THE DEVELOPMENT OF HUMAN SKELETAL MUSCLE AND ITS MOTOR INNERVATION

James Toop

A thesis presented for the degree of
Doctor of Philosophy of the University
of Edinburgh in the Faculty of Medicine

May 1974

ABSTRACT OF THESIS

Name of Candidate James TOOP

Address University Department of Human Genetics, Western General Hospital, Edinburgh.

Degree Doctor of Philosophy *Date* May 1974

Title of Thesis The development of human skeletal muscle and its motor innervation.

1. Some human neuromuscular disorders have a very early age of onset, and in some cases muscular abnormalities are detectable in the neonate. Some abnormalities of development may even be detectable in utero as evidenced by reduced fetal movements. If the events of normal myogenesis were fully understood it might therefore be possible to detect very early microscopic pathological changes in the development of the neuromuscular apparatus in an affected fetus.

2. The aim of the present studies was firstly to investigate the development of normal skeletal muscle and its motor innervation. These studies were based on findings in the soleus and gastrocnemius muscles of 75 normal fetuses of between 5-6 weeks gestation and term. Frozen sections were stained for NADH-diaphorase and myosin ATPase, or with a combined acetylcholinesterase and silver impregnation stain. In the limb-buds at 5-6 weeks there were no discernable muscle cells, but by 7 weeks a few multinucleated myotubes were present. A few of the largest of these showed staining for NADH-diaphorase, but ATPase staining was not visible until 8 weeks. In all cases it was the largest myotubes which showed both histochemical staining as well as the earliest development of neuromuscular junctions. Initially all neuromuscular junctions appeared to consist of one axon ending in one motor endplate on a myotube, but at 12-13 weeks collateral branches of the terminal axons began to appear. Within a few weeks the occurrence of complex terminal axon-motor endplate networks and doubly innervated myotubes was widespread. By 12-13 weeks all myotubes stained either lightly or darkly for NADH-d and ATPase, but no reciprocal pattern was detectable. At 19 weeks there was the first sign of a partially reciprocal pattern of histochemical staining, with only a small proportion of 'type I' cells visible. Up to 30 weeks this partially reciprocal pattern of staining altered little, although the proportion of 'type I' cells was variable. At 24 weeks a few morphologically mature motor endplates were visible although the pattern of terminal innervation was still complex. In the neonate some terminal axon branching was still evident, and there was no truly reciprocal pattern of histochemical staining: adult type I fibres were visible, but not type IIA or IIB. There did appear to be some type IIC fibres.

3. Studies of the histochemical development of anterior horn cells were also carried out. Although it is established that many of the properties of muscle fibres are neurally mediated, these experiments indicated that the histochemical development of muscle could not be simply related to developmental histochemical changes of the anterior horn cells.

4. In a study of the growth and histochemical development of human skeletal muscle cells in vitro, considerable myotube formation was visible in a few cultures,

Use other side if necessary.

but no 'strap' cells with cross-striations were ever seen. Histochemical staining showed the presence of darkly and lightly stained cells, but no true differentiation was evident.

5. A further aim of the present study was to investigate muscle development in anencephalic fetuses and in male fetuses at risk of developing Duchenne muscular dystrophy. Muscles from several anencephalic fetuses were studied, together with the sections of the spinal cord which normally innervate these muscles. Anencephalic fetuses with rachischisis had a greatly reduced number of large neurones in the spinal cord, but despite this apparent deficiency of motor innervation, the skeletal muscles appeared histologically and histochemically normal. The implications of this finding are discussed with reference to the stage of gestation at which the abnormality develops in the spinal cord.

Quadriceps muscles from 5 therapeutically aborted male fetuses at risk of developing Duchenne muscular dystrophy were also studied. No abnormality could be detected in four of the fetuses, but in the fifth the mean and variability of muscle fibre diameter were increased. There were also 2-3% of hyaline fibres present. The changes in the muscle of this 16 week fetus suggest that Duchenne muscular dystrophy may be manifest <u>in utero</u>.

6. The results obtained in the study of the development of normal muscle could usefully be applied to investigations of antenatal pathological processes in other neuromuscular disorders. In addition, experimental studies on the developing histochemical pattern and motor innervation of animal muscle would help to confirm the observations which have been made in the present study. In particular it would be of interest to study the development of muscles which in adult animals are composed almost entirely of one type of fibre.

CONTENTS

SUMMARY

ACKNOWLEDGEMENTS

CHAPTER 1. INTRODUCTION. 1

Histological studies on developing human skeletal muscle 4

Histochemical studies on developing skeletal muscle 7

Studies on the developing motor innervation of skeletal muscle 10

CHAPTER 2. MATERIALS AND METHODS 13

(A) Sources of samples

(1) Normal control samples 13

(2) Samples from anencephalic fetuses and those at risk for DMD 14

(B) Determination of fetal gestation 15

(C) Samples used 15

(1) Skeletal muscle samples 16

(2) Spinal cord samples 16

(3) Samples for tissue culture 17

(D) Processing of samples 17

(E) Staining techniques 19

(1) Histological stains 19

(2) Histochemical stains 20

(3) Stains for demonstration of peripheral motor innervation 21

CHAPTER 3. STUDIES ON THE DEVELOPMENT OF THE MORPHOLOGY, HISTOCHEMISTRY AND PERIPHERAL MOTOR INNERVATION OF NORMAL HUMAN FETAL MUSCLE IN VIVO.

Introduction 23

Materials and Methods 24

Results 25

5 - 6 weeks gestation 25

7 " " 26

8 " " 27

9 " " 28

10 " " 29

11 " " 30

12 " " 31

13 " " 32

14 " " 32

15-21 " " 34

22-24 " " 36

25 " " - neonate 37

Discussion 38

CHAPTER 4. STUDIES ON THE DEVELOPMENT OF THE ANTERIOR HORN CELLS OF THE HUMAN FETAL SPINAL CORD.

Introduction 47

Materials and Methods 49

Paraffin embedded material 50

Frozen material 50

Results

Histochemistry 52

Morphology 53

Conclusion 54

CHAPTER 5. STUDIES ON FETUSES AT HIGH RISK FOR DUCHENNE MUSCULAR DYSTROPHY.

Introduction 58

Materials and Methods 59

Results

Muscle histology 61

Muscle and serum CPK 62

Conclusion 62

CHAPTER 6. STUDIES ON FETUSES WITH CONGENITAL MALFORMATIONS OF THE CENTRAL NERVOUS SYSTEM.

Introduction 64

Materials and Methods 65

Results

Muscle 67

Spinal Cord 68

Conclusion 68

CHAPTER 7. STUDIES ON THE MORPHOLOGY AND HISTOCHEMISTRY OF HUMAN FETAL MUSCLE CELLS IN VITRO.

Introduction 73

Materials and Methods

(1) Setting up and maintenance of primary and subcultures 75

(2) Maintenance of sterility 77

(3) Removal of coverslips and staining 77

(4) Analysis of stained coverslips 78

(5) Analysis of nuclear counts 78

Results

Morphological development 79

Histochemical development 81

Discussion 82

CHAPTER 8. GENERAL DISCUSSION. 87

BIBLIOGRAPHY.

APPENDICES. 1. FETUSES FROM WHICH SAMPLES WERE OBTAINED FOR STUDY.

2. STAINING METHODS USED TO DEMONSTRATE THE PERIPHERAL MOTOR INNERVATION IN HUMAN FETAL MUSCLE.

3. PUBLICATIONS.

SUMMARY

1. Some human neuromuscular disorders have a very early age of onset, and in some cases muscular abnormalities are detectable in the neonate. Some abnormalities of development may even be detectable *in utero* as evidenced by reduced fetal movements. If the events of normal myogenesis were fully understood it might therefore be possible to detect very early microscopic pathological changes in the development of the neuromuscular apparatus in an affected fetus.

2. The aim of the present studies was firstly to investigate the development of normal skeletal muscle and its motor innervation. These studies were based on findings in the soleus and gastrocnemius muscles of 75 normal fetuses of between 5 - 6 weeks gestation and term. Frozen sections were stained for NADH-diaphorase and myosin ATPase, or with a combined acetylcholinesterase and silver impregnation stain. In the limb-buds at 5 - 6 weeks there were no discernable muscle cells, but by 7 weeks a few multinucleated myotubes were present. A few of the largest of these showed staining for NADH-diaphorase but ATPase staining was not visible until 8 weeks. In all cases it was the largest myotubes which showed both histochemical staining as well as the earliest development of neuromuscular junctions. Initially all neuromuscular junctions appeared to consist of one axon ending in one motor endplate on a myotube, but at 12 - 13 weeks collateral branches of the terminal axons began to appear. Within a few weeks the occurrence of complex terminal axon-motor endplate networks and doubly innervated myotubes was widespread. By 12 - 13 weeks all myotubes stained either lightly or darkly for NADH-d and ATPase, but no reciprocal pattern was detectable. At 19 weeks there was the first sign of a partially reciprocal pattern of histochemical staining, with only a small proportion of 'type I' cells visible.

Up to 30 weeks this partially reciprocal pattern of staining altered little, although the proportion of 'type I' cells was variable. At 24 weeks a few morphologically mature motor endplates were visible, although the pattern of terminal innervation was still complex. In the neonate some terminal axon branching was still evident, and there was no truly reciprocal pattern of histochemical staining: adult type I fibres were visible, but not type IIA or IIB. There did appear to be some type IIC fibres.

3. Studies of the histochemical development of anterior horn cells were also carried out. Although it is established that many of the properties of muscle fibres are neurally mediated, these experiments indicated that the histochemical development of muscle could not be simply related to developmental histochemical changes of the anterior horn cells.

4. In a study of the growth and histochemical development of human skeletal muscle cells in vitro, considerable myotube formation was visible in a few cultures, but no 'strap' cells with cross-striations were ever seen. Histochemical staining showed the presence of darkly and lightly stained cells, but no true differentiation was evident.

5. A further aim of the present study was to investigate muscle development in anencephalic fetuses and in male fetuses at risk of developing Duchenne muscular dystrophy. Muscles from several anencephalic fetuses were studied, together with the sections of the spinal cord which normally innervate these muscles. Anencephalic fetuses with rachischisis had a greatly reduced number of large neurones in the spinal cord, but despite this apparent deficiency of motor innervation, the skeletal muscles appeared histologically and histochemically normal. The implications of this finding are discussed with reference to the stage of gestation at which the abnormality

develops in the spinal cord.

Quadriceps muscles from 5 therapeutically aborted male fetuses at risk of developing Duchenne muscular dystrophy were also studied. No abnormality could be detected in four of the fetuses, but in the fifth the mean and variability of muscle fibre diameter were increased. There were also 2 - 3% of hyaline fibres present. The changes in the muscle of this 16 week fetus suggest that Duchenne muscular dystrophy may be manifest in utero.

6. The results obtained in the study of the development of normal muscle could usefully be applied to investigations of antenatal pathological processes in other neuromuscular disorders. In addition, experimental studies on the developing histochemical pattern and motor innervation of animal muscle would help to confirm the observations which have been made in the present study. In particular it would be of interest to study the development of muscles which in adult animals are composed almost entirely of one type of fibre.

ACKNOWLEDGEMENTS

I wish to record my gratitude and thanks to Professor A.E.H. Emery for his constant encouragement and many helpful discussions throughout the course of this project, and for placing the laboratory facilities at my disposal. I also wish to thank Dr. J.N. Webb for his advice and helpful backing during this period of research.

Thanks are also due to Dr. J.B. Scrimgeour, Dr. J.M. Monaghan, Dr. A.D. Bain, and Dr. A.F. Anderson, as well as others, for making available the fetal material on which this study is based.

I also wish to thank Dr. C. Gosden, with whom I cooperated on some of the studies described in Chapter 7, Misses P.D. Jackson and A. Ogg for their contribution to Chapter 4, and Mrs. L. Bain and Mrs. H. Cameron for technical assistance with Chapter 5.

I am also grateful to Miss J.D. Meldrum, Mr. J.E. Pizer, and Mr. W.P. Patterson for preparing the final photographs as well as a figure and table. Many thanks are also due to Mrs. M. Hunter for her excellent typing.

Finally I would like to express my thanks to the Medical Research Council for supporting me during the course of this study.

CHAPTER 1

INTRODUCTION

Human muscular disorders vary in severity from some which have their onset in infancy or childhood, and are rapidly progressive and eventually fatal, to others which are relatively benign and compatible with survival for many years. Muscle disorders either of early or late onset may be genetically inherited, but the fundamental lesion in these diseases is not yet known. However, on the basis of pathological findings, human muscular disorders may also be divided into those of myogenic and those of neurogenic origin. Among the latter are such diseases as the spinal muscular atrophies, motor neurone disease and acute anterior poliomyelitis (GARDNER-MEDWIN and WALTON, 1969: EMERY, 1971). In these disorders there is a defect in the motor innervation, visible as a degeneration of the anterior horn cells of the spinal cord. The members of the former class of diseases include the muscular dystrophies, myotonic dystrophy, central core disease and many other myopathies (GARDNER-MEDWIN and WALTON, 1969), and the disorders have been interpreted as being due to the primary degeneration of muscle.

Studies on patients with genetically inherited muscular disorders have emphasised the need to study the pathological changes both in skeletal muscle and its peripheral motor innervation (COËRS and WOOLF, 1959: COËRS et al., 1973). The possible role of the motor nerve in the pathogenesis of many human muscular disorders is becoming more widely recognised. Studies of human muscle to elucidate the possible role of the motor nerve in initiating and maintaining normal muscle structure and function have been prompted by experiments conducted on animals. In 1904 HARRISON showed that normal motor innervation may not be necessary for normal myogenesis. The importance

of the motor innervation for the normal development of mammalian muscle on the other hand, has been clearly shown: even in the adult the ability of a cross-united motor nerve to produce marked alterations in the properties of the re-innervated muscle is well documented (BULLER et al., 1960b: EDSTRÖM and KUGELBERG, 1969: SAMAHA et al., 1970: BROOKE et al., 1971: BULLER et al., 1971). It has also been shown (KARPATI and ENGEL, 1967: SHAFIQ et al., 1972) that in developing animal muscle, the role of the motor innervation in bringing about normal histochemical development may be particularly important.

Among the many experiments on animals which have demonstrated the possible role of the motor nerve in the pathogenesis of animal analogues of various human muscular and neuromuscular disorders have been those conducted by McCOMAS and MROZEK (1967) and by GALLUP and DUBOWITZ (1973). Although such studies may be valuable, the evidence they provide may not be applicable to human disorders. This is illustrated by the finding of abnormal motor endplates on apparently normal muscle fibres of a dystophic mouse (RAGAB, 1971) although similar changes have never been observed in human muscular dystrophy. Similarly, the abnormalities of peripheral nerves which have been studied in murine muscular dystrophy (BRADLEY and JENKISON, 1973) have never been observed in the human disease.

Despite these findings, recent studies on patients with muscular dystrophy (McCOMAS et al., 1970: DASTUR and RAZZAK, 1973) have led to the suggestion that defective motor innervation may play a significant part in the pathogenesis of the human diseases also. However, the number and variety of conflicting reports on this subject (BRADLEY, 1971) emphasises that a great deal more work will be necessary before the full role of the motor nerve in the pathogenesis of human muscular disorders can be satisfactorily evaluated.

When human muscular disorders become clinically evident, muscle biopsy studies utilising both histological and histochemical stains, as well as those to demonstrate terminal motor innervation, may be used to decide the precise nature of the disease. Occasionally the onset of clinical symptoms may be in the very early neonatal period. One recent study has suggested that it may even be possible to detect abnormal fetal movements during pregnancy in a mother who is a carrier of Werdnig-Hoffmann disease (PEARN, 1973). The possibility that in such cases there are abnormal antenatal fetal movements suggests that at least in these disorders the pathological changes may begin *in utero*.

Very early pathological changes have been found in the muscle of neonates suffering from each of several human muscular disorders (COËRS and WOOLF, 1959: BRADLEY et al., 1972: FARKAS et al., 1974), and muscle biopsy specimens from potentially affected fetuses could provide valuable information concerning the first microscopic pathological changes which occurred. For purposes of comparison, the normal events of myogenesis must be known, and the recent interest in the possible importance of the motor nerve in relation to muscle pathology makes it desirable that the development of the motor innervation be studied also. The primary aim of the present research has thus been to establish some of the parameters of the normal pattern of development of human fetal muscle, to act as the baseline with which comparisons of possibly affected muscle may be made.

During the present period of study, it has been possible in two instances to make comparison between presumably normal and possibly abnormal muscle development. In the first instance, (Chapter 5) comparison between the histological development of normal quadriceps muscle and the pattern of

development found in male fetuses at risk of developing Duchenne muscular dystrophy, enabled the detection of abnormalities in the muscle of one of the fetuses at risk. In a second instance (Chapter 6) several anencephalic fetuses were examined to determine whether an apparent deficiency of the motor innervation to the peripheral musculature was reflected in abnormal development of the skeletal muscle.

Many of the features of human muscle development have been extensively studied, particularly with respect to its histology, and an appreciation of the growth of our understanding of the development of skeletal muscle and its motor innervation will help to highlight areas of these investigations in which there is still uncertainty.

Histological studies on developing human skeletal muscle.

Human skeletal muscle arises from the mesodermal layer of embryonic cells. The muscles of the head, neck and trunk are of somitic origin, but the limb muscles are formed by differentiation *in situ* of mesenchyme cells of the lateral plate mesoderm (HAMILTON et al., 1952). The evidence for the non-myotomic origin of limb musculature in man is good (BARDEEN and LEWIS, 1901-02), and is firmly supported by experimental studies in the chick (STRAUS and RAWLES, 1953). In the limbs, developing muscle is first seen as small rounded uninucleated cells, and in 1839 SCHWANN (cited by TELLO, 1917) was apparently the first to suggest that the morphological development of skeletal muscle came about by fusion of these uninucleated cells. REMAK (1845, cited by TELLO, 1917) disagreed with this concept, suggesting instead that muscle fibres arose by the gradual enlargement of a single cell in which the number of nuclei gradually increased. In 1902, GODLEWSKI suggested that these plump cells with basophilic cytoplasm which he found in the muscle primordia

of rat, sheep and guinea-pig fetuses should be called "myoblasts". TELLO (1917) concurred in this nomenclature of these embryonic precursor muscle cells and further suggested (1922) that there should be separate terms to denote multinucleated cells containing no myofibrils (myocytes) and those with myofibrils (myotubes), but this nomenclature has since fallen into disuse.

Further studies of developing muscle such as those of HEWER (1927-28) did little to resolve the debate concerning the method by which the growth of muscle fibres took place. Hewer's contribution was, however, a study of the development of human skeletal muscle, to confirm that the findings which had been made in animals were true also for man. Hewer also described the appearance of cross-striations and fibrillation of the cytoplasm in muscle cells from the lumbar region of the back of an eight week fetus, and the subsequent maturation of the muscle cells.

The advent of the electron microscope enabled a fuller understanding of cellular structure, and in muscle was used (HUXLEY, 1957) to confirm and expand the suggestion (HANSON and HUXLEY, 1953) that the alternating 'striations' of skeletal muscle were alternate lengths of interdigitating myosin and actin filaments. Subsequently the electron microscope was used to study the ultrastructural development of skeletal muscle (BERGMAN, 1962: ISHIKAWA, 1966: FISCHMAN, 1967: LARSON et al., 1969, 1970). The findings of these investigators agreed in general with those that had been made using light microscopy, but showed in addition other facets of development. For instance, the appearance of cross-striations which can be seen with light microscopy is preceded by several weeks by the formation of larger or smaller sections of actomyosin filaments, associated with groups of ribosomes, at first scattered in the sarcoplasm (LARSON et al., 1969).

In the electron microscope it can be seen that developing skeletal muscle is a far from homogeneous population of cells, being composed both of myoblasts and multinucleated cells of many different sizes. The observation of uninucleated 'satellite cells' present in adult human muscle (MAURO, 1961) led to the suggestion that these cells were the remainder of the embryonic myoblast population, and might function as a reserve for the generation of fresh tissue in the event of muscle injury (MUIR et al., 1965). The basic structure of the satellite cell is now well understood, and experiments on rat muscle demonstrate not only that these cells are the source of nuclei in growing muscle fibres (MOSS and LEBLOND, 1971), but also that their numbers decline throughout life (ALLBROCK et al., 1971). There is also good circumstantial evidence that the satellite cell is the source of myoblasts in regenerating muscle (CARLSON, 1973). In regenerating human muscle, direct observation has indicated fusion between neighbouring myoblasts (MASTAGLIA and WALTON, 1971), and the weight of evidence from tissue culture studies (COOPER and KONIGSBERG, 1961a: STOCKDALE and HOLTZER, 1961) using chick muscle, supports Schwann's original hypothesis that it is cellular fusion which gives rise to the multinucleated skeletal muscle fibres.

The method by which muscle fibres are initially formed is thus fairly well established, but it is not so clear when the number of muscle fibres stops increasing (GOLDSPINK, 1972). MacCALLUM (1898) reported that there appeared to be hardly any postnatal increase in numbers of fibres in the sartorius muscle in man, and more recent counts of muscle fibres (MONGOMERY, 1962) have failed to resolve the situation. In the adult, uninnervated muscle fibres atrophy and die, and in the fetus, the number of muscle fibres may be limited by the availability of motor innervation.

Degenerating muscle fibres in otherwise apparently normal fetal pig muscle were seen by BARDEEN in 1900, and have since been studied with the electron microscope in human fetal muscle (WEBB, 1972). In both these cases, it may be that fibre degeneration occurs as a result of a lack of normal motor innervation.

Histochemical studies on developing skeletal muscle.

The earliest observation that there were two types of muscle fibres, red and white, was made by LORENZINI in 1678 (cited by CIACCIO, 1898). RANVIER (1874) noted the physiological and structural differences between muscles which were related to whether they were primarily red or white, and BULLARD in 1919 and DENNY-BROWN in 1929 saw that different muscle fibres contained different levels of lipid. With the advent of a reliable histochemical stain for succinate dehydrogenase it became possible to demonstrate a consistent variation in the concentration of this enzyme in different muscle fibres (PADYKULA, 1952). Dubowitz and Pearse's discovery of the reciprocal enzyme pattern present in human muscle fibres (DUBOWITZ and PEARSE, 1960) together with the findings of other investigators, established that there was in fact a well-defined two-fibre-type system present in adult human muscle.

