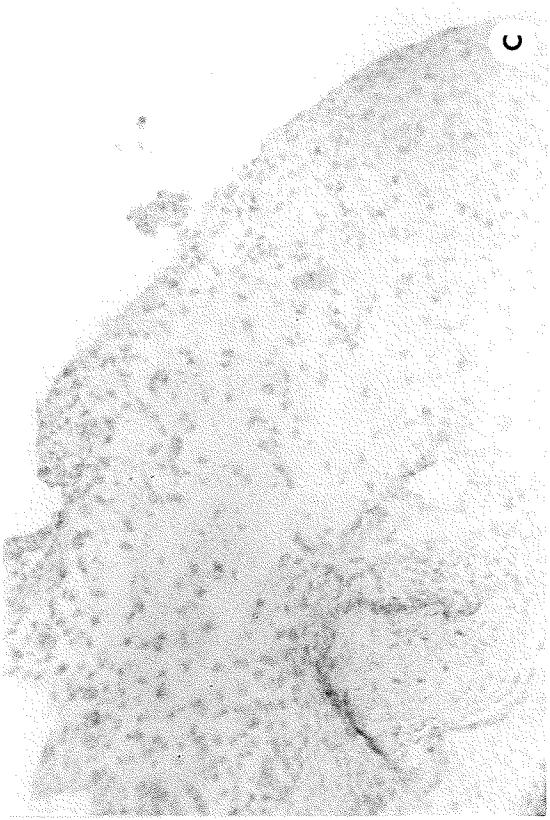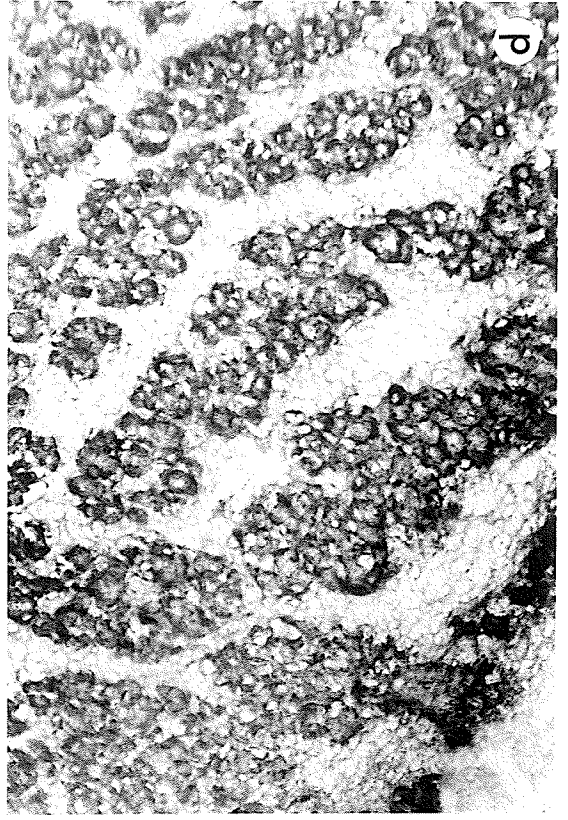
Figure 12: This section of the masseter muscle from a rat at 20 days gestation was stained with H&E, and shows the muscle spindle complex within the paler staining myofibre fascicles of the anterior deep belly of the masseter muscle (X120).
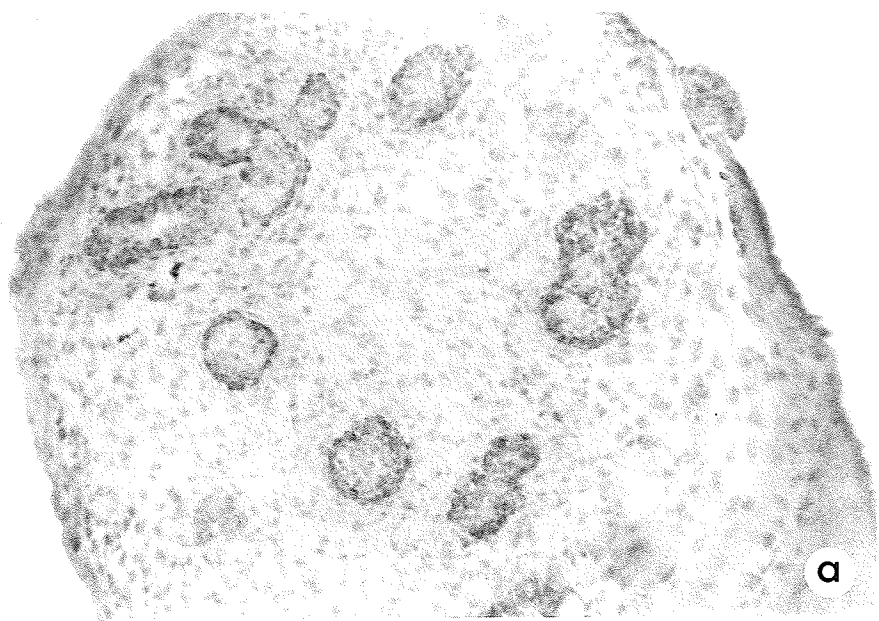
a