Since these early observations there have been several attempts to refine the technique of muscle fibre typing in both human and animal muscle, principally by using a greater number of histochemical reactions. The most extreme example is that of ROMANUL (1964), who by using a battery of histochemical reactions decided that there were eight muscle fibre types. Muscle fibre typing is most meaningful when it is related to the physiological activity of the muscle fibres being typed, and the results of studies both on human subjects (WARMOLTS and ENGEL, 1972) and animals (BROOKE and KAISER, 1970) have shown that there are primarily two types of fibres: those capable

of sustained firing and those active during brief bursts of vigorous contraction. The latter tend to be largely fibres of glycolytic capacity, while the former rely more heavily on oxidative metabolism. Thus a two-fibre-type system does have a basic relevance in histochemical investigation of muscle. More recent studies have shown there to be further divisions of muscle fibre types (BROOKE and KAISER, 1970: DAVIES and GUNN, 1972) and the most recent experiments on rats (KUGELBERG, 1973) have enabled very clear histochemical distinctions to be made between muscle fibres, which are closely related to their speeds of contraction. A pattern of muscle fibre typing similar to that used for animal muscle is now becoming accepted for use in human muscle (DUBOWITZ and BROOKE, 1973).

Muscle fibre typing in animal and human subjects has been extensively studied in the adult, but in 1964 some of the earliest histochemical observations were made on fetal and neonatal muscle, in mice (WIRSÉN and LARSSON, 1964). Following these studies it was suggested that the three different fibre types of adult muscle developed as three distinct populations of cells. In 1963, FENICHEL had reported the results of histochemical and other studies in human fetal muscle to determine the significance of Wohlfart 'B' fibres (WOHLFART, 1937) in fetal and in neonatal muscle. A more thorough histochemical study of developing muscle, both animal and human, was made by DUBOWITZ (1965, 1968). In the neonatal rabbit and less clearly in the guinea-pig, histochemical differentiation appeared almost as in the adult, whereas both in rat and mouse, skeletal muscle was almost wholly undifferentiated at birth. Human neonatal muscle was found to be differentiated at birth, and as in the rabbit and guinea-pig, it was found that samples of skeletal muscle removed from fetuses at various

stages of gestation clearly demonstrated that there was a progression of histochemical differentiation occurring.

Since Dubowitz's studies there have been many reports concerning the histochemical development of animal skeletal muscle *in utero*. Muscle from rhesus monkeys (BEATTY et al., 1967) as well as from bovine fetuses (OMMER, 1971) was found to exhibit a pattern similar to the adult, while Dubowitz's findings have been confirmed for the rat (BROOKE et al., 1971), and a similar pattern seen in the developing jaw muscles of the domestic fowl (DUBALE and MURALIDHARAN, 1970). Recent work on neonatal as well as adult pig muscle (DAVIES, 1972) has shown that considerable histochemical changes take place post-natally in the longissimus muscle of this animal, and that these changes are related to the changing demands made on this muscle as the animal grows.

The fact of histochemical changes occurring in developing muscle is thus well established. It is now generally accepted that each adult motor unit is physiologically (WUERKER et al., 1965) and histochemically (EDSTRÖM and KUGELBERG, 1968: KUGELBERG, 1973) uniform, and controlled by a single large motor neurone. The histochemical changes occurring in the developing muscle of the rhesus monkey (BEATTY et al., 1967), pig (DAVIES, 1972) and man (DUBOWITZ, 1965, 1968) may therefore be the results of a physical rearrangement of the motor innervation, taking place during the maturation of the skeletal muscle.

In the human fetus, the principal study of the histochemical development of muscle remains that of Dubowitz. The emphasis in this study was on fetuses between 12 weeks gestation and term, but little consideration was given to the role of the motor nerve in the changing histochemical pattern

detection of the enzyme acetylcholinesterase at the motor endplate, and the second was the introduction of electron microscopy. These methods have been extensively used to investigate motor endplate structure (COUTEAUX, 1960: COËRS, 1967) in adult animals both in the normal (ZACKS and BLUMBERG, 1961: PADYKULA and GAUTHIER, 1970) and diseased states (ZACKS, 1964: DUCHEN, 1970, 1971). In addition, work has been carried out on the antenatal development of motor endplates in animal muscle (TERÄVÄINEN, 1968: KELLY and ZACKS, 1969). In the human adult, investigations of normal motor endplate structure (ZACKS and BLUMBERG, 1961) and the changes it may undergo in disease (MAIR and TOMÉ, 1972) have added little to our understanding of the role played by motor innervation in the overall context of human muscle development.

Only two reports have appeared to date of human antenatal endplate development, which make use of the two new methods mentioned above. The first was an electron microscope study in which muscle from both upper and lower extremities of 7 human fetuses of between 9 and 16 weeks gestation was examined (FIDZIAŃSKA, 1971, 1973). This demonstrated the presence of immature motor endplates on muscle cells in the myotube stage. The second report contained the results of studies of the appearance of acetylcholinesterase in the soleplate of the human motor endplate (JUNTUNEN and TERÄVÄINEN, 1972) and was complementary to previous studies of a similar nature which had been conducted on the rat (TERÄVÄINEN, 1968). Muscle from 38 fetuses and 4 still born infants was used to trace the development of the motor endplate in the intercostal and tibialis anterior muscles from the earliest stages of motor endplate formation up to the features visible in the neonate.

Many of the studies of the developing motor innervation which have been conducted have been valuable in the present project because of the

parallels which have been shown to exist between developing animal and human muscle. The same is true also of the histochemical and histological studies of developing muscle. However, the relationship of the pattern of developing motor innervation to the histochemical development of the muscle has not been explicitly investigated. This has been simply understood in terms of the events which are known to occur in adult human or animal muscle when either experimental or disease-induced changes occur in the pattern of terminal innervation of skeletal muscle. The studies which have been conducted in the present investigations have sought to explore more fully the influence of the motor innervation in the histochemical development and differentiation of human fetal muscle. The main body of the studies has involved the making of frozen sections, principally of the soleus and gastrocnemius muscles of 75 presumed normal human fetuses of a wide gestational age range. The frozen sections were stained either for histochemical reactions, or with a specially adapted combined acetylcholinesterase and silver impregnation stain, and the findings obtained by the different staining methods correlated as far as possible.

CHAPTER 2

MATERIALS AND METHODS.

This chapter is concerned in general with the materials and methods used in the series of studies reported here. Details of particular materials or methods not included will be found in the appropriate chapters.

(A) Sources of Samples.

All the materials used in this series of studies were of human origin, and came either from fetuses which had been aborted, or from neonates which had gone for post-mortem examination. The abortions were in most cases artificially induced or carried out surgically, but in addition there were samples from spontaneous abortions. All the samples studied were either skeletal muscle, or spinal cord, from specific sites which will be indicated in the appropriate sections.

(1) Normal control samples.

In none of these was there a known genetic history of muscular or neuromuscular disease, and no spontaneous abortion specimens were used in which there was a possibility or overt evidence of muscular or chromosomal abnormalities. The majority of samples were from abortions in which pregnancy had been terminated by a variety of medical or surgical means. Up to approximately 12 weeks gestation, the majority of specimens were obtained following intrauterine curettage and suction. From the fetal parts thus produced one or occasionally both legs and at least one foot were removed. These specimens were among the least autolysed that were obtained.

Between 10 and 24 weeks gestation many samples were obtained from fetuses whose abortions had been induced by the administration of either intra- or extra-amniotic physiological saline, or prostaglandin E_2 or $F_{2\alpha}$, either alone

or together with either buccal pitocin or syntocinin I.V. In these cases fetal death preceded abortion by up to 24 hours, and at first sight tissue preservation in these specimens did not appear good. However subsequent microscopic examination indicated that in the majority of these cases this damage had not been so great as to markedly alter the histological or histochemical profile of the specimen.

A third method of termination of pregnancy was by hysterotomy. In the early phase of the study the majority of samples were obtained from hysterotomy specimens since this was the principal method in use: since then it has been superseded by the use of prostaglandin. Samples from hysterotomy specimens were extremely fresh and free from autolysis. Samples from fetuses which had aborted spontaneously were generally fairly well preserved, although in cases in which the fetus had evidently been dead in utero, the same sample criteria were applied as for those fetuses whose abortions had been induced by prostaglandin.

Finally, samples were obtained after postmortem examinations from one 24 week fetus and one neonate which had each lived for only three days after delivery. These had been kept, prior to examination, at $4^{o}C$ for two days, but deterioration of the histological or histochemical profile of the tissues examined proved to be minimal.

The samples referred to above were all from local hospitals, and were used in the study of the normal histological and histochemical development of skeletal muscle and anterior horn cells of the spinal cord, and for most of the study of the growth of human skeletal muscle cells in vitro. Details of these fetuses are in Appendix 1.

(2) Samples from anencephalic fetuses and those at risk for DMD.

(a) Anencephalic fetuses. 10 anencephalic fetuses were obtained

at the time of therapeutic abortion, after the diagnosis had been suspected on the basis of clinical findings, X-radiography, sonography or α-fetoprotein estimations on the amniotic fluid. With prior warning of an anencephalic fetus it proved possible in most cases to arrange a postmortem examination, where necessary, soon after delivery, so that the samples obtained were extremely fresh. All these fetuses were delivered in local hospitals.

(b) Fetuses at risk for DMD. These were obtained through the cooperation of several doctors in different parts of the country. The abortions were performed either by hysterotomy, the use of prostaglandin, or intra-uterine suction and curettage. Details of these fetuses will be found in Chapter 5.

(B) Determination of fetal gestation.

Where possible this was done by each of three methods ie. heel - toe and crown - rump length measurements, as well as menstrual age. The heel-toe measurements used as standard were from a table compiled by STREETER (1921). This estimate of gestation was used more than any other, and therefore relied on most, because it was the only measurement of the three which was available for all fetuses. When possible the crown-rump length was also measured and gestation estimated from data given by Hamilton (HAMILTON et al., 1952). The gestation as determined from the first day of the last menstrual period was not always reliable, sometimes due to an obviously inaccurate date given by the mother, but more seriously, in the very young fetuses, because it is the post-conceptional and not menstrual age which is related to the stage reached in fetal development.

(C) Samples used.

The samples used in these studies were either portions of several skeletal muscles or sections of the spinal cord.

(1) Skeletal muscle samples.

(a) Normal controls. The tissue used in this part of the study was from the soleus and gastrocnemius muscles. These muscles were selected for three reasons. Firstly, these are muscles which in animals have distinctly different histochemical profiles, and it was considered interesting to see whether, if this were true in man, their patterns of histochemical development would be different. Secondly, these are the two largest muscle masses in the lower leg, and, in very young fetuses, should therefore be more easy to see than most other leg muscles. Thirdly, they lie in close apposition to one another and are therefore easily removed together.

(b) Anencephalic fetuses and those at risk for DMD.

(i) Anencephalic fetuses. A variety of skeletal muscles was studied in this series, of which details will be found in Chapter 6.

(ii) Fetuses at risk for Duchenne Muscular Dystrophy. Samples for study were taken from the quadriceps femoris muscle. This was selected since in the pathology of DMD, it is one of the first muscles to become clinically affected by the disease.

(2) Spinal cord samples.

The study of the spinal cord development was not as extensive as that of the skeletal muscle development, and the only spinal cords sectioned were those from normal controls and anencephalic fetuses.

(a) Normal controls. In each case the caudal end of the spinal cord was used, being removed from just above the lumbar enlargement. Further details will be found in Chapter 4.

(b) Anencephalic fetuses. Seven of the spinal cords of anencephalic fetuses were studied. Sections were made both of rachischitic portions of the spinal cord, as well as of apparently normal segments: in

each case the level at which the cord was sectioned was related to the skeletal muscles which had been investigated. Further details will be found in Chapter 6.

(3) Samples for tissue culture.

The tissue grown was exclusively skeletal muscle, which was taken from the quadriceps femoris. In very immature fetuses, ie. 10 weeks gestation and below, it was often not possible to obtain sufficient muscle from the quadriceps alone to set up the required number of cultures, and in these cases the skeletal muscle was taken from wherever it was available. Full details of tissue culture methods will be found in Chapter 7.

(D) Processing of samples.

Whenever possible, samples of muscle and spinal cord were removed and frozen on the day on which the fetus was delivered. On the occasions on which this was not possible, the samples were stored at 4°C until they could be frozen (see footnote). Autolysis in the spinal cord is more rapid than in muscle and all but 2 spinal cords were kept for a maximum of 2 days before freezing. Muscle is less liable to autolytic damage, and was kept at 4°C. for a maximum of 4 days before freezing.

Footnote: In order to study the changes that occur in skeletal muscle which is not frozen upon receipt but left at room temperature, a small piece of quadriceps muscle from an anencephalic fetus was left on the bench in a sealed tube, and samples removed from it daily for up to five days. After 3 days some nuclear degeneration could be seen, and this rapidly progressed to total fibre destruction. Despite this, it was still possible to see after 4 days at room temperature the few scattered dark fibres which are characteristic of fetal skeletal muscle of this gestation, when frozen sections were stained for myosin ATPase at pH 4.3.

In fetuses of up to about 14 weeks gestation the bones of the lower leg were sufficiently cartilaginous, and the limb cross-section of such an area that it was possible to make sections of the whole leg. Between about 14 and 18 weeks gestation the fetal triceps surae (soleus and gastrocnemius) was removed whole by cutting the Achilles tendon and freeing the muscles at their proximal ends, before freezing them together. After about 18 weeks gestation the soleus and gastrocnemius were frozen separately since they were of such a size that it was impractical to handle them together.

Both muscle and spinal cord were frozen in isopentane chilled in liquid nitrogen. In a few cases freezing was done in liquid nitrogen alone, but this resulted in a less well preserved tissue with more holes as a result of ice crystal formation. After freezing, the majority of specimens were kept frozen at $-20^{\circ}C$, and latterly at $-70^{\circ}C$, in sealed tubes. If the tubes remained well sealed, storage, even for as long as one year, produced no detectable change in the histology or histochemistry of the muscle. Spinal cords were stored at $-70^{\circ}C$ for up to four months. (Some spinal cords were fixed in formalin and embedded in paraffin for sectioning. Details will be found in Chapter 4.)

For sectioning, the frozen muscle and spinal cord samples were attached to chucks by means of Ames O.C.T. compound taking care not to allow the frozen specimen to unfreeze. Sections were made at $-20^{\circ}C$ either on a Cambridge rocking microtome or on a Slee retracting microtome, and attached to glass slides at room temperature. The thickness of the sections varied from 10μ for histological and histochemical studies, to $20\mu - 30\mu$ or occasionally 50μ when studying the peripheral motor inervation.

Whole transverse sections of the lower leg were taken at a point approximately equivalent to that found 10 cm. below the tibial tuberosity in an adult, and transverse sections of the soleus and gastrocnemius muscles from

the point at which muscle cross-sectional area was greatest.

(E) Staining techniques.

The following stains were used either on muscle or spinal cord. The duration of each stain was kept constant, and not altered for fetuses of different gestations.

(1) Histological stains.

(a) Haematoxylin and eosin. (Muscle and spinal cord) Most of the staining was done using 1% aqueous eosin, but in later sections it was found that 5% eosin in 25% ethanol produced a stronger stain which differentiated the cytoplasm more clearly, especially in the young fetuses.

(b) Toluidine blue. (spinal cord)

- fix fresh frozen sections 1 hour in 10% formol saline.
- rinse well in distilled water.
- stain in 1% Toluidine blue in 1% borax at 56°C for $\frac{1}{2}$ - 1 minute.
- differentiate in 95% ethanol.
- dehydrate, clear and mount.

(c) Gomori-trichrome. (muscle) Staining was carried out as described by ENGEL and CUNNINGHAM (1963).

(d) Phosphotungstic acid-haematoxylin. (muscle) This was a modified version of Mallory's stain (MALLORY, 1897, 1900-01).

An amount of haematein sufficient to produce a final concentration of 0.08% w/v is mixed to a paste with a very small amount of water, and dissolved in 0.9% phosphotungstic acid. This mixture is brought to the boil, cooled and filtered before use.

- place sections in 0.25% $KMnO_4$ for 5 minutes
- wash in distilled water for 5 minutes

- place in 5% aqueous oxalic acid for 5 minutes
- wash in distilled water for 5 minutes
- stain in PTAH 16 - 24 hours
- rinse very briefly in 95% ethanol
- dehydrate very rapidly in absolute alcohol, clear in xylene and mount in DPX.

(2) Histochemical stains.

(a) NADH-diaphorase. (muscle) The method used was Barka's modification (BARKA and ANDERSON, 1963) of that developed by Scarpelli (SCARPELLI et al., 1958). A stock solution was made up which contained everything except NADH, substituting water for the NADH solution. This stock solution could be stored indefinitely at -20°C. For use, a small amount of the stock solution was melted, and NADH added to a final concentration of approximately 1 mg/ml. Slides which had been allowed to dry for approximately 15 minutes (so that the sections would not become detached from the glass) were incubated in the above medium at 37°C for 1 hour. In control sections, incubation was carried out in a medium from which NADH had been omitted.

After incubation, the sections were briefly rinsed in distilled water, and then placed in acetone for 5 minutes to remove the unreduced tetrazolium. Sections were mounted in a 100% w/v aqueous polyvinylpyrrolidone medium, as described by BURSTONE (1957), since this results in less granularity in the muscle fibres than is the case if the section is dehydrated in the normal way.

(b) Calcium-activated myosin ATPase. (muscle) The basis of the method used was that described by PADYKULA and HERMAN (1955) for the stain of myosin ATPase at pH 9.4, but with the substitution of 1M Tris for 0.1M sodium barbitone (Dr. A.S. Davies, personal communication). It was found that unless the muscle had been fairly fresh when it was frozen, the

differential in staining intensity between muscle fibres was not as great as expected. This was sometimes improved by pre-incubating the sections in a carbonate/bicarbonate buffer at pH 9.4. For the demonstration of myosin ATPase at pH 4.3, sections were pre-incubated in Walpole's acetate buffer at pH 4.3 for 10 minutes, then washed in distilled water prior to the normal incubation for 40 minutes at 37°C. Despite great care in doing the staining, a surface precipitate on the section often made it very difficult to distinguish clearly how darkly the muscle fibres underneath had been stained.

(c) Succinate dehydrogenase and (d) phosphorylase stains were also used, in staining anterior horn cells of the spinal cord. Details will be found in Chapter 4.

(3) Stains for demonstration of peripheral motor innervation.

This was done in one of two ways: either an acetylcholinesterase (AChE) stain was used to stain for motor endplates (soleplates) alone, or a combined silver impregnation and AChE stain was used.

(a) Acetylcholinesterase stain. (muscle and spinal cord) The stain used was one described by PAGE (1971), which is itself a much modified version of Koelle's stain (KOELLE and FRIEDENWALD, 1949). In about half of the sections stained for AChE, ammonium sulphide was used to produce a deposit of black copper sulphide. However, the stain produced at the motor endplate by this method could be seen clearly for only a few months. A more satisfactory method was used latterly, in which silver is deposited at the site of the motor endplate. Details of this method are in Appendix 2.

(b) Combined acetylcholinesterase and silver impregnation. (muscle). The first method used was one described by Namba (NAMBA et al., 1967), and was used as they recommended, although occasionally with minor modifications. Although some good results were obtained, they were

never entirely consistent when used to stain fetal muscle, and another method was developed after much experimentation. This was a combination of Page's method for acetylcholinesterase (PAGE, 1971), and Palmgren's for the staining of axons (PALMGREN, 1960). Details will be found in Appendix 2.

CHAPTER 3

STUDIES ON THE DEVELOPMENT OF THE MORPHOLOGY, HISTOCHEMISTRY AND PERIPHERAL MOTOR INNERVATION OF NORMAL HUMAN FETAL MUSCLE IN VIVO.

Introduction.

The structure of the motor endplate and organisation of the motor innervation in animals has been investigated by many workers since the first studies in 1836 (cited by ZACKS, 1964). The embryogenesis of the motor endplate has also been studied in detail, and the first observations on human motor endplate development were made by TELLO (1917, 1922). A major contribution to our understanding of the development of human motor innervation was Cuajunco's detailed study in 25 human fetuses (CUAJUNCO, 1942). However the technique used was straightforward silver staining, since there was no reliable method for demonstrating acetylcholinesterase (AChE) in the soleplate of the neuromuscular junction. Recent studies of the development of the fetal neuromuscular junction have made use of this stain (JUNTUNEN and TERÄVÄINEN, 1972), and enabled the morphological development of the neuromuscular junction to be studied.

In the present study it has been possible to confirm many of both Cuajunco's and Juntunen and Teräväinen's findings, and in many cases to combine their different types of observations in the same specimen. In addition, histochemical staining of muscle from the same fetuses in whose muscle the pattern of developing motor innervation was observed, has enabled these two facets of muscle development, which are so closely interrelated, to be studied together.

The results presented here differ from previous reports in several

respects. In 1963, FENICHEL recorded the results of studies of the development of the 'B' fibre in human fetal muscle (WOHLFART, 1937), which included a brief histochemical survey based on the ATPase reaction at pH9.4 in 5 fetuses. In 1965 and more fully in 1968, DUBOWITZ presented data on the histochemical development of human muscle based on studies from 17 fetuses between 11 and 25 weeks gestation, and 33 premature or neonatal infants. In contrast to these cases, the present study is based on results of histochemical staining of muscle from 75 fetuses between 6 weeks gestation and term, with the emphasis on the period of development up to 25 weeks. On the other hand, while previous authors have studied as many as 18 muscles (DUBOWITZ, 1968) in any one fetus, the results presented here are based principally on findings in only two major leg muscles, originally selected because of the different physiological and histochemical staining properties of these muscles in the adult.

In the present report, more emphasis has been placed on the study of muscle histochemistry and motor innervation during development than on histology, since the histological and ultrastructural development of human fetal muscle has been previously reported in great detail (ISHIKAWA, 1966: LARSON et al., 1969). In addition, the light microscope is restricted in its usefulness in the investigation of developing muscle since many important events take place intracellularly in the maturing cells. Comment on and illustration of the muscle histology have been included principally to place the other observations in context.

Materials and Methods.

Most of the material relevant to this section has been discussed in Chapter 2. In all, 75 normal fetuses were involved, ranging from 6 weeks

gestation to the neonate (Appendix 1). Studies were based mainly on the soleus and gastrocnemius muscles, although for comparative purposes a few thigh muscles were studied in the younger fetuses. Frozen sections were stained with H and E and for NADH-d and myosin ATPase either at pH9.4 or 4.3, and the structure of terminal motor innervation investigated using one or other of two combined AChE and silver impregnation stains, also as described in Chapter 2. Occasionally sections were stained with PTAH in order to demonstrate cross-striations. All finished slides were viewed either on the projection screen or through the eyepieces of a Projectina microscope (Projectina, Heerbrugg, Switzerland) or photographed using a Leitz Orthomat automatic camera fitted to a Leitz Ortholux research microscope. Measurements were made by using an eyepiece graticule which was calibrated against a graduated 1 mm. slide whose smallest division was 10μ.

Results.

Many of the results will be presented in this section according to the events which occurred during a particular week of gestation. Although this method may make for some difficulty, it has been selected because the chapter is more concerned with understanding how the individual parts contribute to the whole process of muscle development than with presenting each aspect of the development separately.

5 to 6 weeks gestation. (1 fetus)

The limb buds of this fetus were so small that they could not be sectioned individually and the whole fetus was sectioned longitudinally. There were no identifiable myoblasts in the limb buds, and the only tissue recognisable as developing muscle was found in the vicinity of the fore-limb.

Since this was associated with a profusion of red blood cells it is most likely that this was developing cardiac muscle. Many small myoblasts were found together in groups, and some of the cells were large for this gestation, being up to 20μ in diameter. By means of a silver stain it was possible to see also some large tracts of nerve growing out into the extremities. Most of the axons in these tracts appeared at this stage to end blindly in unspecialised tissue. However, on axons associated with the developing cardiac muscle there did appear to be a number of very immature neuromuscular contacts which consisted principally of very thin terminal axons, whose bulbous endings were closely apposed to myoblasts.

7 weeks gestation. (1 fetus)

Transverse sections of the lower leg showed that many of the muscle groups were distinguishable, although the muscle cells themselves were rather disorganised with scattered small groups of myoblasts, each up to 10μ diameter, interspersed with nuclei, some of which were nearly as large as the muscle cells themselves. In addition, there were a few young myotubes present, up to 60μ in length, with central chains of up to seven nuclei: in one 10μ section a total of five such cells were visible in the soleus and gastrocnemius muscles.

Histochemical staining with NADH-d showed that the large majority of cells reacted only very slightly, and generally remained very pale over their entire area (Plate 1a). However, some of the larger cells, which were probably also the more mature ones, became more heavily stained mainly in the perinuclear region, with a clear ring of sarcoplasm around. When stained for ATPase at pH9.4 the majority of the cells in this case also became only very faintly stained, a pale brown colour. Again some of the larger cells

showed a different pattern from that seen in the smaller cells.

The distribution of stain appeared to be slightly different in the cells of the soleus from those of the gastrocnemius. In the gastrocnemius the larger cells usually had a darker ring of sarcoplasm with a pale nucleus at the centre, while in the soleus the cells appeared somewhat darker over almost their entire surface. The mean diameters of these large cells in the two muscles were however very similar, at 9 - 10μ. In all cases the larger cells appeared to be randomly scattered over the area of the muscle.

The combined silver and AChE stain demonstrated the presence of several very small and very immature neuromuscular connections in both the soleus and gastrocnemius muscles (Plate 1d). Many of these connections were similar to those described in cardiac muscle at 5-6 weeks, but in addition to bulbous nerve endings there were also longer terminal axon processes, which were attached to a much larger area of a cell circumference. Such connections were not seen in longitudinal sections of myotubes but the diameters of the cells on which these connections were seen suggested that they were among the larger cells, similar if not identical to those described above, and could well have been myotubes cut transversely. In addition to axons participating in actual neuromuscular contact, there were others which appeared to be undergoing branching and terminal ramification.

8 weeks gestation. (5 fetuses)

The degree of organisation of the blocks of muscle tissue was not uniform in all 5 specimens, and varied from two in which the demarcation was extremely poor, to two specimens in which the individual muscles were very clearly delineated. The one muscle specimen from the thigh was more mature than any other. It was also found that the muscles whose organisation was

Plate 1

a. Transverse section of the gastrocnemius muscle of a 7 week fetus (72/48). A few stained muscle cells are visible. NADH-d, x 135.

b. Transverse section of the soleus muscle of an 8 week fetus (72/96). More mature muscle cells have a darkly-staining rim of cytoplasm with a pale centre. NADH-d, x 330.

c. Transverse section of the leg muscle of an 8 week fetus (73/134). Only a few muscle cells are stained. ATPase pH9.4, x 135.

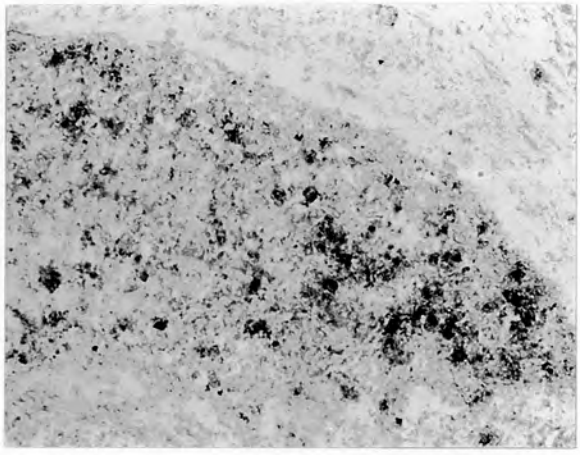
d. Upper. A fine terminal axon with its bulbous ending closely apposed to a myotube.

Lower. A terminal axon (possibly branched) making contact with a small group of myotubes.

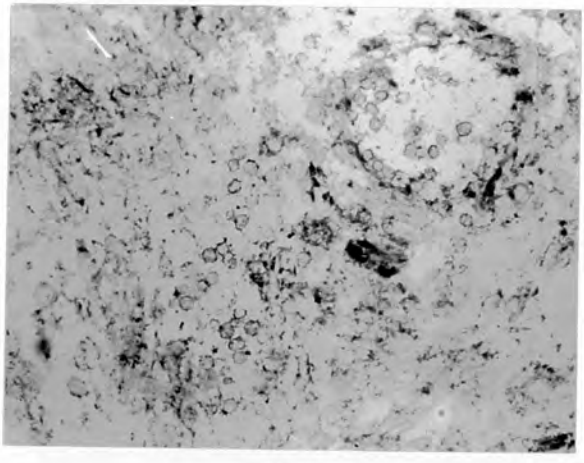
7 week fetus (72/48). AChE and silver impregnation, x 1450.

e. Section of the thigh muscle of an 8 week fetus (1006). Several terminal axons making contact with myotubes, forming very early motor endplates. AChE and silver impregnation, x 520.

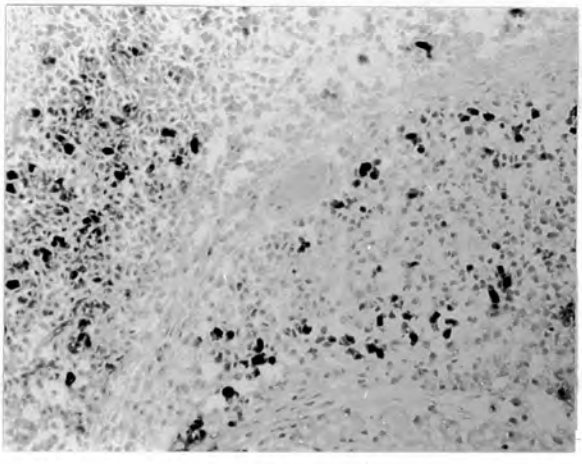
PLATE 1



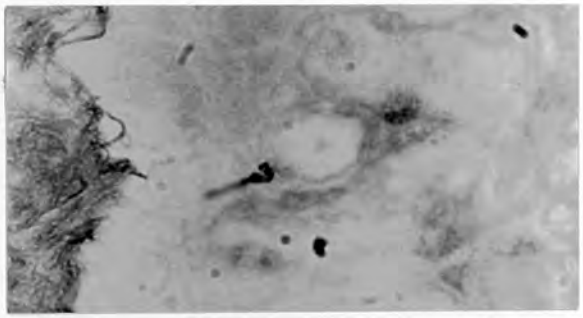
a

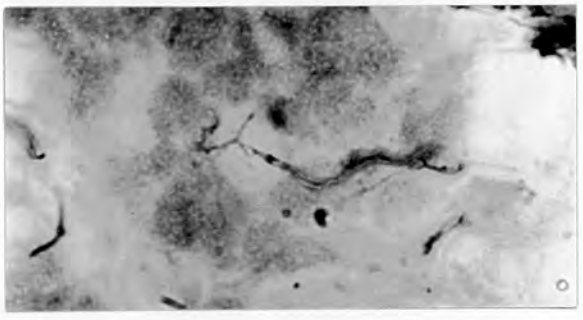


b

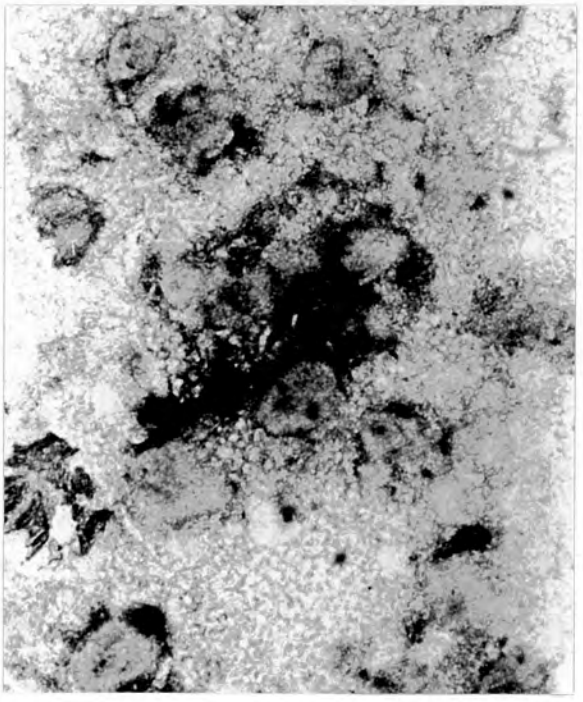


c





d



e

poor had very few myotubes present, and these were nearly all very small, while the more well defined muscle groups had more myotubes, often found in groups of 3-4, some of which contained chains of 15 central nuclei. In transverse sections such cells were larger than the majority of myoblasts around them, became more heavily stained with eosin, and were seen in all the sections. There also appeared to be preferential histochemical staining of these larger diameter cells, while the smaller cells had only very faint general staining.

With NADH-d the larger cells showed two patterns of staining. Some of them had a strong perinuclear stain, as at 7 weeks, although, in some thigh muscle, some also had stain present in the nuclear region. Other cells, nearly all perfectly circular, had become darkly stained only in a narrow ring at their peripheries (Plate 1b). ATPase at pH4.3 demonstrated no staining present in any of the muscle cells, but at pH9.4 scattered darker cells could be seen among a large number of very light, less mature ones (Plate 1c). Many of the large cells were stained over their entire cross-sectional area, while others, similar to those at 8 weeks, had a pale nucleus bounded by a dark ring of sarcoplasm.

In the study of the motor innervation, the sections from the legs still showed only rudimentary neuromuscular connections, similar to those visible at 7 weeks, while in the thigh muscle there was clear evidence of a more mature degree of neuromuscular junction formation (Plate 1e). Occasionally several neuromuscular junctions at similar stages of development were seen on neighbouring muscle cells, and appeared to have been formed as a result of collateral or ultraterminal branching of the terminal axon.

9 weeks gestation. (3 fetuses)

Long myotubes were scattered throughout the thigh muscle, and in

the muscles of the legs of these fetuses, although the muscle groups themselves were not at all clearly demarcated. Transverse sections showed the presence of many scattered larger cells which stained intensely with eosin, the cells themselves being similar to those seen at 8 weeks, although their numbers had increased.

The stains for NADH-d and ATPase, showed essentially the same as was seen at 8 weeks (Plate 2 a,b), but there were some apparent centres of aggregation where groups of more darkly staining cells were found.

The motor innervation at 9 weeks (Plate 2 c - e) had progressed to a stage where there were comparatively many neuromuscular junctions together with many axons, whose terminal ramifications and divisions were seen in greater numbers. The neuromuscular connections themselves often had bizarre appearances, and could be seen as either many-fingered attachments, each up to 10μ long, closely applied to the surface of a myotube, or occasionally bearing projections which themselves appeared closely attached to the muscle cell. In some cases, when the neural part of the neuromuscular apparatus could not be seen, the stain showed many very small closely-packed areas of AChE activity on the cell surface, to which small processes from the immature neuromuscular junctions may well have been attached.

<u>10 weeks gestation</u>. (5 fetuses)

Muscle histology again indicated considerable variability between the same muscle blocks in different specimens. Some muscles were composed of loosely connected cells at different stages of maturation, while others had many larger cells which stained darkly with eosin, and contained up to 10% of the nuclei in the muscle (Plate 3 a). As in younger fetuses, some large

Plate 2

Sections of the soleus muscle of a 9 week fetus (73/124).

a. A few stained cells present. NADH-d, x 330.

b. None of the muscle cells is at all stained. ATPase pH 4.3, x 135

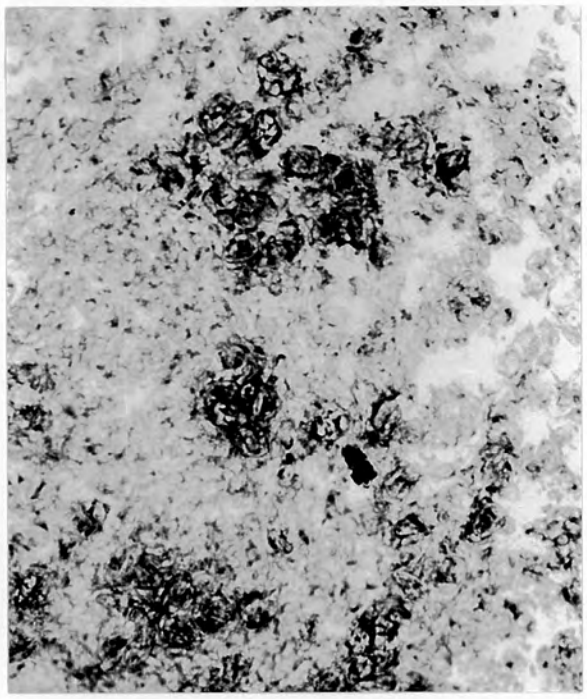
c-e. AChE and silver impregnation.

c. A single terminal axon ending in 1 motor endplate on a myotube. x 1450.

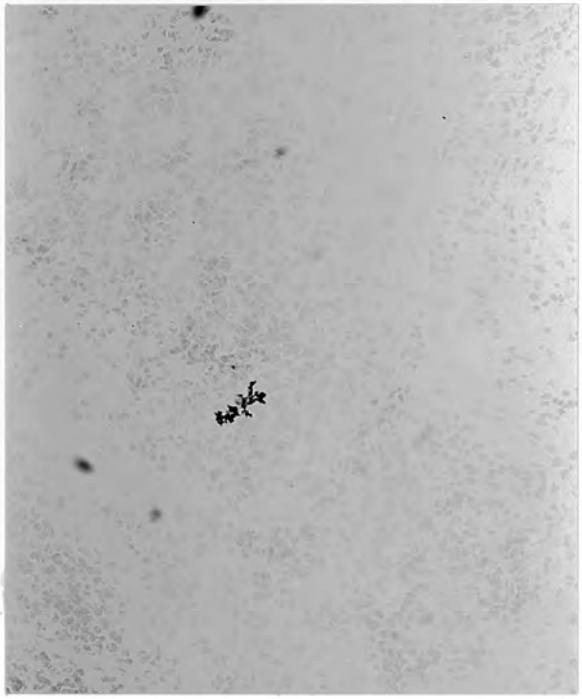
d. Primitive motor endplates of different shapes on a group of myotubes. x 1450.

e. Possible double innervation of a myotube by two separate motor endplates. x 1250.

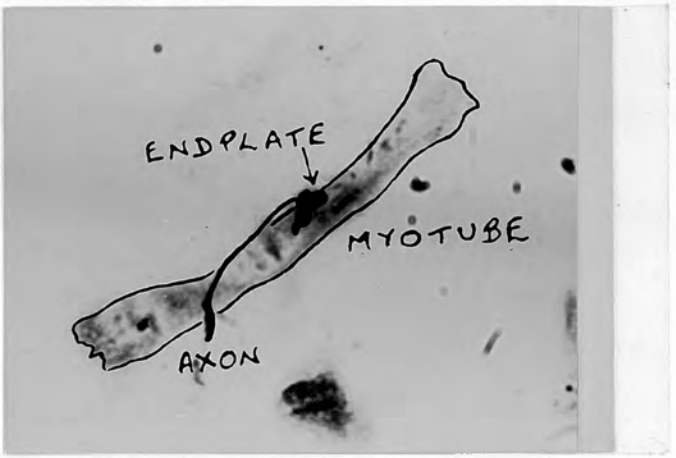
PLATE 2



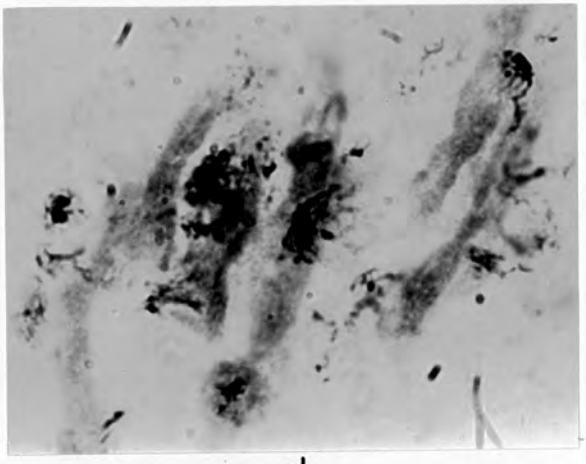
a



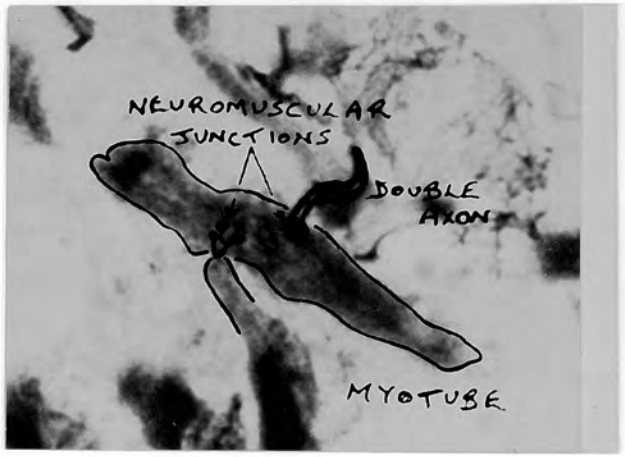
b



c



d



e

cells were clearly myotubular with chains of central nuclei, while others contained as few as 2 nuclei, and were evidently the most recently formed multinucleated cells. The large cells were at this stage of gestation 10-15μ in diameter. By means of the PTAH stain, it was possible to see a few peripheral myofibrils in some of the larger cells, although at this stage no cross-striations could be seen.

At this stage NADH-d staining of the larger cells (Plate 3 b) showed a light blue reaction product over their entire area. Most of these cells still showed a darkly stained ring at the periphery of the sarcoplasm, and either did or did not have a dark stain in the nuclear region in the centre of the cell. Using ATPase at pH4.3, there was still very little stain at all on any of the muscle cells, but at pH9.4 (Plate 3c, d), in muscle from the fetus in which myofibrils could be seen, the darker staining appeared to have become concentrated in certain areas within the sarcoplasm which could be related to myofibril development. Otherwise the staining at this stage was very similar to that seen at 9 weeks gestation. In addition the motor innervation appeared to have changed little (Plate 3 e), except that there were sufficient motor endings present for them to show up as distinct bands of motor innervation in most muscle blocks.

11 weeks gestation. (4 fetuses)

In these fetuses, the development of the thigh muscles was again seen to be more advanced than that of the leg muscles. In the legs the soleus muscles contained generally more, and more mature, multinucleated cells than the gastrocnemius muscles, and this was reflected by the degree of staining present in the multinucleated cells of those muscles (Plate 4 a). Staining for NADH-d, showed that about 50% of the cells in the soleus

Plate 3

Sections of gastrocnemius muscles of 10 week fetuses.

a. Myotubes are present in small groups. (Fetus 988). H and E, x 330.

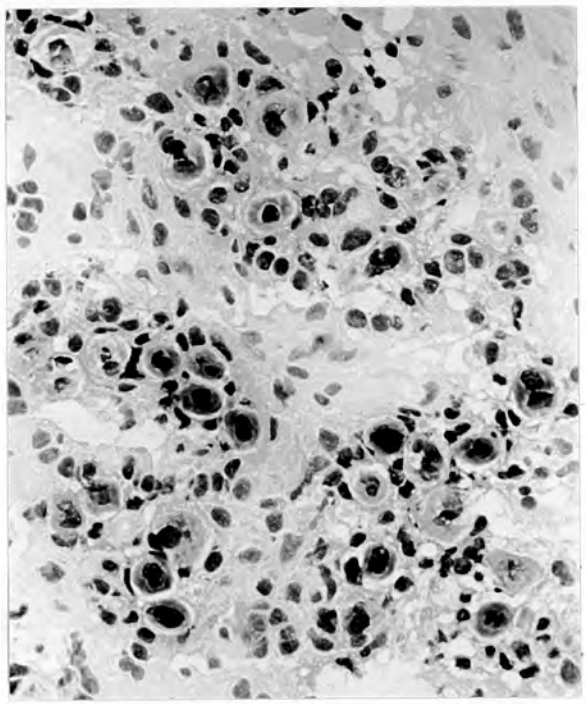
b. Strong perinuclear stain in myotube groups. Cytoplasm of myotubes is unstained. (Fetus 73/138). NADH-d, x 330.

c. A few cells both in longitudinal and transverse section are heavily stained. (Fetus 72/61). ATPase pH 9.4, x 135.

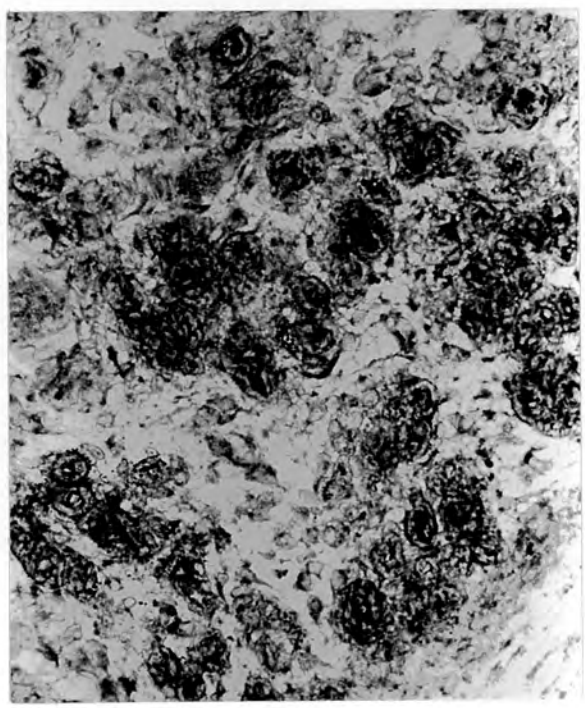
d. Particulate staining of some muscle cells, possibly associated with developing myofibrils. (Fetus 73/138). ATPase pH 9.4, x 330

e. A single axon terminating in 1 motor endplate on a myotube. (Fetus 73/138). AChE and silver impregnation, x 520.