Figure 13: Micrographs of sections of the masseter muscle (superficial belly) taken from one animal in the 6-week-old control group. (X120)

a) This section was stained with anti-fast MHC, and shows that all myofibres in the belly are postively stained.

b) This section, stained with anti-slow MHC, demonstrates no positively stained fibres.

c) This section was stained with anti-neonatal MHC, and exhibits no positive staining of myofibres.

d) This section was stained with anti-embryonic MHC. There are no postively stained myofibres present.
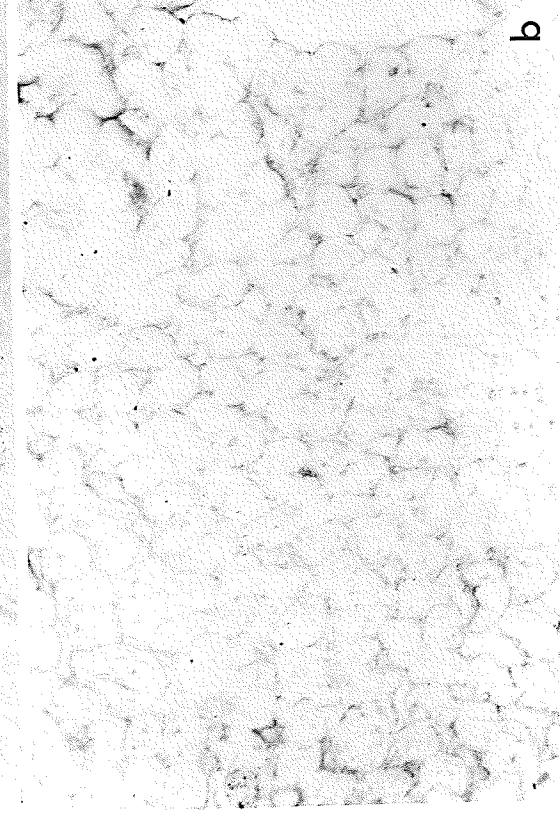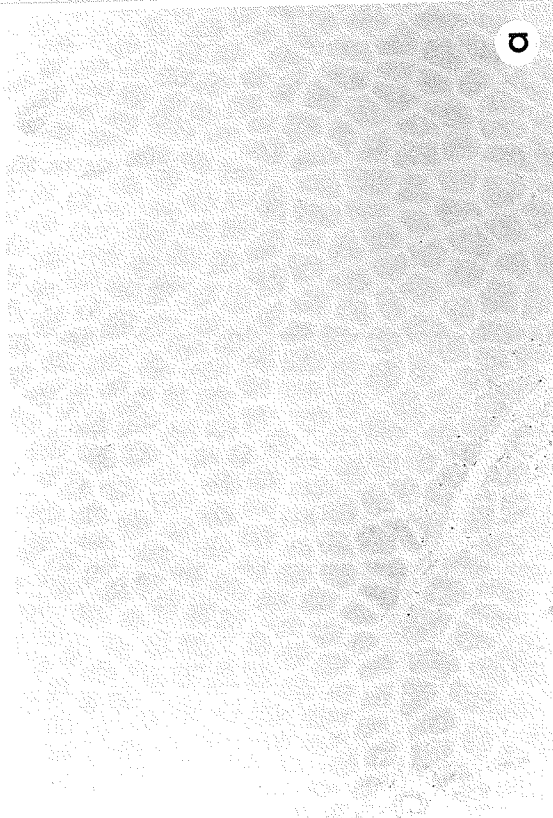
Figure 14: Micrographs of sections of the masseter muscle (superficial belly) taken from one animal in the 6-week-old experimental (enameloplasty) group. (X120)