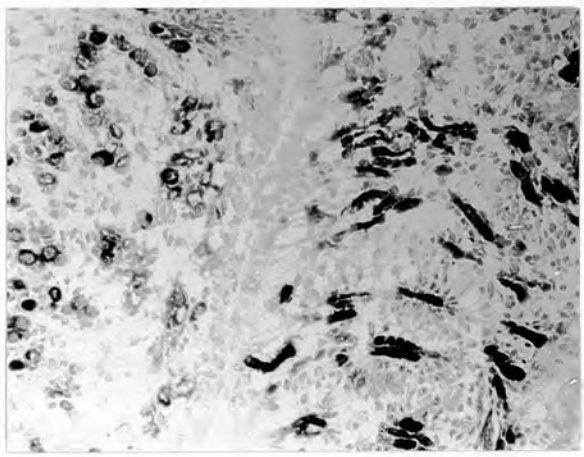
PLATE 3



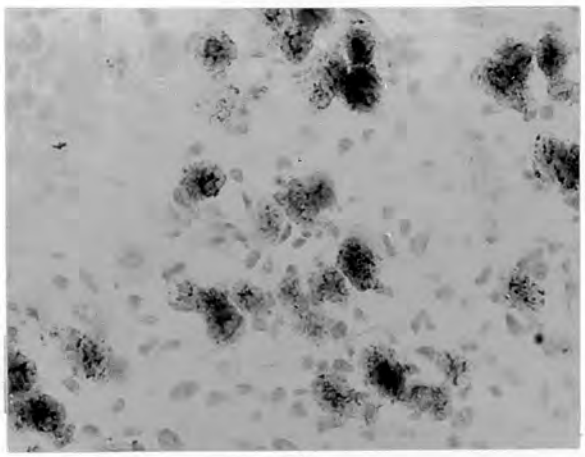
a



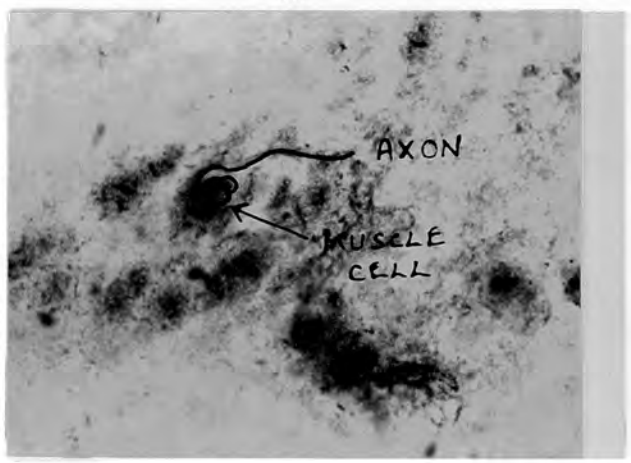
b



c



d



e

reacted in the manner characteristic of the more mature multinucleated cells, while the percentage in the gastrocnemius was lower: but in both muscles the large multinucleated cells were found in groups of up to 10 cells together (Plate 4 b). Staining for ATPase at pH9.4 (Plate 4 c) emphasised that the muscle cells' histochemical pattern was far from stable, since even among the larger cells which were relatively heavily stained, even this staining was patchy on some cells.

The motor innervation to the muscle cells did show one new feature at this stage (Plate 4d, e), which was the presence of two distinct areas of AChE positivity on the circumference of some of the larger cells, although in general the neuromuscular junctions were still very immature. In one fetus there was clear evidence of an adult-like band of motor innervation on several longitudinally sectioned myotubes lying side by side.

12 weeks gestation. (9 fetuses)

At this period of development, some muscle cells were seen for the first time to have peripheral nuclei and a very few cross-striations. It was still found that there was a higher proportion of multinucleated cells in the soleus than in the gastrocnemius (Plate 5a). Histochemically, further maturation of the larger cells was evident by means of NADH-d staining, since small, discrete, very dark particles could be seen in the sarcoplasm of some cells. These particles may represent the earliest mitochondrial aggregates normally present between the myofibrils of adult muscle (Plate 5b).

By means of ATPase staining at pH9.4, three distinct types of large cells were visible in one fetus (Plate 5c). The presumably least mature of these had a narrow ring of dark sarcoplasm around a pale or colourless nuclear region. The second cell type had a much thicker rim of darkly

Plate 4

Sections of muscle from 11 week fetuses.

a. Long myotubes present in the soleus muscle of fetus 987.
H and E, x 135.

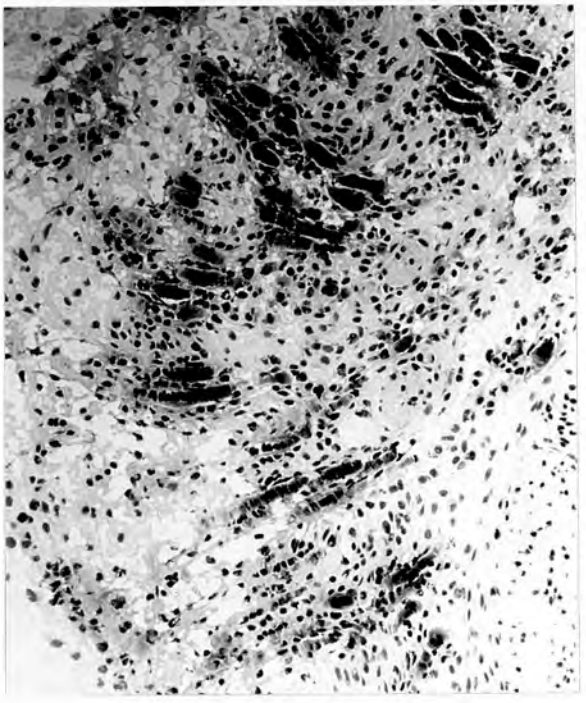
b. A few darkly-staining myotubes among many pale ones in the gastrocnemius muscle of fetus 987. NADH-d, x 330.

c. Muscle cells from the leg of fetus 72/207. ATPase pH9.4, x 135.

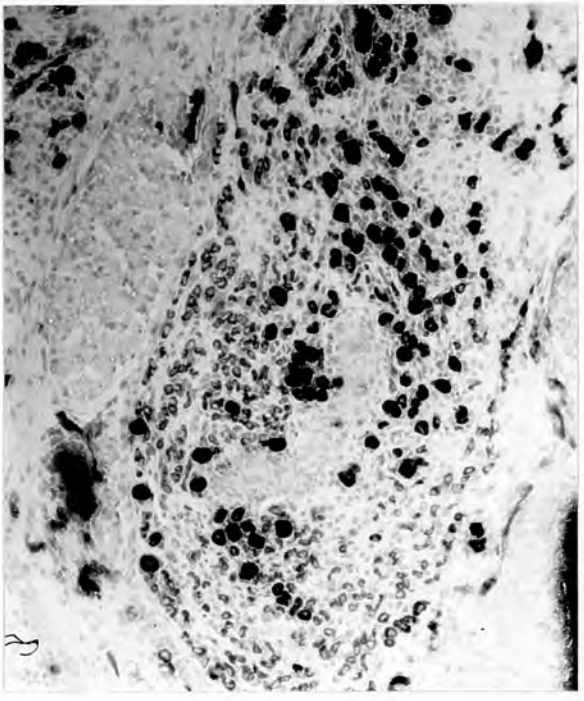
d. Double innervation of a myotube, by 2 terminal axons. (Fetus 73/181)
AChE and silver impregnation, x 1095.

e. AChE positive sites on neighbouring myotubes. Some myotubes have 2 such sites on their circumference. (Fetus 73/181).
Silver-intensified AChE, x 520.

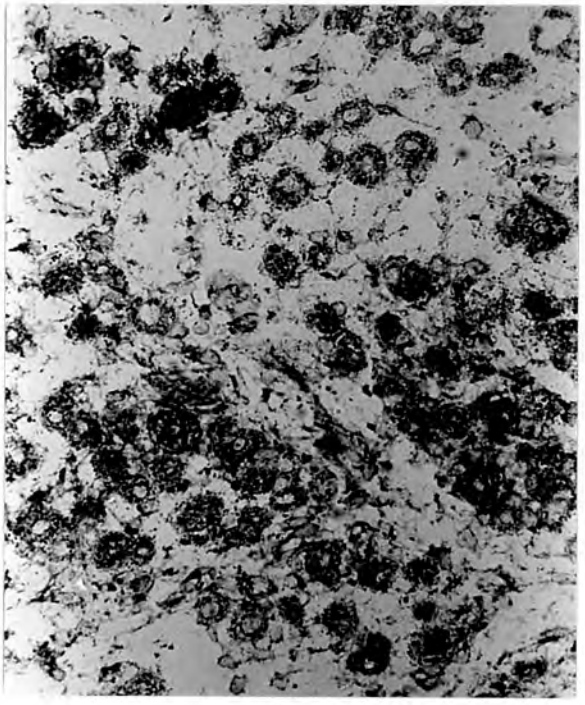
PLATE 4



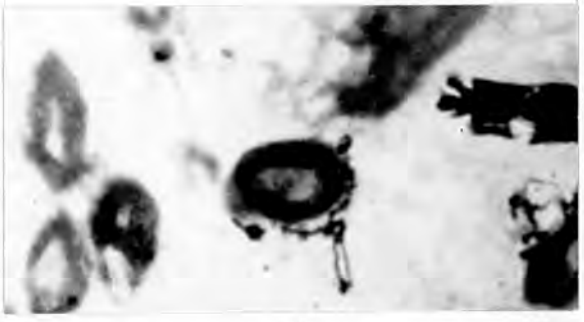
a



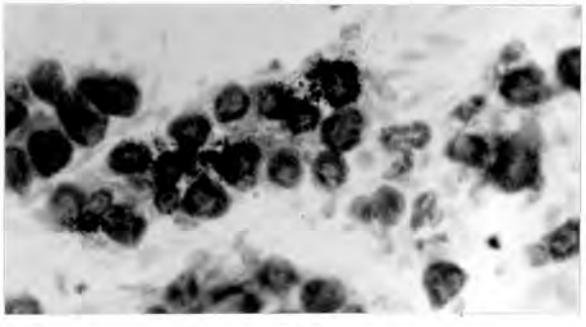
c



b



d



e

Plate 5

Sections of muscle from 12 week fetuses.

a. The soleus and gastrocnemius muscles from fetus 72/20.
H and E, x 135.

b. Myotubes in the gastrocnemius muscle of fetus 73/88. NADH-d, x 330

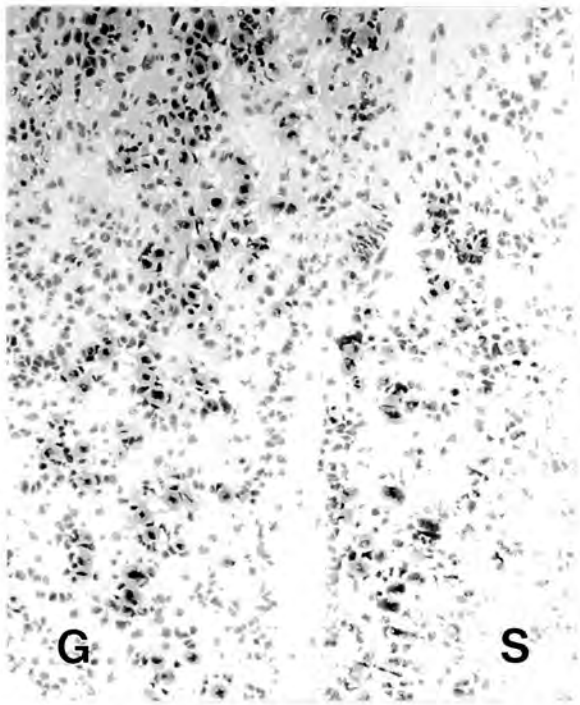
c. Darkly and lightly staining myotubes in the soleus muscle of fetus 72/56. ATPase pH 9.4, x 330.

d. and e. Terminal motor innervation in the gastrocnemius muscle of fetus 73/221.

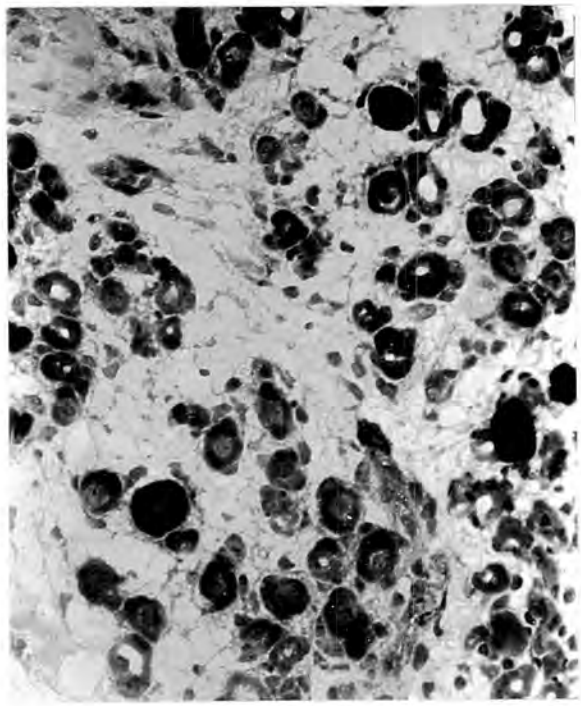
d. Double innervation of a myotube, and terminal axon branching. AChE and silver impregnation, x 520.

e. Motor endplates formed on groups of neighbouring myotubes. Silver-intensified AChE, x 520.

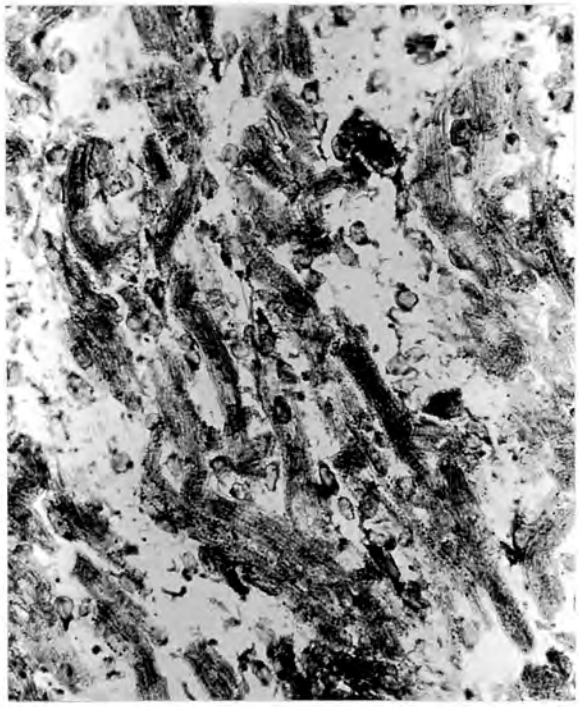
PLATE 5



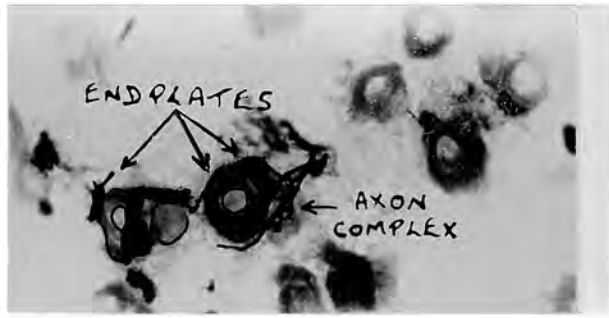
a



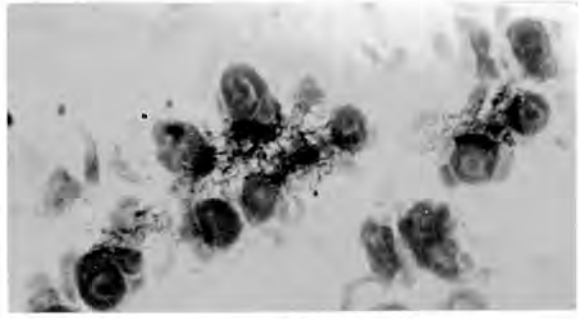
c



b



d



e

stained sarcoplasm, with the nuclear region staining only palely, and the most mature was the largest and was stained over its entire cross-sectional area.

The motor innervation of the muscle at this stage (Plate 5d, e) was not found to have altered, but rather to have been consolidated, with more neuromuscular junctions present, innervation bands clearly evident in nearly all muscles, and the presence in the majority of cases of one terminal axon ending in one motor endplate on a muscle fibre. Occasionally, however, there was evidence of some less restricted areas of AChE positivity, possibly indicating the formation of a second neuromuscular junction on that muscle cell.

13 weeks gestation. (6 fetuses)

The histological and histochemical observations in the muscles of these fetuses were very similar to those at 12 weeks gestation. The terminal motor innervation was slightly different in that small tenuous ultraterminal and collateral branches of terminal axons could be clearly seen. In addition the basic pattern of the motor innervation was slightly different, as one or more AChE positive sites could now be clearly seen on many myotubes (Plate 6 a).

14 weeks gestation. (8 fetuses)

The proportion of myotubes was found to be continuing its steady increase, and although, probably for technical reasons, cross-striations were not always obvious, the muscle cells were becoming grouped into definite fascicles, and some myotubes were beginning to show peripherally displaced nuclei.

ATPase staining at pH9.4 demonstrated that there was a wide range of fibre types, similar to the three first described at 12 weeks (Plate 6 b). The most darkly staining cells may well have included most of the mature cells

whose nuclei now occupied a peripheral position, having been displaced by the rapidly increasing number of myofibrils. NADH-d staining also showed the continued progression to maturity of more and more myotubes. Increasing numbers of these in 5 of the fetuses of this gestation, had in their sarcoplasm the intensely darkly staining discrete particles which were first seen at 12 weeks gestation (Plate 6 c), and in at least one of these five fetuses, parallel chains of these particles could be seen in myotubes visible in longitudinal section.

This period of gestation, at 14 weeks, marked a new phase in the development of the motor innervation, insofar as the establishing of neuromuscular contacts was concerned. Up to this stage the number of multinucleated cells had been increasing rapidly, and the motor innervation had kept pace with this development in the constant and rapid formation of new neuromuscular connections. As early as 11 weeks gestation, it had been noted that some of the myotubes may well have had two AChE positive areas on their surface, and this was found in an increasing proportion of myotubes up to the present stage. At 13 weeks the observation had been made that there was ultraterminal and collateral branching of some terminal axons, and the major difference at 14 weeks was that this complex pattern of branching was becoming characteristic of more and more of the terminal motor innervation (Plate 6 d).

The development of the motor innervation in this respect, which has been described in some detail up to 14 weeks gestation, will not be described so fully from this stage on, since its increasing ramification and complexity has made quantitation of the development on subjects the size of these neuromuscular junctions and terminal axons impossible. However, at 14 weeks

Plate 6

a. Double innervation of some myotubes in the soleus muscle of a 13 week fetus (73/210). Silver-intensified AChE, x 520.

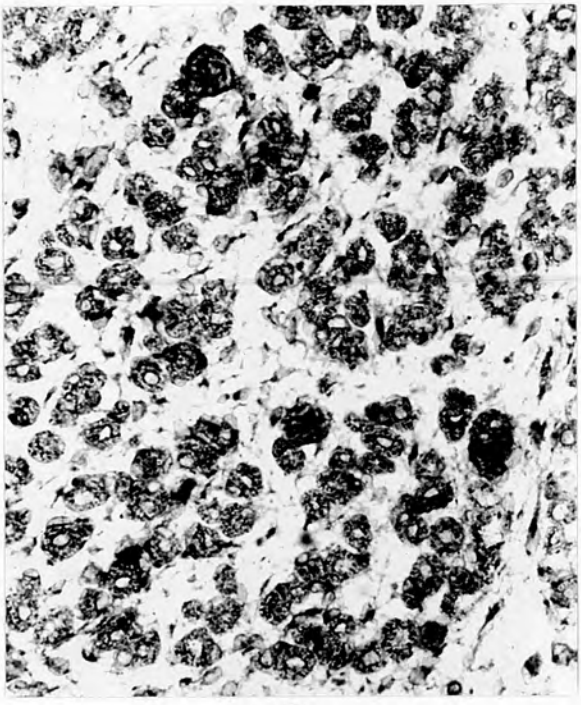
b. Darkly and lightly staining myotubes in the soleus muscle of a 14 week fetus (73/16). ATPase pH 9.4, x 360.

c. Groups of myotubes, nearly all with some cytoplasmic stain, in the gastrocnemius muscle of a 14 week fetus (72/202). NADH-d, x 330.

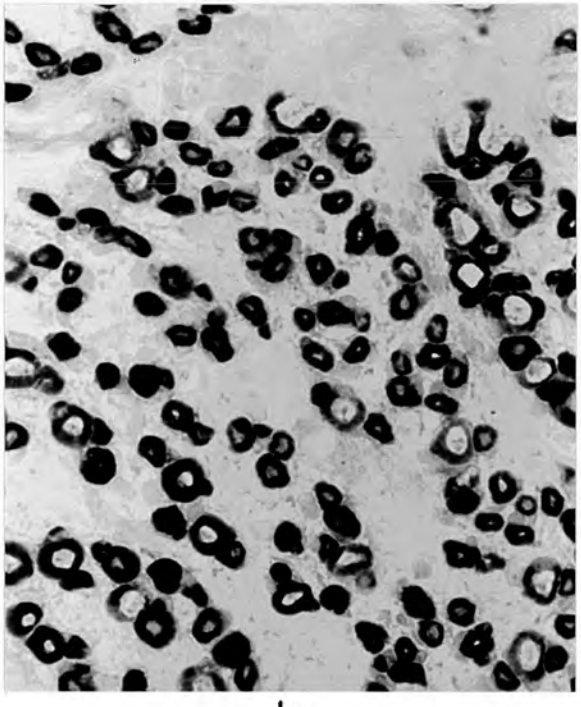
d. Complex terminal axon-motor endplate interconnections in the leg muscle of a 14 week fetus (72/23). AChE and silver impregnation, x 1095.