a)This section was stained with anti-fast MHC, and shows a mosaic pattern of myofibre staining.

b)This section was stained with anti-slow MHC, and shows a number of positively stained myofibres.

c)This section was stained with anti-neonatal MHC, and shows a few foci of myofibres stained within the belly of the masseter.

d)This section was stained with anti-embryonic MHC, and shows two positively-stained myofibres in the section.
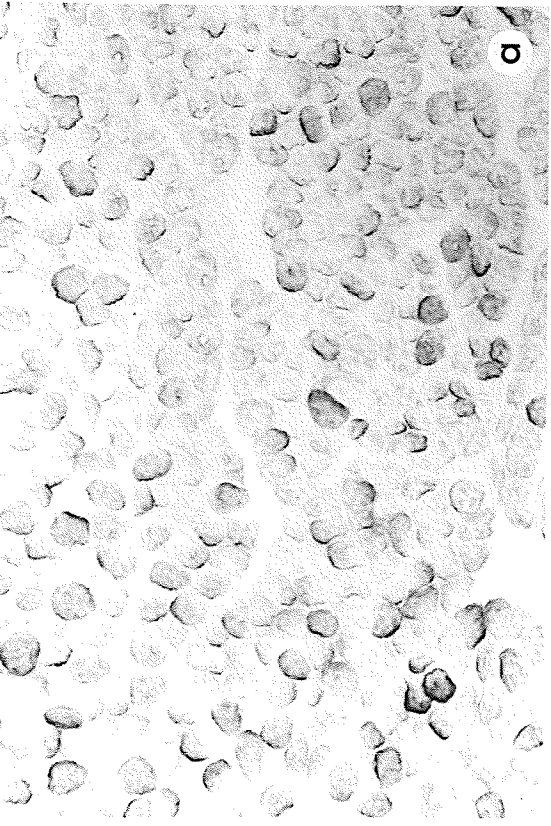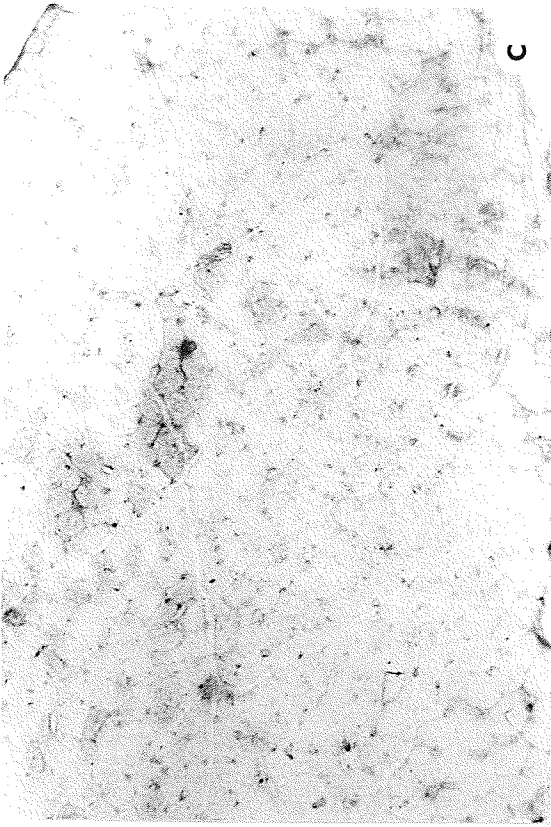
Figure 15: Micrographs of histological sections of masseter muscle stained with H&E (X120). The H&E stained sections were used for morphometry of fibre diameter.

a) This micrograph shows the superficial belly of masseter muscle from a 6-week-old control rat, and demonstrates the fascicular arrangement of myofibres, each with darker-staining peripherally located nuclei.

b) This micrograph shows the superficial belly of the masseter muscle from a 6-week-old experimental rat (enameloplasty group). The fascicular arrangement of myofibres and their peripheral nucleation are not different from the control group, but the fibres are generally smaller in size than observed in Figure 15a.
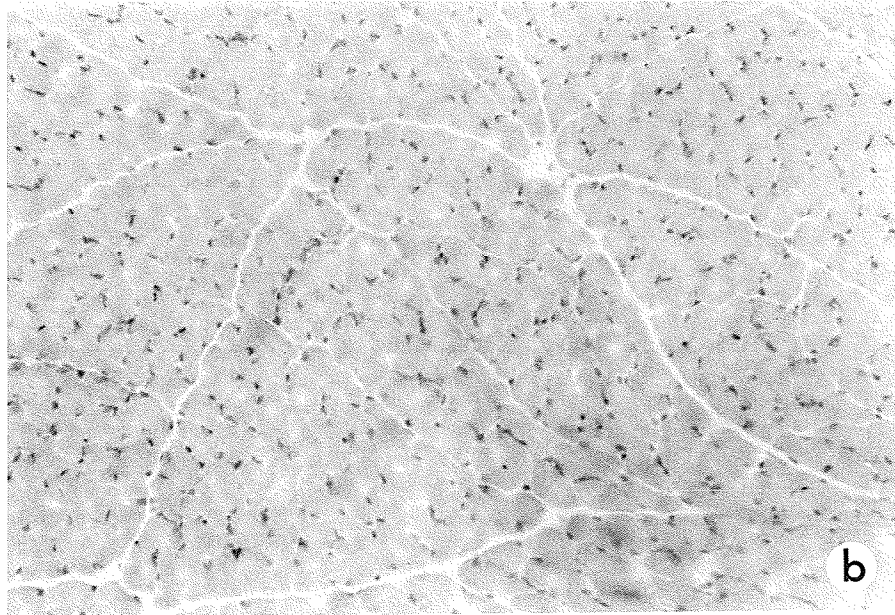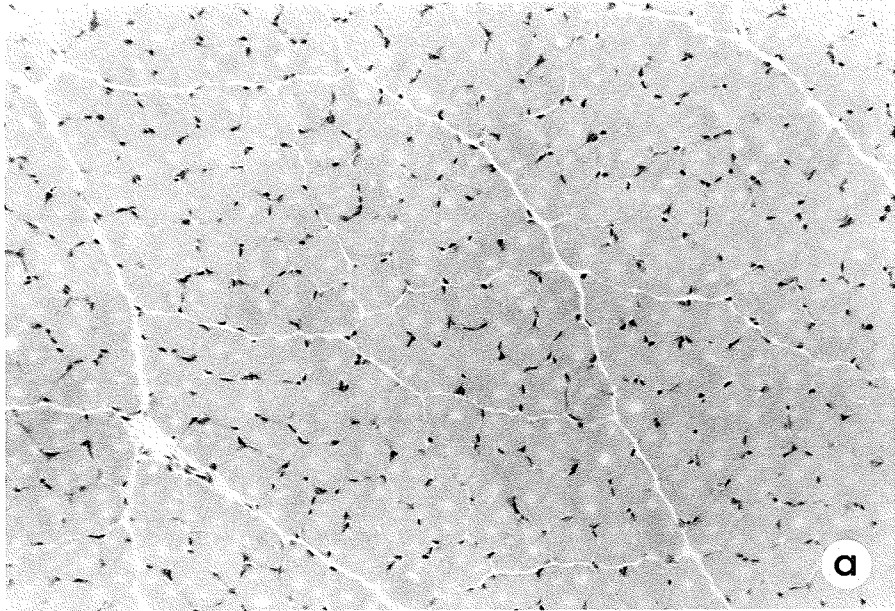
Figure 16: Micrographs of maxillary molar teeth in sagittal section, H&E stained (X16).