PLATE 6

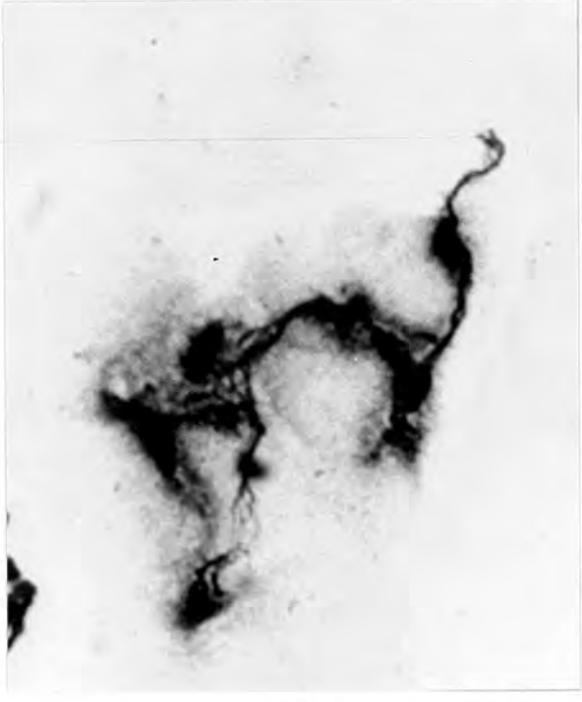
a



c



b



d

it was also seen that there were more comparatively small neuromuscular junctions, in addition to those which had become well established and were considerably larger. This development of the actual area of close neuromuscular contact will, on the other hand, be described in detail as changes occur in increasing proportions of the numbers of motor endplates involved.

15-21 weeks gestation. (15 weeks - 4 fetuses: 16 weeks - 3 fetuses: 17 weeks - 3 fetuses: 18 weeks - 3 fetuses: 19 weeks - 2 fetuses: 20 weeks - 2 fetuses: 21 weeks - 2 fetuses).

During this period of development, gradual histological changes were seen in the skeletal muscle (Plates 7c, 9c) which resulted, except for their diameter, in the almost completely adult appearance of the fibres. At 15 weeks the soleus was composed largely of the adult type of muscle fibre, although these comprised only about half of the muscle cells of the gastrocnemius, the rest being myotubes at different stages of maturation. At 16 weeks gestation, the development of the muscle cells of the gastrocnemius still lagged behind that of the soleus fibres. By 18 weeks, there were few myotubes present at all in either the soleus or gastrocnemius, and between this stage and 21 weeks gestation it could be seen in transverse section that the muscle fibres, now accompanied by extremely few myotubes, became packed into fascicles within the muscles. At this period of development, although there was still variation in fibre size, the diameters of the muscle fibres were not very different from those seen in fetal muscle between 10 and 12 weeks gestation. Between 18 and 21 weeks gestation, there was also the first appearance in the soleus and gastrocnemius muscles of several scattered fibres which had larger diameters than any of the other cells.

Histochemically the skeletal muscle underwent considerable changes during the 15 - 21 week period of development. At 15 weeks it could be seen that the sarcoplasm of the majority of the fibres and myotubes present in the muscle reacted strongly for NADH-d, and contained some of the small intensely stained particles already described (Plate 7 a). At 16 weeks the findings were similar, except that the perinuclear stain, similar to that in the larger cells at 7 - 8 weeks gestation, was decreasing in intensity. ATPase staining at pH4.3, showed instead of a few dark cells as at 12 - 13 weeks, that all the muscle cells had become darkly stained (Plate 7 e). At pH9.4 (Plate 7 d) the staining intensity was still uneven on some of the cells present, and there were dark and light patches on both the larger and smaller diameter cells.

By 17 - 19 weeks the histochemical pattern of the muscle, now nearing adult form histologically, began at first very slowly to take on a more stable appearance. The histochemical stains which in the earlier stages of development first demonstrated the presence of enzymatic activity in only a few of the larger cells, and only later in all the cells, now began to adopt a pattern of stain distribution which, with NADH-d, was similar to that seen in those early stages. Between 17 - 19 weeks it became apparent that there were two cell populations with only a few darker cells among a large excess of light ones (Plates 8 a, 9 a). Using ATPase at pH9.4 the appearance was almost the exact opposite of this (Plate 9 b). Instead of the few darker cells which had been evident at earlier stages of gestation, there was now a large excess of the dark cells, and only a few lighter ones. Using ATPase at pH4.3 the opposite was the case (Plate 8 c).

Plate 9a,b demonstrates that by 19 weeks gestation there is a

Plate 7

a. Occasional very darkly stained myotubes in the gastrocnemius muscle of a 15 week fetus (980). NADH-d, x 330.

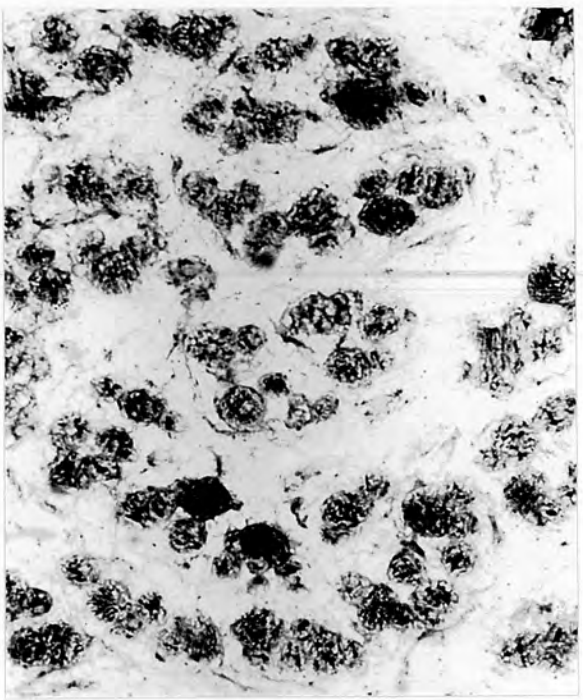
b. Tenuous terminal axons connecting motor endplates on different myotubes in the gastrocnemius muscle of a 15 week fetus (73/32). AChE and silver impregnation, x 585.

c. Groups of myotubes with central and peripheral nuclei in the gastrocnemius muscle of a 16 week fetus (938). H and E, x 330.

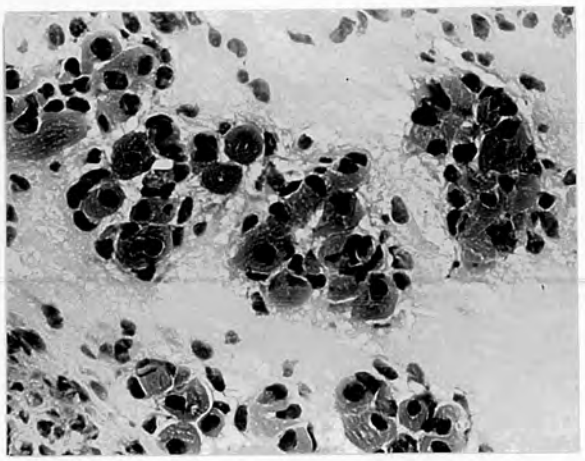
d. Excess of darkly staining myotubes in the triceps surae of a 16 week fetus (73/55). ATPase pH 9.4, x 360.

e. Darkly staining myotubes in the triceps surae of a 16 week fetus (73/55). ATPase pH 4.3, x 330.

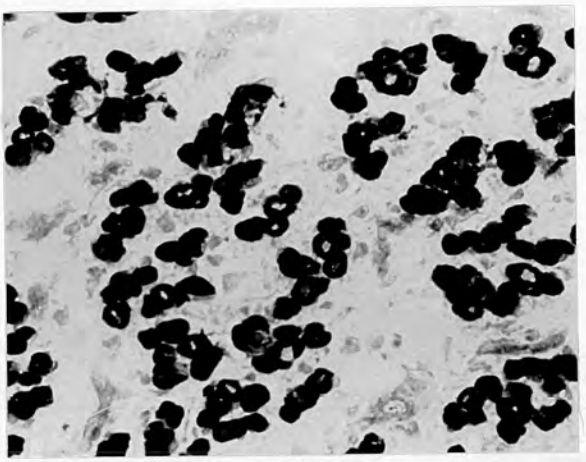
PLATE 7



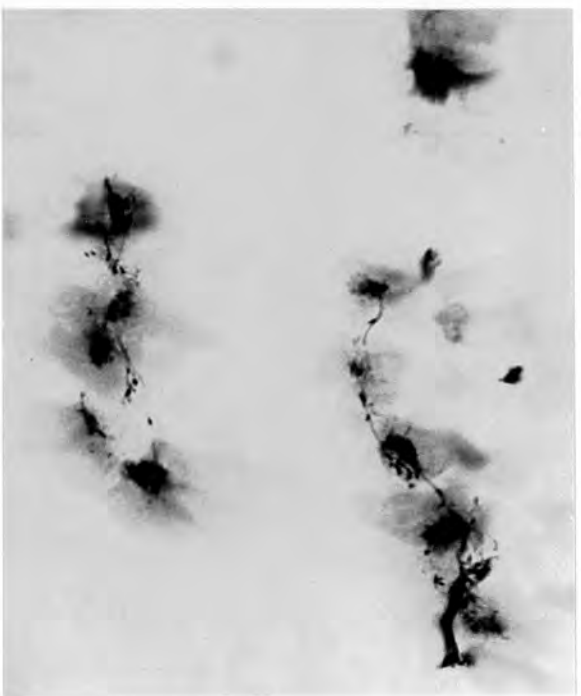
a



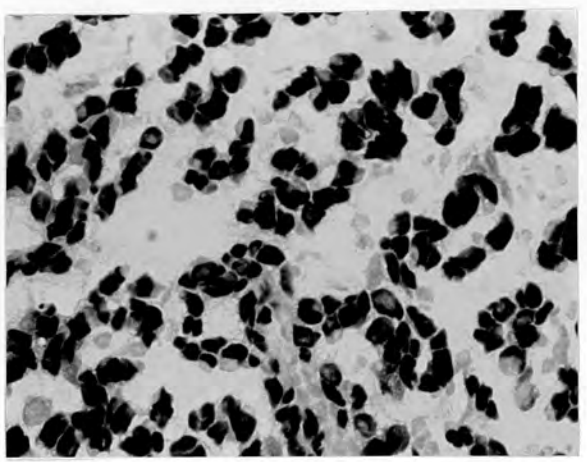
c



d



b



e

Plate 8

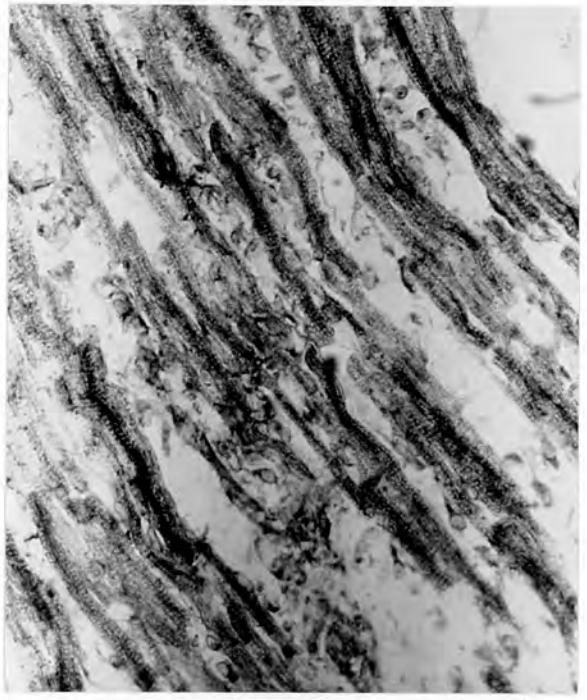
a. Darkly and lightly stained fibres in the gastrocnemius muscle of a 17 week fetus (73/259). NADH-d, x 135.

b. 4 or 5 motor endplates interconnected by fine terminal axons in the triceps surae of a 17 week fetus (72/345). AChE and silver impregnation, x 1500.

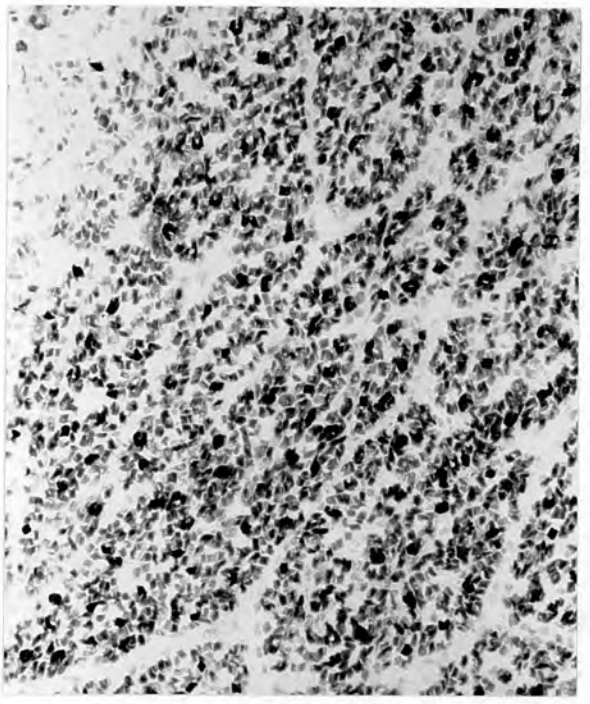
c. Scattered, darkly staining fibres in the gastrocnemius muscle of an 18 week fetus (73/130). ATPase pH 4.3, x 135.

d. Complex terminal axon-motor endplate connections in the triceps surae of an 18 week fetus (73/81). AChE and silver impregnation, x 330.

PLATE 8

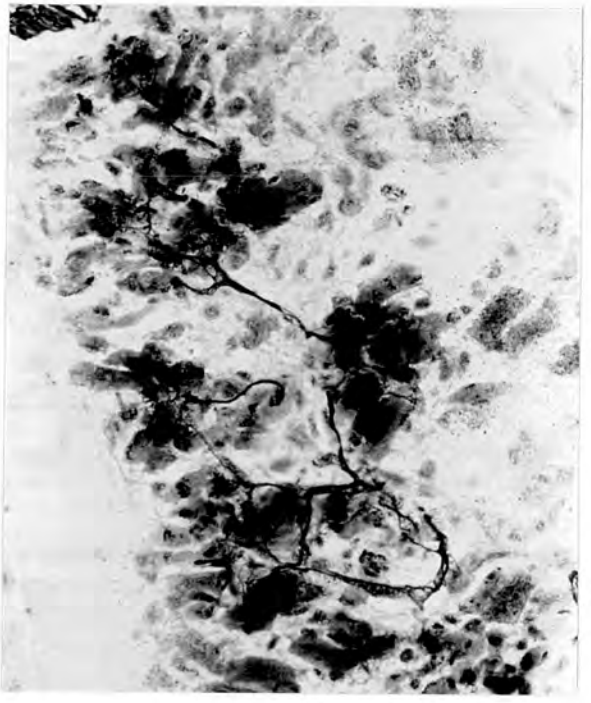


a



c

b



d

Plate 9

a and b. Partially reciprocal pattern of histochemical staining visible in the soleus muscle of a 19 week fetus (72/210). x 280. Single arrows, type I fibres: double arrows, possibly type IIC fibres.

a. NADH-d

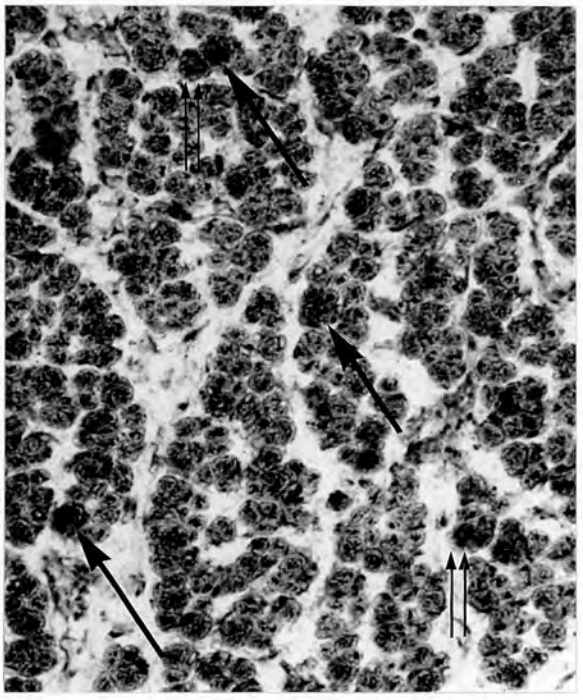
b. ATPase pH 9.4.

c. Mature form of muscle fibres in transverse section in the soleus muscle of a 19 week fetus (72/210). H and E, x 330.

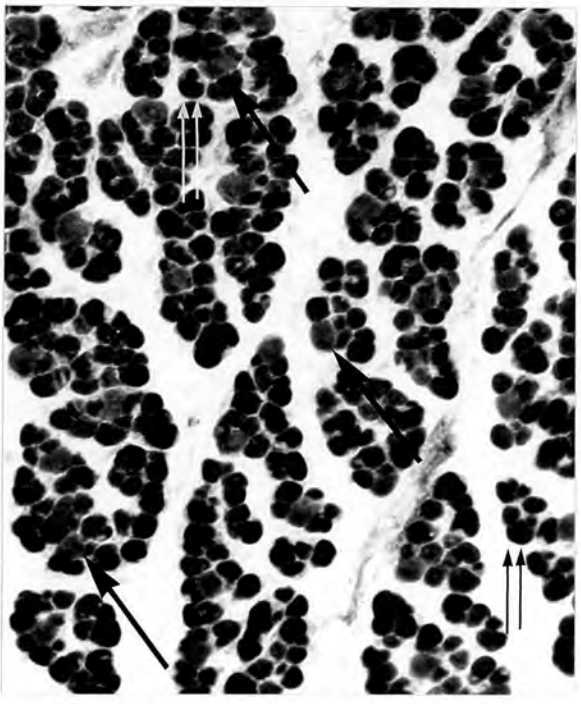
d. Terminal motor innervation in the soleus muscle of a 20 week fetus (73/125). AChE and silver impregnation, x 520.

e. Darkly and lightly staining fibres in the gastrocnemius muscle of a 20 week fetus (73/125). The staining properties are consistent along the length of the muscle fibres. NADH-d, x 135.

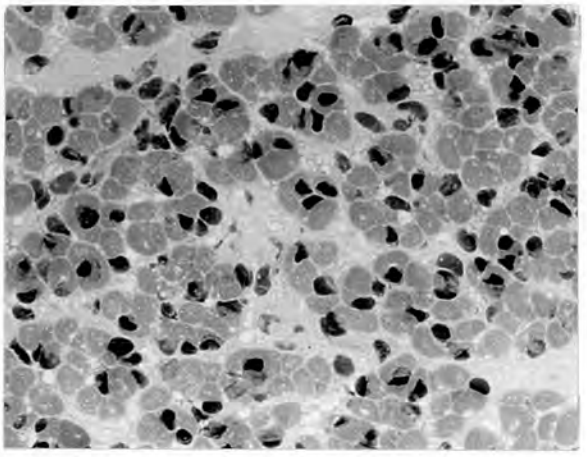
PLATE 9



a

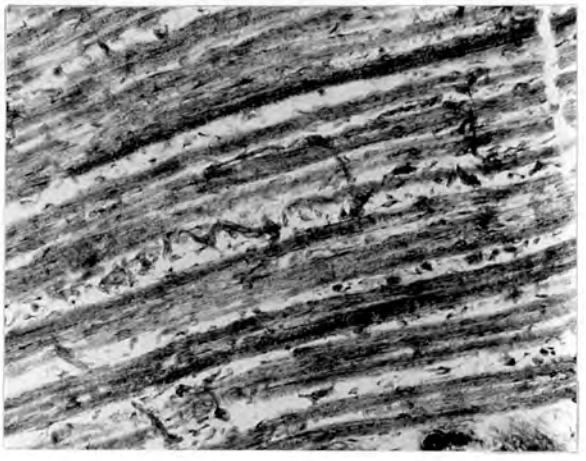


b



c

d



e

Plate 10

a and b. Partially reciprocal pattern of histochemical staining visible in the soleus muscle of a 21 week fetus (72/198). x 225.

Single arrows, type I fibres: double arrows, possibly type IIC fibres.

a. NADH-d

b. ATPase pH9.4.

c. Terminal motor innervation complex in the soleus muscle of a 21 week fetus (72/198). AChE and silver impregnation, x 520.

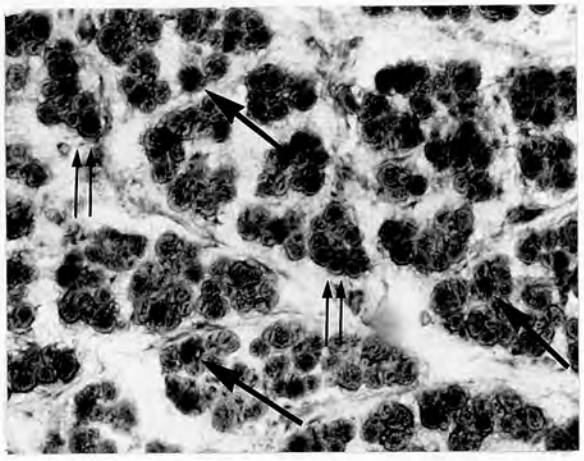
d and e. Partially reciprocal pattern of histochemical staining in the soleus muscle of a 22 week fetus (73/127). x 225.

Single arrows, type I fibres: double arrows, possibly type IIC fibres.

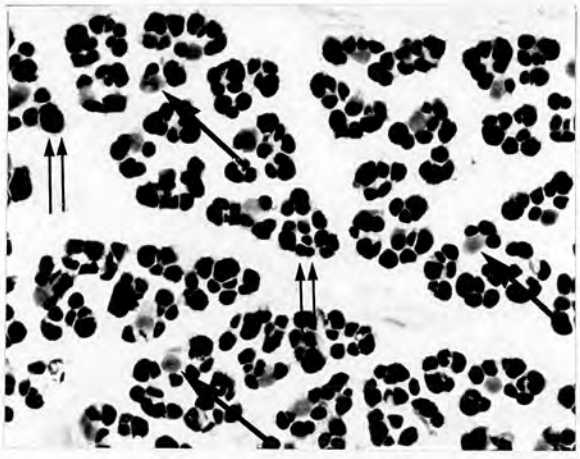
d. NADH-d

e. ATPase pH9.4.