a) Occlusal surface of a molar from a rat in the 6-week-old control group, showing typical rounded tooth margins.

b) Occlusal surface of a molar from a rat in the 6-week-old experimental group, 6 days after the previous occlusal adjustment (enameloplasty). The surface is flattened in comparison with the molar seen in Figure 16a.

c) The apex of the molar in Figure 16b, demonstrating the location of the section used for morphometry of distance from the apical foramen to the mesial and distal aspects of the occulsal surface.

a

b

c
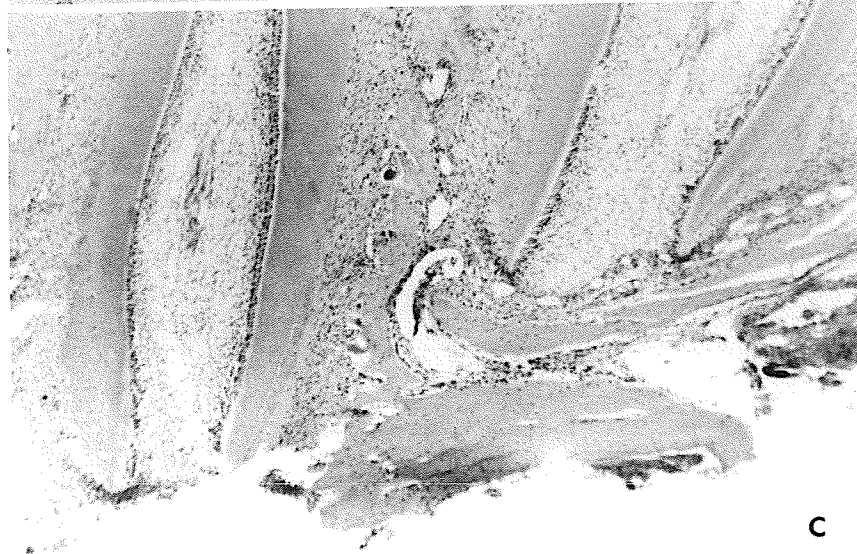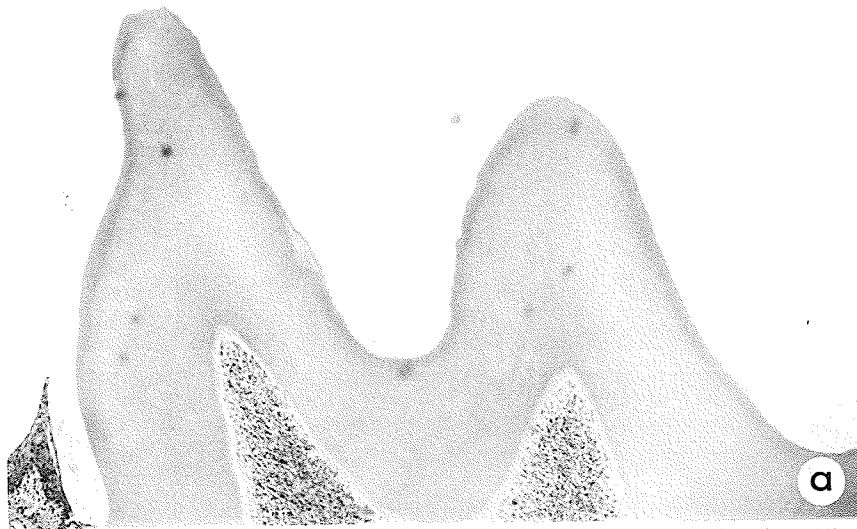
TABLE 1