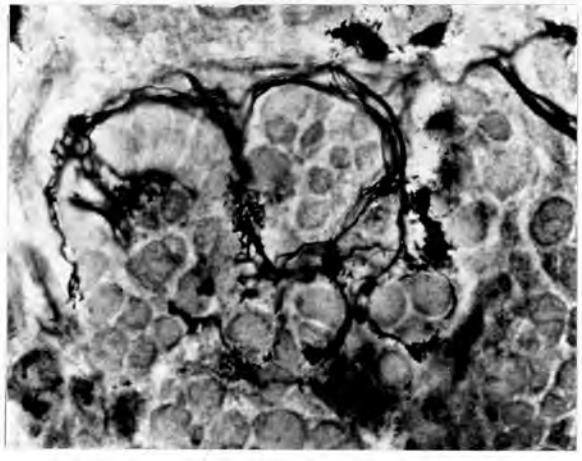
PLATE 10



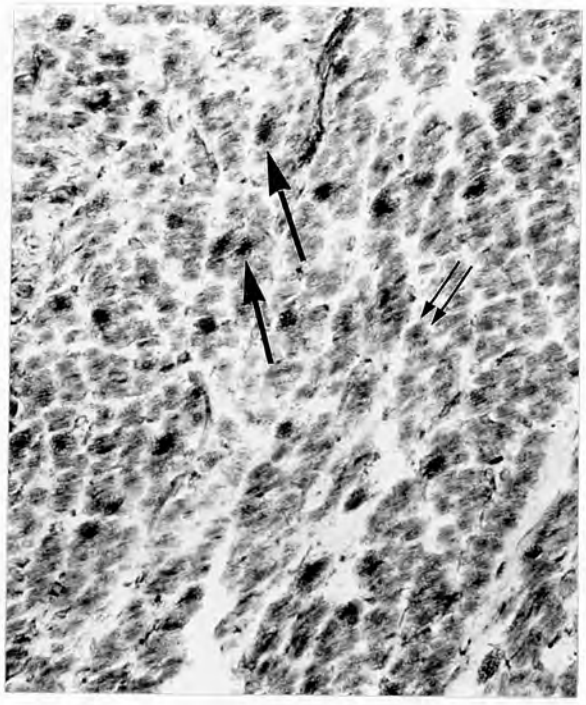
a



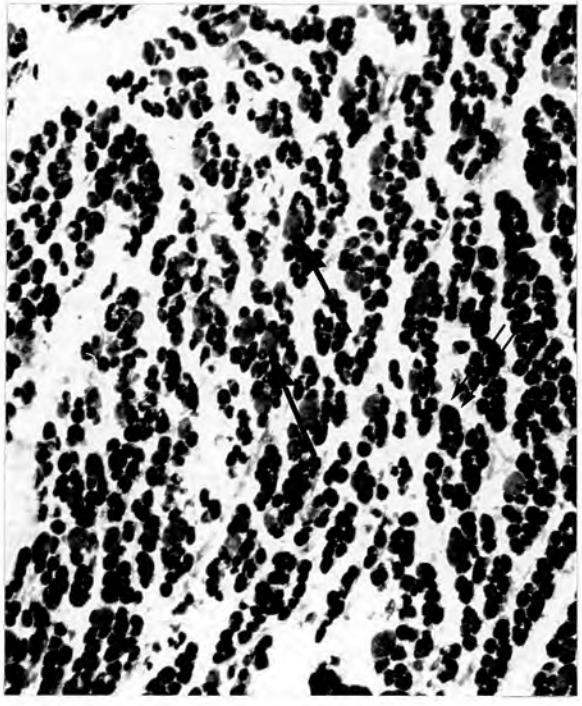
b



c



d



e

Plate 11

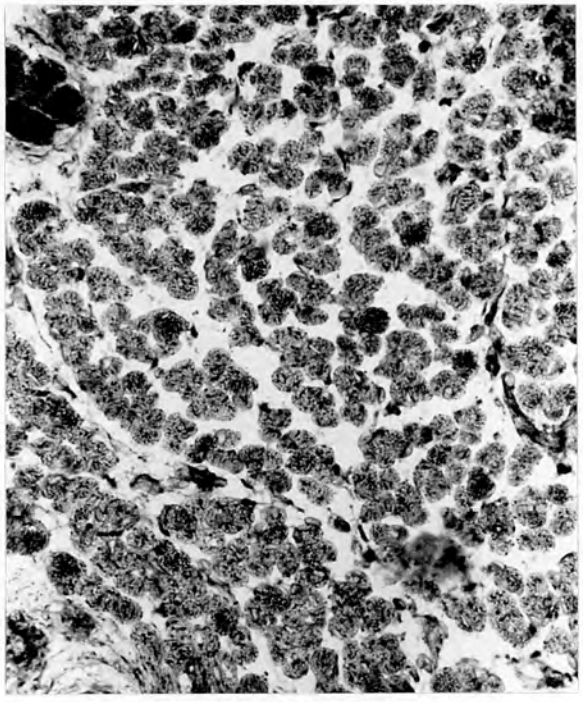
a. Excess of palely staining fibres in the soleus muscle of a 23 week fetus (994). NADH-d, x 330.

b. Terminal motor innervation in soleus muscle of a 24 week fetus (73/31). Note presence of some motor endplates (arrowed) with indentations of soleplate. AChE and silver impregnation, x 605.

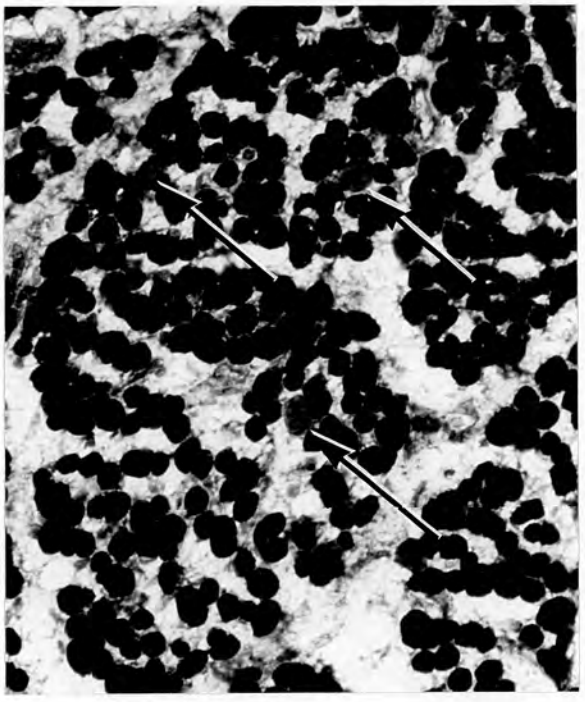
c. Histochemical staining of the triceps surae of a 28 week fetus (1015). Note the few palely stained fibres (arrowed). ATPase pH9.4, x 330.

d. Terminal motor innervation in the soleus muscle of a 28 week fetus $(73/90)_p$ - palely staining motor endplate. AChE and silver impregnation, x 770.

PLATE 11

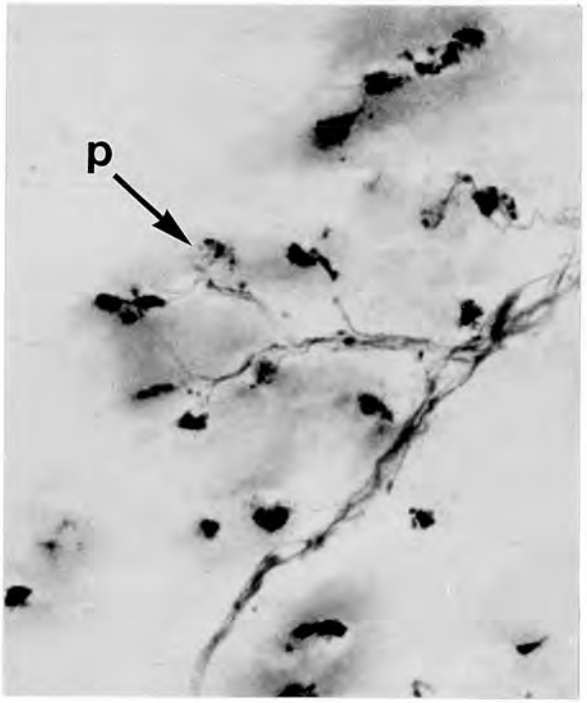


a



c

b



d

partially reciprocal histochemical pattern in the muscle. However, in addition to the fibres which stain darkly for NADH-d and lightly for ATPase, there are some which stain darkly for both enzymes.

These findings were confirmed in fetuses at 20 and 21 weeks gestation (Plates 9 e, 10a, b) but were very difficult to quantify meaningfully, to the extent that the proportions of light and dark cells as seen with either NADH-d or ATPase were not at all constant, or even similar in fetuses of the same or different gestations. In keeping with the histological findings at this period of gestation, it was possible in sections stained either for NADH-d or ATPase to see scattered in the muscle, cells which were larger than average, which stained darkly for NADH-d, and lightly for ATPase at pH9.4.

During this period of development the nature of the motor innervation appeared hardly to alter at all (Plates 7 b, 8b,d, 9 d). There were still many tenuous terminal axon branches whose interconnections with motor endplates and other axons continued to increase in number. There still existed also a profusion of types and sizes of motor endplates, incorporated into this maze of the terminal motor innervation, but AChE positive areas were at this stage hardly ever seen outside the closely defined territory of the motor endplate.

22 - 24 weeks gestation. (22 weeks - 3 fetuses: 23 weeks - 2 fetuses: 24 weeks - 2 fetuses)

The only differences from earlier periods in the histology of the muscle, were that the muscle fibres became even more tightly packed in their fascicles, and the proportion of myotubes shrank to almost nil, although some could still be seen in one 23 week fetus.

Histochemical differentiation was also clearly evident in the

muscle from these fetuses (Plates 10d, e, 11 a), although the staining for NADH-d and ATPase at pH9.4 still showed an only partially reciprocal pattern, very similar to that at 19 to 21 weeks, and unlike the adult pattern.

The 24 week stage of development, however, marked the beginning of another period of development for the motor endplate, though not the nature of the ramifying neuromuscular interconnections (Plate 11 b). Although there was still a profusion of motor endplates of many sizes, some were seen here for the first time, which appeared to have small indentations of the soleplate, which were similar to, although far less developed than, those found in more mature motor endplates.

25 weeks gestation - neonate. (25 weeks - 2 fetuses: 28 weeks - 3 fetuses: 29 weeks - 1 fetus: Neonate - 1)

Histologically the characteristics of skeletal muscle altered very little during this final period of development, and in the neonate the average muscle fibre diameter was only slightly larger than at 12 weeks gestation. In the one neonatal specimen available there did not appear to be any of the distinctively large muscle fibres which have already been described at stages around 20 weeks gestation. The main difference between this neonatal muscle and that of the adult lay in the shapes of the cells, which were somewhat rounded in outline, and appeared to have considerably more connective tissue around them than is found in adult muscle.

Histochemically the soleus and gastrocnemius muscles underwent a marked change during this final phase of development. The nature of the staining pattern altered slightly as far as the reciprocity between serial sections stained for NADH-d and ATPase at pH9.4 was concerned, and the actual proportions of dark and light cells changed considerably. By 29

weeks gestation, there was still a fairly large excess of fibres which stained lightly with NADH-d (Plates 11 a, 12 a), but in the neonate the proportion of these fibres had decreased considerably (Plate 13a, b).

Careful analysis in the neonatal specimen showed that the muscle cells which were stained most darkly for ATPase at pH4.3 were also the darkest when stained for NADH-d. The rest of the muscle cells, however, could be divided into several classes on the basis of their histochemical staining properties. Thus there appeared to be adult type I fibres visible in the neonate, but no distinct groups of cells recognisable as adult type II.

Finally, maturational changes of the neuromuscular apparatus were evident between 25 weeks gestation and birth. Up to 29 weeks gestation, there were still several different sizes of motor endplates visible. A few of the motor endplates appeared to have less distinct outlines than the rest (Plate 11 d) although the proportion of such endplates was not great. There was still clear evidence of double innervation of some muscle fibres (Plate 12b, c) and branching of some of the terminal axons (Plate 12 d). In the neonate the number and variety of motor endplate sizes was less than that seen in the muscle of fetuses of 29 weeks gestation, and there was a higher proportion of large motor endplates (Plate 13c, d). In addition, the ultraterminal and collateral terminal axon branching was reduced, there were fewer 'exploratory' tenuous axons, and nearly every terminal axon now terminated in one large motor endplate on a muscle fibre. The pattern of terminal motor innervation was thus more like that seen in adult muscle than in developing fetal muscle.

Discussion.

A consistent observation in studies of fetal muscle development has been the presence of a degree of asynchrony. Thus, it is seen not only that

Plate 12

a. Occasional darkly stained fibres in the gastrocnemius muscle of a 29 week fetus (73/384). NADH-d, x 135.

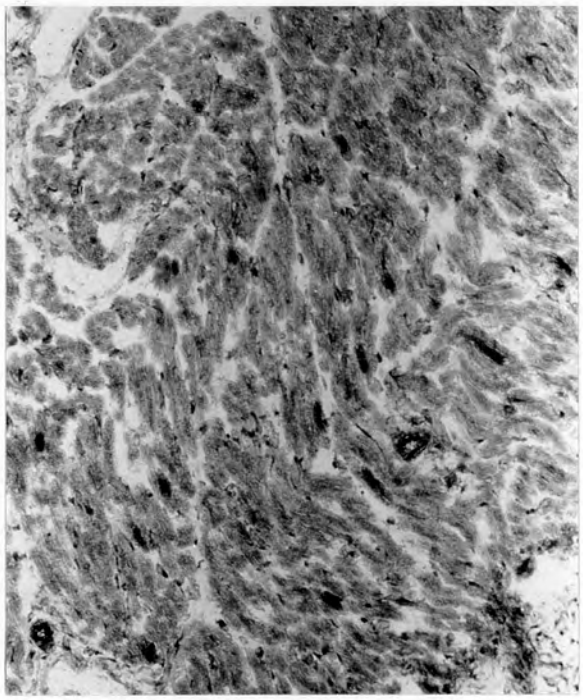
b. Motor endplates in the soleus muscle of a 29 week fetus (73/384). Note double innervation of some fibres (arrowed) Silver intensified AChE, x 520.

c and d. Terminal motor innervation in the soleus muscle of a 29 week fetus (73/384). AChE and silver impregnation, x 930.

c. note double innervation of some muscle fibres (arrowed).

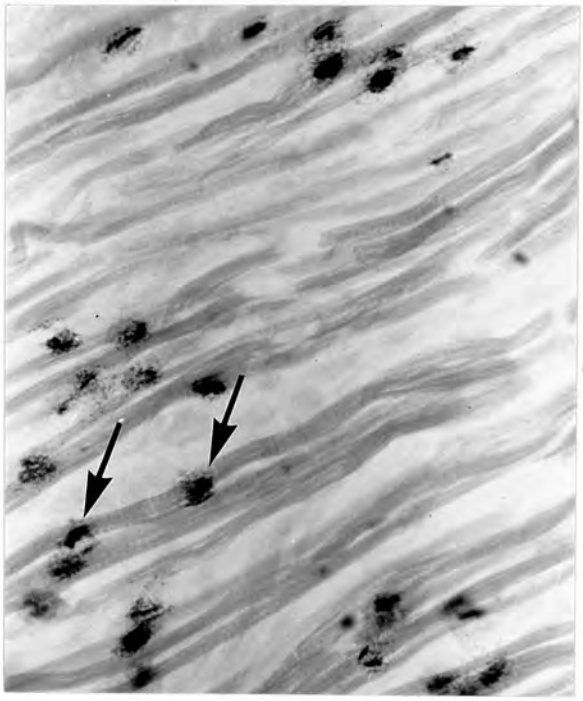
d. note extensive branching of terminal axons (b - arrowed).

PLATE 12

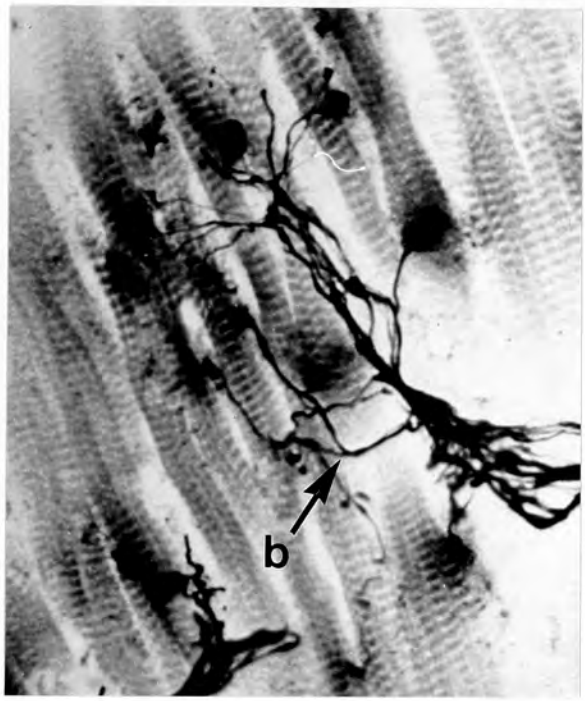


a

c



b



d

Plate 13

a and b. Partially reciprocal pattern of histochemical staining in the gastrocnemius muscle of a neonate (73/89). x 330
Single arrows, type I fibres: double arrows, possibly type IIC fibres.

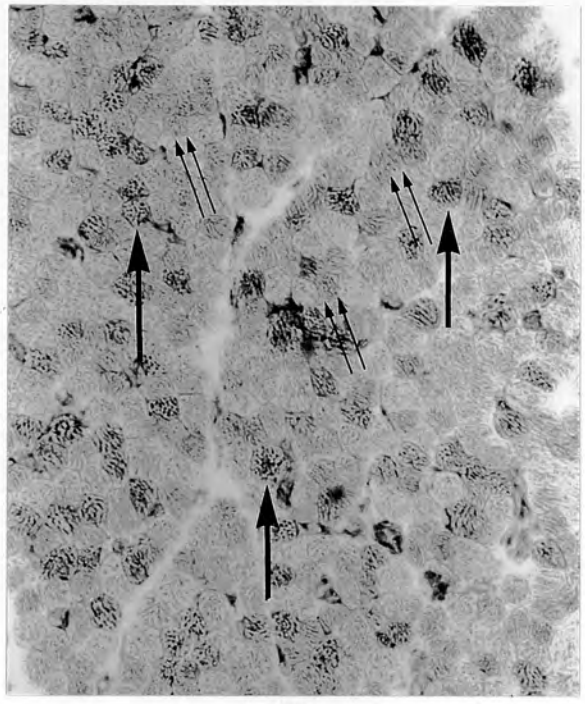
a. NADH-d

b. ATPase pH4.3.

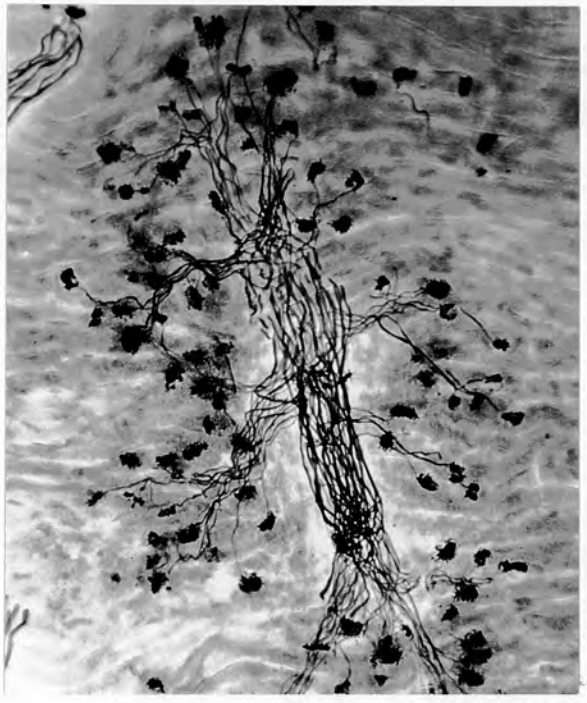
c. Terminal motor innervation in the soleus muscle of a neonate (73/89). AChE and silver impregnation, x 255.

d. Terminal motor innervation in the gastrocnemius muscle of a neonate (73/89). b - branched terminal axons are visible. AChE and silver impregnation, x 605.

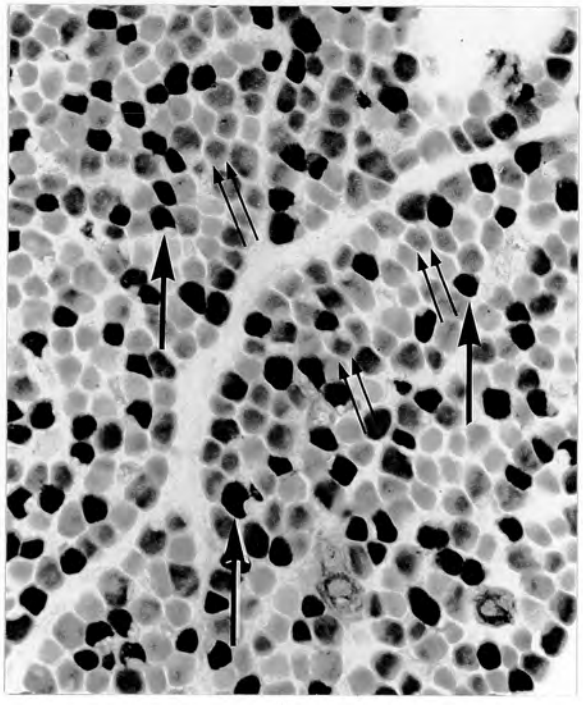
PLATE 13



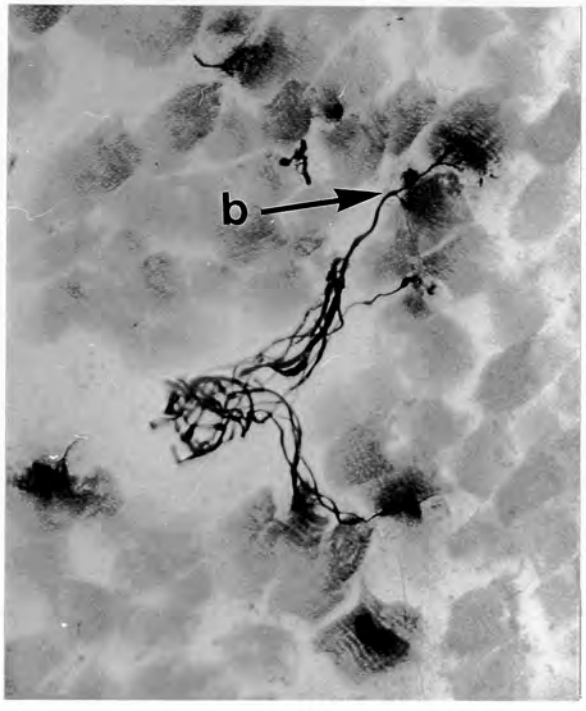
a



c



b



d

cephalic skeletal muscles mature at an earlier stage of development than do caudal ones (BOËTHIUS, 1969), and that not all parts of any individual muscle develop at the same rate (FISCHMAN, 1972: KONIGSBERG, 1965) but also that the motor endplates of the peripheral motor innervation in the intercostal muscles mature before those in the leg muscles (JUNTUNEN and TERÄVÄINEN, 1972). Evidences of this asynchrony are present in all the aspects of muscle development which have been considered in the present study. The observation that all fetuses nominally of the same gestation are not at the same developmental stage is doubtless due partly to the difficulty of estimating fetal gestation accurately, as well as to the asynchronous development of the muscle. However, it has been found to be generally true that the histochemical development of the muscle cells appears to be tied to their histological maturation, and both in turn, are linked to the developmental changes occurring in the neuromuscular apparatus. This similarity of developmental patterns for these different aspects of muscle development suggests that they may be interdependent.