DATA FOR BODY WEIGHTS OF RAT GROUPS

GROUP (n)	BODY WEIGHT IN GRAMS[a]
3 WEEK CONTROL (6)	43.3 ± 1.6
3 WEEK EXPERIMENTAL (6)	41.2 ± 1.4
4 WEEK EXPERIMENTAL (6)	76.7 ± 2.3
5 WEEK EXPERIMENTAL (6)	107 ± 2.5
6 WEEK CONTROL (4)	159.5 ± 4.1
6 WEEK EXPERIMENTAL (6)	160 ± 3.6
ADULT (4 MONTH) (6)	326.2 ± 7.5

a: data are mean ± standard error of the mean

TABLE 2

MASSETER MUSCLE FIBRE DIAMETER DATA FOR EACH AGE GROUP

GROUP (n)	FIBRE DIAMETER (mm)[a]
GESTATION (20 DAYS) (5)	0.0084 ± 0.0005
3 DAY (6)	0.0087 ± 0.0007
3 WEEK - SUPERFICIAL MASSETER (6)	0.0135 ± 0.0007
- DEEP ANT. MASSETER (6)	0.0151 ± 0.0008
- DEEP POST. MASSETER (6)	0.0153 ± 0.0007
6 WEEK CONTROL- - SUPERFICIAL MASSETER (3)	0.0245 ± 0.0009
- DEEP ANT. MASSETER (3)	0.0255 ± 0.0007
- DEEP POST. MASSETER (4)	0.0246 ± 0.0008
6 WEEK EXPERIMENTAL - - SUPERFICIAL MASSETER (4)	0.0172 ± 0.0005
- DEEP ANT. MASSETER (5)	0.0180 ± 0.0006
- DEEP POST. MASSETER (4)	0.0176 ± 0.0004
ADULT (4 MONTH) - - SUPERFICIAL MASSETER (6)	0.0317 ± 0.0014
- DEEP ANT. MASSETER (7)	0.0290 ± 0.0015
- DEEP POST. MASSETER (6)	0.0309 ± 0.0004

a: data are mean ± standard error of the mean

TABLE 3

PROPORTION OF MASSETER MUSCLE FIBRES STAINED POSITIVELY FOR NEONATAL-EMBRYONIC, NEONATAL, SLOW, AND FAST MYOSIN HEAVY CHAIN (MHC) IN EACH GROUP.

RAT GROUP		NEONATAL-EMBRYONIC	NEONATAL	SLOW MHC	FAST MHC
20 DAYS GESTATION		(5)[a] .530±.124[b] 930[c] 1695[d]	(5) .632±.122 1210 1845	(5) .274±.084 486 1875	(5) .593±.070 1065 1875
3 DAYS		(5) .205±.023 290 1400	(5) .232±.096 230 935	(5) .190±.035 193 1040	(5) .841±.058 1065 1240
3 WEEKS	SM	(5) .023±.011 18 730	(5) .119±.058 168 987	(5) .041±.025 40 760	(5) .790±.085 665 865
	DAM	(5) .074±.039 66 825	(5) .189±.069 137 721	(5) .065±.032 56 730	(6) .742±.082 540 775
	DPM	(5) .110±.035 69 605	(4) .154±.062 96 610	(5) .077±.034 39 512	(6) .731±.112 600 805
6 WEEK CONTROL	SM	(2) .010±.010 4 520	(3) .023±.015 12 500	(3) .043±.022 36 730	(4) .950±.050 980 1020
	DAM	(2) 0 ± 0 0 590	(3) .015±.015 9 440	(2) 0 ± 0 0 520	(2) .900±.100 480 500
	DPM	(2) 0 ± 0 0 350	(3) .032±.016 25 630	(2) 0 ± 0 0 320	(2) 1.00±0 435 435