In 1942, CUAJUNCO described the development of the neuromuscular junction in biceps brachii from 25 human fetuses. He too noted the asynchronous nature of muscle development in the muscle of 15 week fetuses, and described the appearance of three different cell types, which he suggested were separate generations of muscle cells, each arising by longitudinal division of the preceding generation of cells. His assessment of the development of the neuromuscular interconnections in muscle was, in many respects more accurate, and is paralleled by some of the findings made in the present investigations. Cuajunco considered that the small dotlike terminal enlargements of the motor axons, which he first saw at 10 weeks

gestation, did not constitute true neuromuscular connections. Similar terminal expansions seen in the present muscle at the same stage, should be regarded as no more than the very earliest non-functional neuromuscular contacts. In a few cases, histochemical staining alone demonstrated a diffuse, generalised staining on neighbouring myotubes, but not the small darkly stained areas which would indicate the concentration of the enzyme into soleplate regions. In addition, control sections of muscle, pre-incubated in 10^{-5}M eserine before AChE staining and silver impregnation, also showed the presence of small clublike terminal expansions of some motor axons. Cuajunco's methods suffered from a lack of definitive information in this respect, since as TIEGS (1953) has pointed out, each of the traditional cytological methods for motor endplate staining could demonstrate only one part of the motor endplate's make-up, and Cuajunco had no reliable method for the demonstration of AChE.

Between 10 - 14 weeks gestation, Cuajunco did not fail to observe that often small groups of myotubes become innervated at the same time. If only one motor axon is in the vicinity of such a group, it appears to branch so as to innervate all the nearby cells. Cuajunco also described the very rapid development both of the morphology of the motor endings, as well as that of the network of tenuous axons which connected them to one another, and found that by 14 weeks the motor endings had begun to show some of the complexities of the mature form. The present results do not show this, in part certainly because the leg muscles and not biceps brachii are being considered.

Cuajunco did not remark on the small neuromuscular junctions which in the present study were found at so many stages of development, although he did point out that after 14 weeks gestation some of the muscle fibres appeared

to have more than one neuromuscular junction; the second ones which he saw may well have been similar to the smaller, less mature ones seen in the present studies. The formation of a second motor endplate on a muscle fibre could indicate either the insufficiency of the first neuromuscular connection, or merely that the muscle fibre is not yet mature enough to prevent the formation of the second connection, as occurs in normal adult muscle.

Other studies, although not concerned with the numbers of neuromuscular connections present on an individual muscle fibre, provide evidence on other aspects of motor endplate maturation. JUNTUNEN and TERÄVÄINEN (1972) described a variety of motor endplates with different degrees of maturity present in any one fetus, and found that the morphology of the motor endplates remained very simple up to 14 weeks. This is very different from an earlier report (HEWER, 1934-35) which suggested that motor endplate development occurred at the end of the second trimester of pregnancy, but tends to substantiate the one electron microscope observation made on human muscle (FIDZIAŃSKA, 1971,1973), in which only rudimentary secondary synaptic clefts were seen at 10 - 11 weeks gestation. After 14 weeks, Cuajunco describes the continuing development of the motor endplate to its almost mature form before the end of gestation, while Juntunen and Teräväinen emphasise the incompleteness of this development even in the neonate. Studies by COËRS and WOOLF (1959) have also demonstrated that motor endplate maturation is far from complete in infants, and the present results agree more with the latter observations than those of Cuajunco.

On the other hand, the terminal axon branching and nerve network formation described by Cuajunco is very evident in the present results, although beginning at 13 - 14 weeks as it does, and continuing until at least

25 weeks gestation, it covers a much longer period than he suggested. The precise nature of these multiple anastamosing and ramifying axons is impossible to determine, particularly as their arrangement appears to be so unstable. As pointed out by Cuajunco, uninnervated muscle fibres generally do not survive, and developing muscle fibres appear to become innervated by the nearest motor axon. Such an apparently haphazard occurrence would inevitably lead initially to a very complex innervation pattern, in which there would be numerous ultraterminal and collateral terminal axon branches, not at all like that found in the adult.

Cuajunco also described the change from this unstable pattern to that found late in gestation, which involved the breakdown of the network of anastamosed motor nerves. This rationalisation, as well as possible motor endplate destruction as shown by the presence of some poorly outlined motor endplates at this stage of development, is also seen in the present studies. Cuajunco suggested that the multiple innervation of some individual muscle fibres persisted at least until the neonatal period, thus adding weight to the then current theory of the plurisegmental innervation of some human voluntary muscle fibres. It is now known both from electrophysiological evidence (DIAMOND and MILEDI, 1972) as well as from very many histochemical studies, that each mature muscle fibre has only one point of neuromuscular contact, but the present evidence suggests that the innervation of neonatal human muscle is more complex. This appears to agree with Coërs' and Woolf's very brief observations on the pattern of terminal motor innervation in infants (COËRS and WOOLF, 1959).

During the last trimester of pregnancy there is some simplification of the motor supply to the muscle, but the way in which it occurs is still

unknown. However, there is now evidence, other than histological, for this process, from animal experiments. The clearest evidence has come from the study of endplate potentials in newborn rats (REDFERN, 1970). Most endplate potentials at birth appeared to be the result of the summation of between 2 and 4 units, but the pattern became simpler during the second week of life, and by 16 - 18 days consisted only of single units. It was proposed that the complex endplate potential units resulted from the stimulation of separate motor axons each terminating at a different point on the muscle fibre, and that during subsequent development, all but one of the synapses on each muscle fibre was lost. Some credence has been given to this hypothesis following recent observations of spontaneously degenerating peripheral nerves in 7 - 14 day old rats (REIER and HUGHES, 1972). If allowance is made for the fact that at birth rat muscle is considerably less mature than human, it is conceivable that a similar process may take place in the antenatal development of human muscle.

If the process of simplification of the motor innervation pattern does occur in rats as suggested by Redfern, and is applicable to human fetal development, there are several implications for the histochemical development of fetal muscle. The first is that while the complex network of interconnected axons exists, if the neuromuscular connections are random there is likely to be no stable histochemical pattern, if it is the trophic effect of the nerve on the muscle which determines its biochemical properties. The present results have shown this to be so in developing human muscle. The second implication is that only when the pattern of motor innervation becomes stable will there be histochemical stability, and certainly at birth, when the pattern of motor innervation is becoming more mature, there is a degree of histochemical

stability. There is some stability, however, at the 20th-25th week of gestation, when the first partially reciprocal pattern of histochemical staining is seen, and the subsequent alteration in the proportion of fibre types may well reflect changes occurring in the motor innervation during this period.

In young fetuses, it is interesting to note, and perhaps significant, that it is only the larger diameter cells which stain darkly with H and E and for NADH-d and ATPase, and these cells are also those which appear to become innervated first. Whether or not the cells could have survived uninnervated in the human fetus up to this myotube stage is a matter for conjecture, since in practice it has not been possible to determine for any individual myotube, or group of myotubes, whether the histochemical dark staining properties developed first, or whether the myotubes had first received their motor innervation.

The staining properties of these immature muscle cells, as seen in the present studies, place them outside the normal adult classification of types I and II, since cells may stain darkly for both enzymes. However, in fetal muscle, as Guth and Samaha have pointed out (GUTH and SAMAHA, 1972), the pH profile of the ATPase stain is unlike that in the adult, and in man it may not be until approximately 25 weeks gestation that the fibre types begin to have meaning. At this stage the proportion of type II fibres appears much higher than type I and as described above, and suggested by DUBOWITZ (1965, 1968), the change to a higher proportion of type I fibres at birth may well be neurally mediated.

It has long been known (BANU, 1922 cited by CLOSE, 1972) that newborn mammalian limb muscles are at first uniformly slow. More recent studies on cats (BULLER et al., 1960a) and on rats, to judge either by the fibre types

present in the neonatal muscle (BROOKE et al., 1971) or by the muscle contraction times (CLOSE, 1964), have confirmed this in some animals. This would appear to contradict the present findings, since in the human fetus, at the developmental stage equivalent to the maturity of the neonatal rat, there seems to be a large excess of type II fibres. Although these fibres stain darkly for ATPase, they may in fact not be fast contracting and measurements of contraction times have obviously never been carried out on human fetal muscle. However, the presence of reciprocally stained sections of muscle for NADH-d appears to confirm that the apparent fibre types do represent the actual situation. The subsequent change in the relative proportions of the different fibre types results in the situation found in the neonatal specimen, where some type I muscle fibres are apparently differentiated and stable, but the type II are still subdivided into several classes. One of these classes appears to be similar to that designated fibre type II C in either rat (BROOKE et al., 1971) or human (DUBOWITZ and BROOKE, 1973) muscle. It is thought that type II C fibres are probably only precursor fibres, and give rise to mature II A and II B fibres. All neonatal specimens will probably not be at the same stage of development as the one studied here, since as has already been noted, fetuses do not mature at the same rate, but it is probable that in general the findings in neonatal specimens will not be very different from these.

In the adult human, different kinds of muscular atrophy have high-lighted the fact that there is more selective atrophy of type II muscle fibres than of type I (DUBOWITZ and BROOKE, 1973), which has led to the suggestion that the type I muscle fibres are of a more fundamental and more stable type than the type II muscle fibres. Such a suggestion, which might also follow from studies conducted on degenerating muscle in the dystrophic hamster (JOHNSON and PEARCE, 1971) finds an echo in the present results. This might possibly

explain the observation in many fetuses in the present studies, that the development of the soleus muscle, which in the adult, has a high proportion of type I fibres (JOHNSON et al., 1973) is more rapid than the development of the gastrocnemius muscle, which has a higher proportion of type II fibres.

CHAPTER 4

STUDIES ON THE DEVELOPMENT OF THE ANTERIOR HORN CELLS OF THE HUMAN FETAL SPINAL CORD

Introduction.

The purpose of this part of the study was to try to relate the normal development of the large neurones in the anterior horn of the spinal cord (anterior horn cells, AHC's) both temporally and histochemically to the development in the peripheral parts of the motor units that comprise skeletal muscle.

The results of nerve cross-union experiments (BULLER et al., 1960b: CLOSE, 1972) in the partial reversal of the normal histochemical pattern in adult cat muscle, suggest that the biochemical aspects of muscle are under direct neuronal control. Experiments in which twitches of individual motor units of cat gastrocnemius muscle were studied (WUERKER et al., 1965), as well as experiments in which single motor units of adult rat skeletal muscle were depleted of glycogen through prolonged direct axonal stimulation (EDSTRÖM and KUGELBERG, 1968: KUGELBERG, 1973), suggest also that each motor unit, controlled by one α motor neurone (AHC) is biochemically and physiologically homogeneous.

It is possible to divide the α motor neurones into 'fast' and 'slow' on the basis of the twitch properties of their respective motor units (WUERKER et al., 1965: BURKE, 1967), but the neurones themselves cannot be unambiguously divided into 'fast' and 'slow' on an electrophysiological basis. However, on a basis of cell size and histochemical staining intensity differentials, CAMPA and ENGEL (1970a, b, 1971) and ODUTOLA (1972) have been able to distinguish several populations of neurones in the spinal cords of adult cats and monkeys respectively. One of the stains used by Campa and

Engel was SDH, and the validity of some of their findings has been disputed by PYSH and KHAN (1972), who point out that the failure of some cells in the cat CNS to stain strongly for SDH does not necessarily mean that the cells concerned are not involved in oxidative metabolism.

There have been several previous studies of the development of anterior horn cells in the human fetus, on one 14 week fetus by ROMANES (1940 - 41), and on the lumbo-sacral spinal cords of six fetuses of between 14 and 28 weeks gestation, by ELLIOTT (1943), although no histochemical studies have yet been carried out. Studies of the development of AHC's in rabbits (ROMANES, 1941 - 42) and cats (ROMANES, 1951) demonstrate that very early in fetal development the large cells of the anterior horn become aggregated into columns whose arrangement is very similar to that seen in the adult. Similarly, in the human material, the morphological studies mentioned above showed that the AHC's of the lumbo-sacral spinal cord develop in well-defined columns. It is not possible to say with certainty which of the cell columns in the fetus supply which muscles, although in adults it has been possible to relate particular cell columns functionally to certain skeletal muscles, through a parallel study of paralysed limb muscles and areas of degenerate anterior horn cells in a post mortem study of poliomyelitis patients (SHARRARD, 1953, 1955). By extrapolation from animal studies (ROMANES, 1941 - 42) it may be suggested that to study the development of the anterior horn cells innervating the soleus and gastrocnemius muscles in the fetus, one may study these cells in the lumbo-sacral spinal cord which occupy the same relative positions as these cells do in the adult.

A study similar in concept to the present one was carried out by ATSUMI (1971 a, b, c) who studied the relationship between the histogenesis of motor neurones, and the motor endplate development of the intercostal muscles

in the chick embryo. An interesting conclusion from this work was that the appearance of Nissl bodies in the large motor neurones occurs at nearly the same time as the localisation of acetylcholinesterase at the motor endplates. A similar observation was made in rabbit by ROMANES (1941 - 42) who noted that Nissl granules appeared in the neurones at the time when they first began to show signs of functional activity. Nissl bodies consist of discrete zones of endoplasmic reticulum (PALAY and PALADE, 1955), or cisternae (PANNESE, 1968) with scattered ribosomes, and thus their appearance may signal the initiation of particular enzyme or protein synthetic pathways in the neurone. In the present study an attempt was made to discover whether there are any histochemically demonstrable biochemical differences between individual anterior horn cells in the human fetus. It was also hoped to determine, if two α motor neurone species could be demonstrated, whether one cell type or the other became active first, and if this was related to the histochemical observations in human myogenesis. Simultaneously, it was possible to describe more fully the morphological and numerical development of the anterior horn cells of the lumbo-sacral spinal cord.

A preliminary report of the present findings has already appeared (TOOP and JACKSON, 1973).

Materials and Methods.

Studies were carried out both on frozen spinal cords and those embedded in paraffin. A record of those studied by each method and the gestation of the fetus from which they came appears in Appendix 1. In each case the lumbo-sacral spinal cord was carefully dissected free from the spinal column, except from fetuses of 9 weeks gestation or less, since at this age the spinal cord alone was too soft to be handled. In these latter cases the whole lumbo-sacral spinal column was frozen or embedded in paraffin wax.

as appropriate. Since autolysis is rapid in the spinal cord every effort was made to use cords which were as fresh as possible, and although the data in Appendix 1 show that the total delay between fetal death and fixation was sometimes more than a day, there was no marked degeneration in the AHC's, since they were stored at 4°C in the interim.

Paraffin embedded material.

The spinal cords remained in 10% formol saline for up to 11 months. Processing was then carried out by automatic schedule on a Histokinette, and the specimen double embedded in paraffin wax. Either transverse or coronal sections of a spinal cord were cut at 40-50 μ , and attached to albuminised slides for staining. The stains used were 1% toluidine blue in 1% borax,and haematoxylin and eosin, which were made up and used as described in Chapter 2. A combined Luxol fast blue and cresyl violet stain was also used, following a method described by Duchen (Dr. L. Duchen, personal communication). Each of these stains demonstrated adequately the morphology of the anterior horn cells, and the paraffin embedded material was used to study the increase in cell number and size with increasing gestational age.

Frozen material.

Spinal cord lengths were frozen in isopentane chilled in liquid nitrogen and stored in sealed containers at -70°C until required. Transverse sections were cut at 40 or 50 μ in cords from fetuses over 11 weeks gestation, and at 30 μ from those of 7-11 weeks gestation. The slides were allowed to dry for 10-30 minutes at room temperature, and in most cases stained the same day. Occasionally, dried slides were stored overnight at -70°C. The following stains were used: Histology: as for paraffin embedded material; Histochemistry: succinate dehydrogenase and phosphorylase

(CAMPA and ENGEL, 1970b), and silver-intensified acetylcholinesterase (Appendix 2). These frozen sections were not used to count total numbers of AHC's as in the paraffin-embedded material, but only to establish the relative proportions of the different types of cells.

The motor neurones which invest the triceps surae muscle are found, in the adult, in the dorsomedial region of the pool of anterior horn cells, extending in a column in each half of the spinal cord from the middle of the lumbar enlargement to the caudal end of the spinal cord (SHARRARD, 1955). Both frozen and paraffin sections were therefore taken only between these two extreme points. Initially two methods were used to try to localise the precise cell columns of interest. Firstly, cell charts were constructed (ELLIOTT, 1942) by drawing on paper the outline of the spinal cord from a projection of the first of a series of serial sections, each 20μ thick. The anterior horn cells from this first section and up to five subsequent sections were then also drawn in, until the cell column could be distinguished. Since this process took a long time and was not always accurate, it was found preferable to cut sections 40 - 50μ thick, which contained a sufficient number of anterior horn cells to show the cell columns without the need for constructing a cell chart. Although the spinal cords had not been marked before sectioning with respect to the level relative to spinal nerves, it proved possible to estimate the level at which sections occurred by comparing the numbers and patterns of the groups of AHC's to those mapped by SHARRARD (1955). For each spinal cord, two different points were studied along the length of the triceps surae cell columns, to avoid sampling errors which might arise if only one region were considered.

Cell measurements in paraffin sections were made by the method of GIACOBINI and HOLMSTEDT (1958) in which the long and short 'diameters'

(m_1 and m_2) of a cell are multiplied, and the square root extracted to give a computed diameter, m: i.e. $m = \sqrt{m_1 m_2}$. Fifty cells in each fetus were measured in this way, measurements being made on randomly selected cells on a projected image at a magnification of x 400. Counting also was done using a projected image, since this enabled positive identification of each cell. Cells were counted in both halves of a spinal cord section.

Results.

Histochemistry.

The most useful results obtained were from the acetylcholinesterase staining. In fetuses of 12 weeks gestation and over, it was possible with this stain to distinguish two types of cells, light and dark. With the SDH stain, but not with the phosphorylase, it was also possible to distinguish two cell types. AChE and SDH were therefore the stains used in differentiating cell types.

Before 8½ weeks gestation, no AChE or SDH staining could be seen in or near the area in which the anterior horn cells are found in the young fetus. At 8½ weeks the only staining associated with the anterior horn was a band of AChE stain around the periphery, which although it did not appear to be closely associated with specific cells, was well localised (Plate 1a). This same banding pattern was visible in fetuses of up to 10 weeks gestation, after which the band appeared to migrate medially, so that by 11 - 12 weeks the staining was in the region associated with the AHC's in the adult (Plate 1b). By this time also individual AHC's were visible in this region. Meanwhile the nerve roots and dorsal columns of the spinal cord stained normally for AChE, although the intensity of stain in the dorsal columns increased with gestation.

By 12 weeks the AHC's stained for AChE could be clearly divided into

Plate 1

Transverse frozen sections of spinal cord,

stained with silver-intensified AChE.

a. From 8 week fetus, x 53

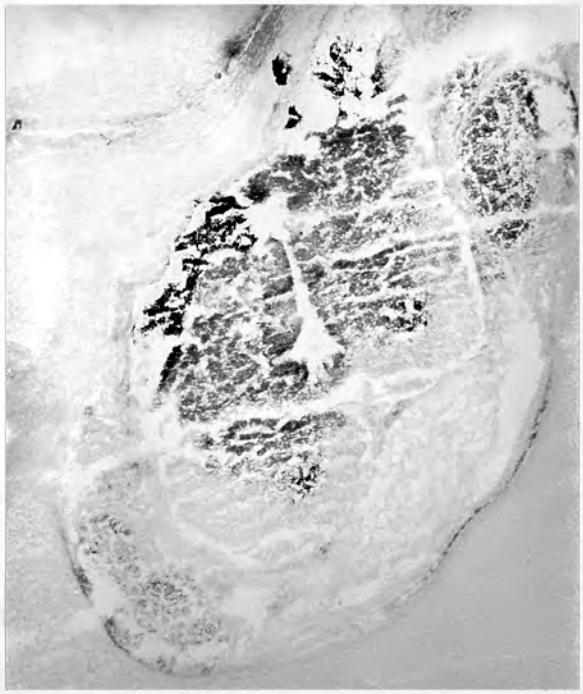
b. From 12 week fetus, x 53

c and d. Lightly (arrowed) and darkly stained AHC's present, x 330.

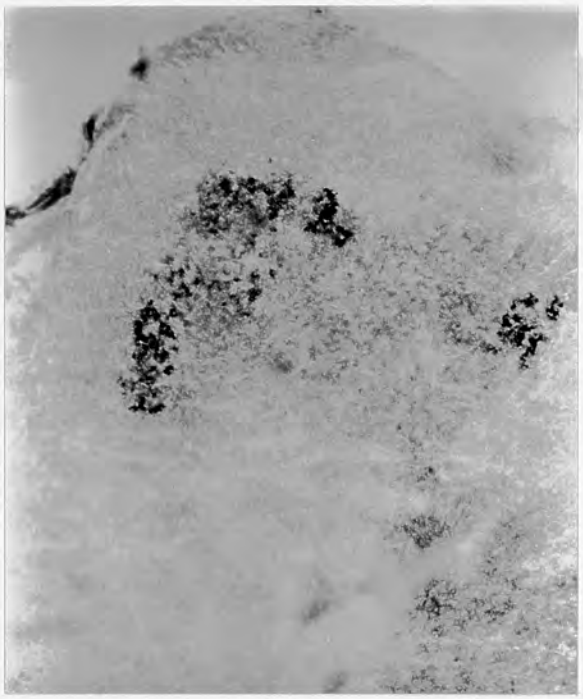
c. - from 13 week fetus.

d. - from 16 week fetus.