CONTINUED ......

6 WEEK EXPERI- MENTAL	SM	(6) .147±.066 475 2105	(5) .006±.006 8 1040	(5) .076±.039 175 1860	(5) .759±.132 1115 1615
	DAM	(6) .023±.023 30 1700	(5) .055±.016 47 1010	(5) .204±.154 203 1590	(5) .697±.126 1320 1860
	DPM	(6) .043±.031 62 1180	(5) .126±.046 87 720	(5) .071±.063 127 1350	(6) .953±.023 2175 2260
ADULT	SM	(6) 0 ± 0 0 920	(7) .010±.009 23 1140	(5) .044±.034 35 710	(5) 1.00±0 880 880
	DAM	(6) 0 ± 0 0 920	(7) .001±.001 1 1070	(6) .066±.029 68 930	(7) 1.00±0 840 840
	DPM	(6) 0 ± 0 0 950	(6) .010±.010 22 1100	(4) .005±.005 3 650	(6) 1.00±0 900 900

SM:Superficial Masseter Belly
DAM:Deep Anterior Masseter Belly
DPM:Deep Posterior Masseter Belly
a:indicates sample size (n)
b:indicates mean ± standard error of the mean
c:indicates number of stained fibres
d:indicates number of fibres counted

TABLE 3A

MHC ANTIBODY STAINING OF CONTRALATERAL (LEFT HAND SIDE)
SUPERFICIAL MASSETER MUSCLE OF 6 WEEK EXPERIMENTAL RAT

FAST MHC	SLOW MHC	NEONATAL MHC	EMBRYONIC MHC
1.00^a	0	0.12	0
$\dfrac{90^b}{90^c}$	$\dfrac{0}{60}$	$\dfrac{5}{60}$	$\dfrac{0}{90}$

a: indicates proportion of fibres stained positively, n=1.
b: indicates number of stained fibres
c: indicates total number of fibres counted

TABLE 4

BLIND STUDY OF EXPERIMENTAL AND CONTROL RAT FEEDING AND ANCILLARY ORAL HABITS

		CONTROL RAT				EXPERIMENTAL RAT			
		IP	IF	MF	GM	IP	IF	MF	GM
DAY 1	am	/	/	/	x	x	x	x	/
	pm	/	/	/	x	x	x	x	/
DAY 2	am	/	/	/	x	/	x	/	/
	pm	/	x	/	/	/	x	x	x
DAY 3	am	/	x	x	/	x	x	/	/
	pm	/	x	/	/	x	/	/	x
DAY 4	am	x	/	/	/	/	/	/	/
	pm	/	x	/	/	x	x	/	/

OBSERVATION COMMENCED 36 HOURS POST-OCCLUSAL ADJUSTMENT (20 min each time)

x - indicates no observation of this activity
/ - indicates observations were made during this period

IP - Incising paper

IF - Incising food

MF - Masticating food

GM - Grooming

TABLE 5

COMPARISON OF MAXILLARY MOLAR TOOTH LENGTH FROM CONTROL AND
EXPERIMENTAL (ENAMELOPLASTY) GROUPS

CONTROL	EXPERIMENTAL
0.79 ± 0.0425[a]	0.64 ± 0.0268

a: data are mean $\pm$ standard error of the mean

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