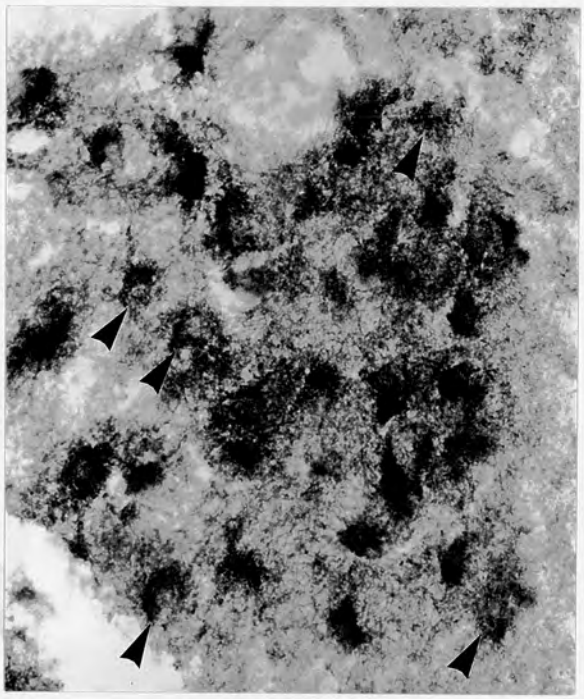
PLATE 1



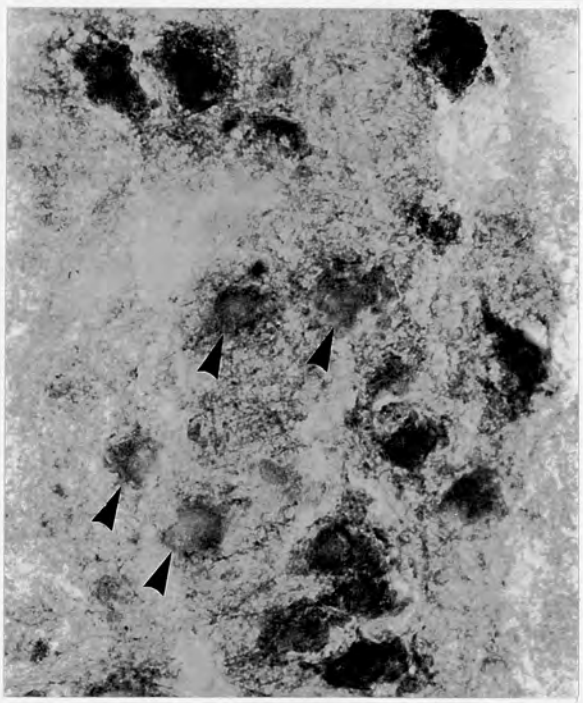
a



b



c



d

two groups, which subjectively were judged to be lightly and darkly staining. These cells were in groups similar in position to those seen in the adult, although much smaller. During gestation the total numbers of AHC's increased, but at the same time the proportions of dark and light cells hardly changed at all (Plates 1 c, d, 2 a - c : Table 1).

The SDH staining, in spite of incubation being continued for 1½ hours, was faint in all sections. However, it seemed in some sections that the cells which stained most heavily for SDH appeared to be similar in number, size and position within the anterior horns to those cells which stained lightly for AChE (Plate 2 d). In addition these two different stains each showed cells which appeared to occur more centrally in the columns of anterior horn cells.

Morphology.

The numbers and sizes of cells in the anterior horns of the spinal cord were counted throughout the same gestational range for which the histochemistry of the AHC's was studied. During development there were increases both in cell size and number at both of the levels of the spinal cord which were studied. Before 12 weeks gestation it was extremely difficult to clearly identify individual AHC's and be able to count them; Table 1 gives the results of counts carried out after 12 weeks gestation. It can be seen that the numbers of cells in the lumbar region increased by about 50% between 12 weeks and birth, but remained relatively static in the sacral region during the same period (Plate 3).

The increasing diameters of the AHC's with gestation are illustrated by the photographs in Plate 4. At each gestation, the AHC diameter computed from cell measurements was used to calculate cell surface area and volume, and the figures for each of these in the lumbar and sacral regions

Plate 2

Frozen sections of human fetal spinal cord

a, b and c. AHC's stained with silver-intensified AChE.

a. - 18 week fetus, x 135.

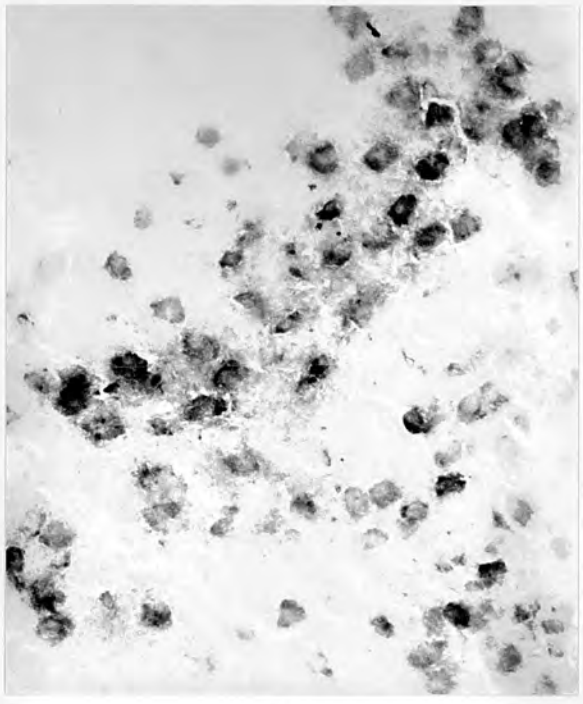
b. - 22 week fetus, x 135.

c. - 28 week fetus, x 330.

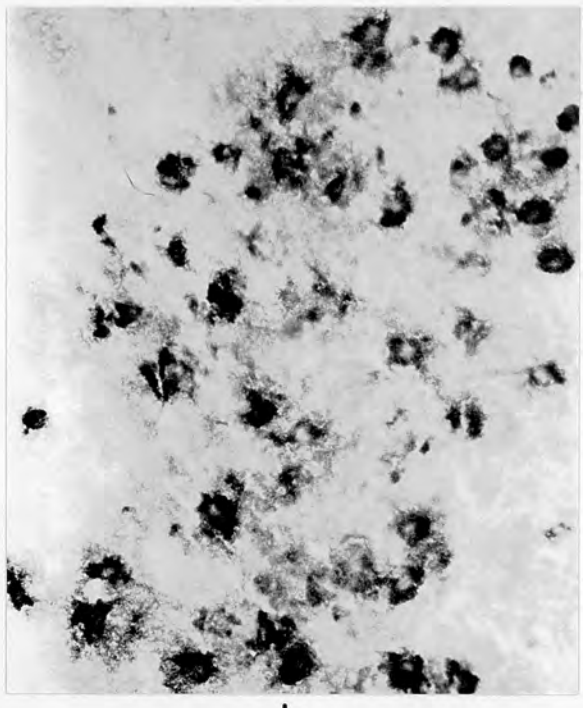
d. - AHC's in the spinal cord of a 20 week fetus.

SDH and safranin, x 135.

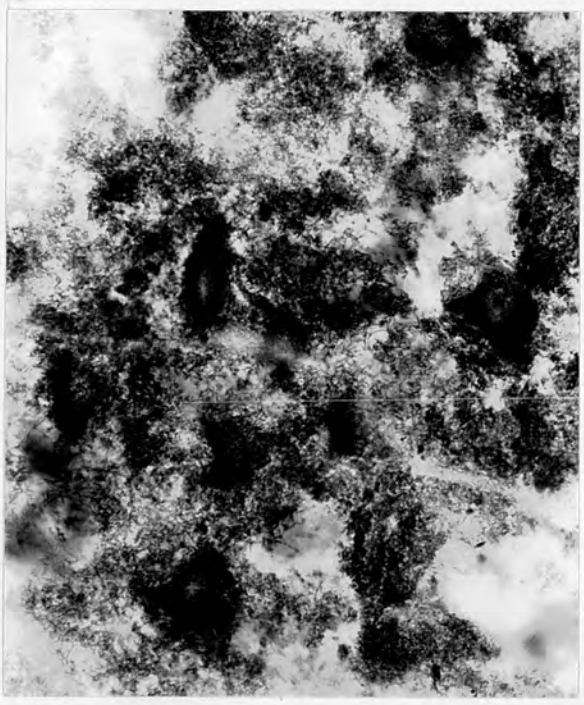
PLATE 2



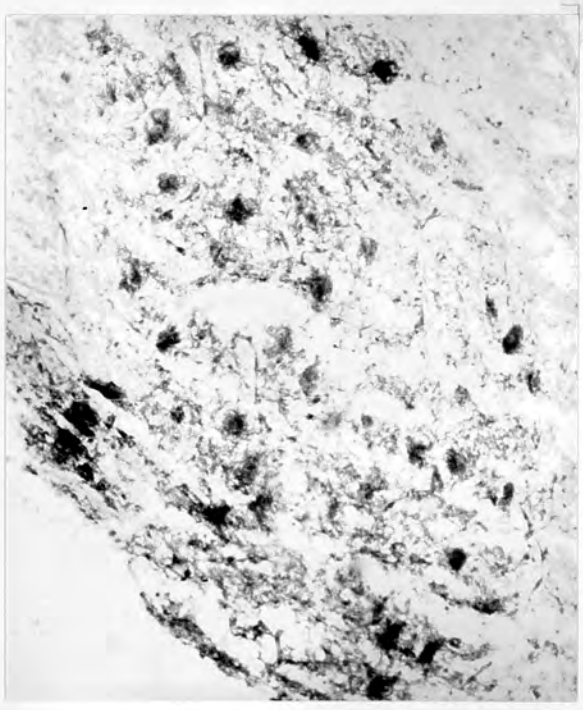
a



b



c



d

Plate 3

Paraffin sections of human fetal spinal cord.

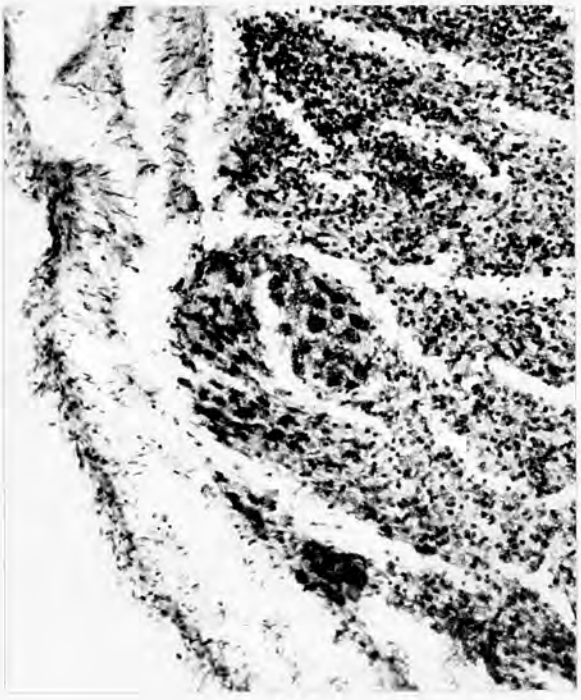
a. AHC's from 14 week fetus. Toluidine blue, x 135.

b. AHC's from 17 week fetus. H and E, x 53.

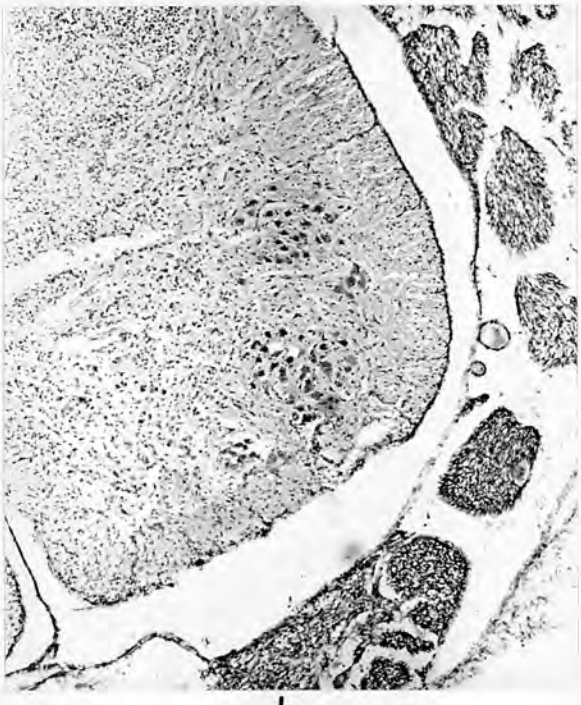
c. AHC's from 24 week fetus. Luxol fast blue and cresyl violet, x 53.

d. AHC's from neonate. H and E, x 53.

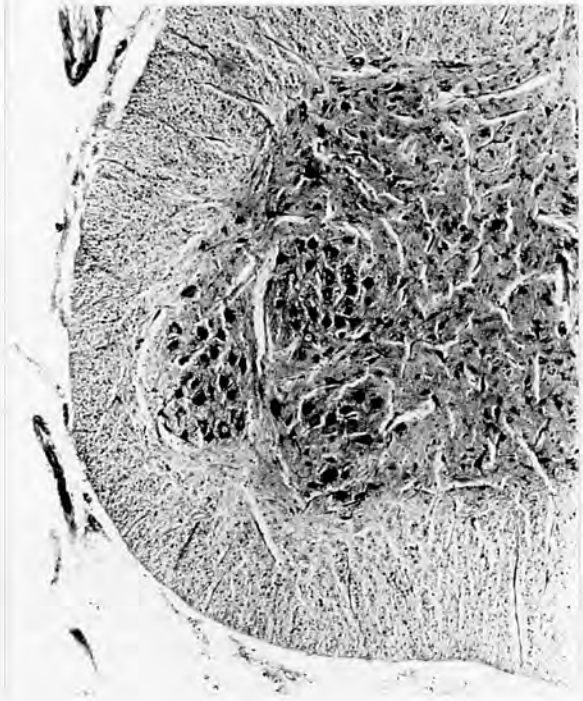
PLATE 3



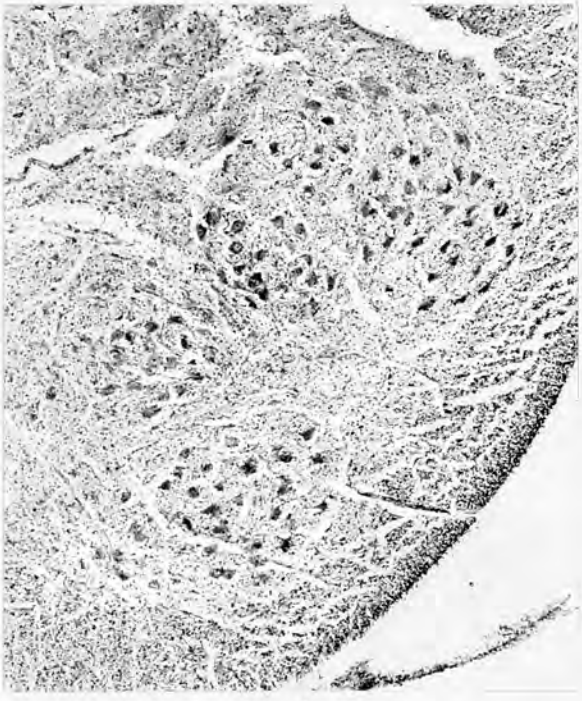
a



b



c



d

TABLE 1

ANTERIOR HORN CELLS IN THE HUMAN FETAL SPINAL CORD

Gestation (wks.)	Cells stained for AChE (%)		No. of cells per section.	
	Light	Dark	Lumbar	Sacral
12	65	35		
13	61	39	224	224
14	66	34	218	228
15	69	31	260	232
16			260	230
17	66	34	276	
18	69	31		
19			300	
20	61	39		
22	70	30	302	232
23			306	236
24			314	236
28	72	28		
Neonate			340	244

showed the opposite trend from that visible in the cell number counts; cell surface area and volume increased more rapidly relative to initial size, in the sacral region than in the lumbar (Figure 1). Cell surface area was also plotted in histogram form for all gestations, using data for the lumbar and sacral regions separately. The results were more confusing than helpful, since each histogram for both lumbar and sacral regions had between one and four peaks. None of the distributions was normal, although a few could have been described as uni- or bi-modal. In addition to these, there were some distributions which had more than two 'peaks' each; but without a sufficiently large sample of cells it was not possible to decide whether or not these were true representations of the actual distributions of AHC sizes.

Conclusion.

If it is true that by exertion of a trophic influence, the large motor-neurones of the anterior horn of the spinal cord dictate the physiological and biochemical properties of the skeletal muscle fibres which they innervate, it would not be unreasonable to expect to see some correlations during development between the maturity of the anterior horn cells and that of the skeletal muscle fibres in their motor units.

This may to some extent be true with regard to numbers of anterior horn cells. Spinal nerves from the region of the lumbar enlargement supply most of the muscles of the lower limb, where the number and bulk of muscle fibres increases dramatically after the formation of the early myotubes. The present results show that at the same time there is an increase in the number of anterior horn cells. Conversely, spinal nerves from the sacral region of the spinal cord, especially S2 or S3, innervate a relatively small muscle mass, and the increase in the number of anterior horn cells in the

Plate 4

Paraffin sections of human fetal spinal cord.

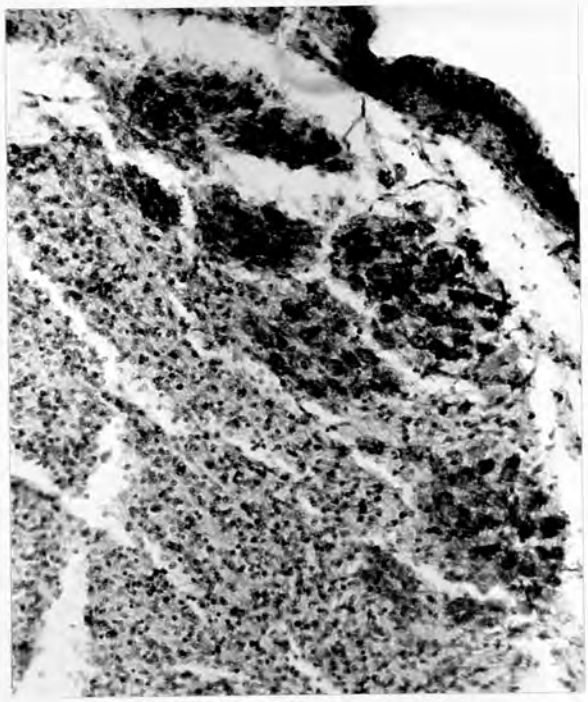
a. AHC's from 14 week fetus. Toluidine blue, x 135.

b. AHC's from 17 week fetus. H and E, x 135.

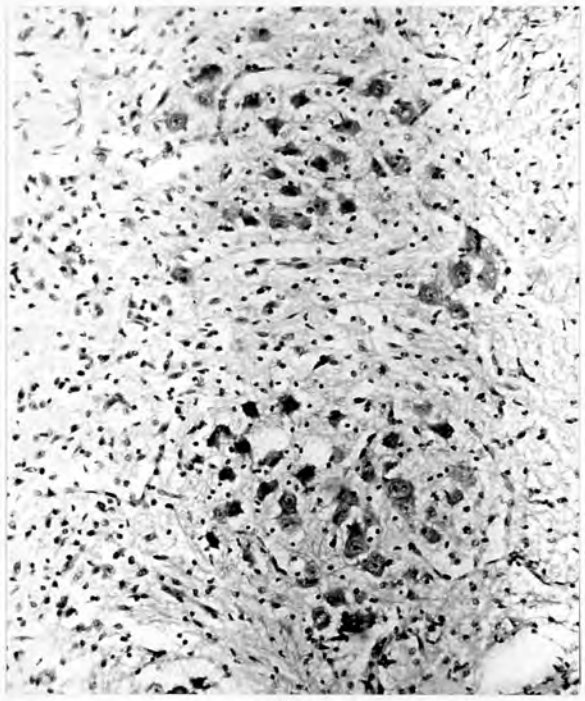
c. AHC's from 24 week fetus. Luxol fast blue and cresyl violet, x 135.

d. AHC's from neonate. H and E, x 135.

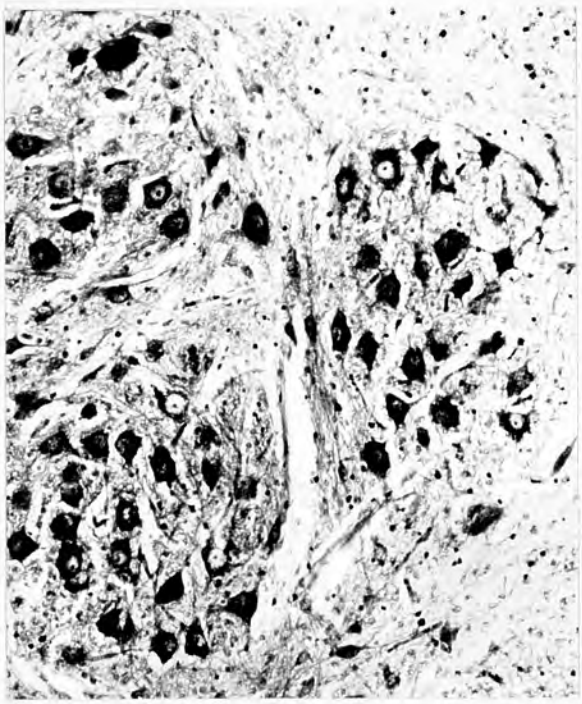
PLATE 4



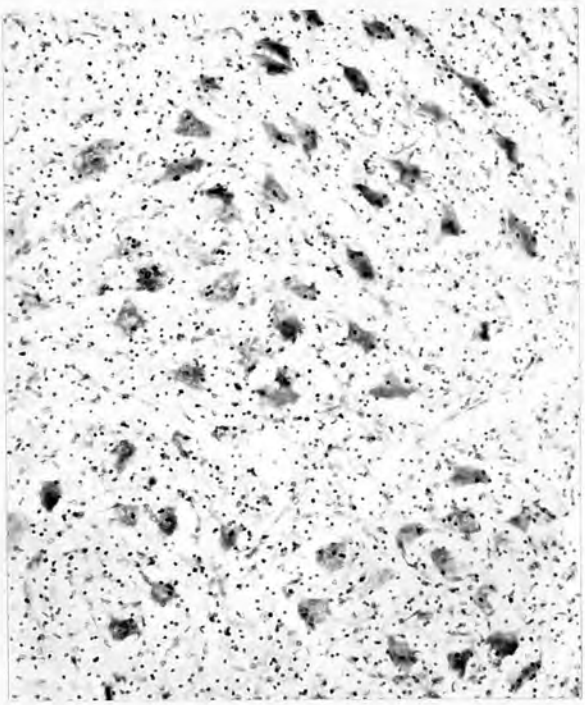
a



b



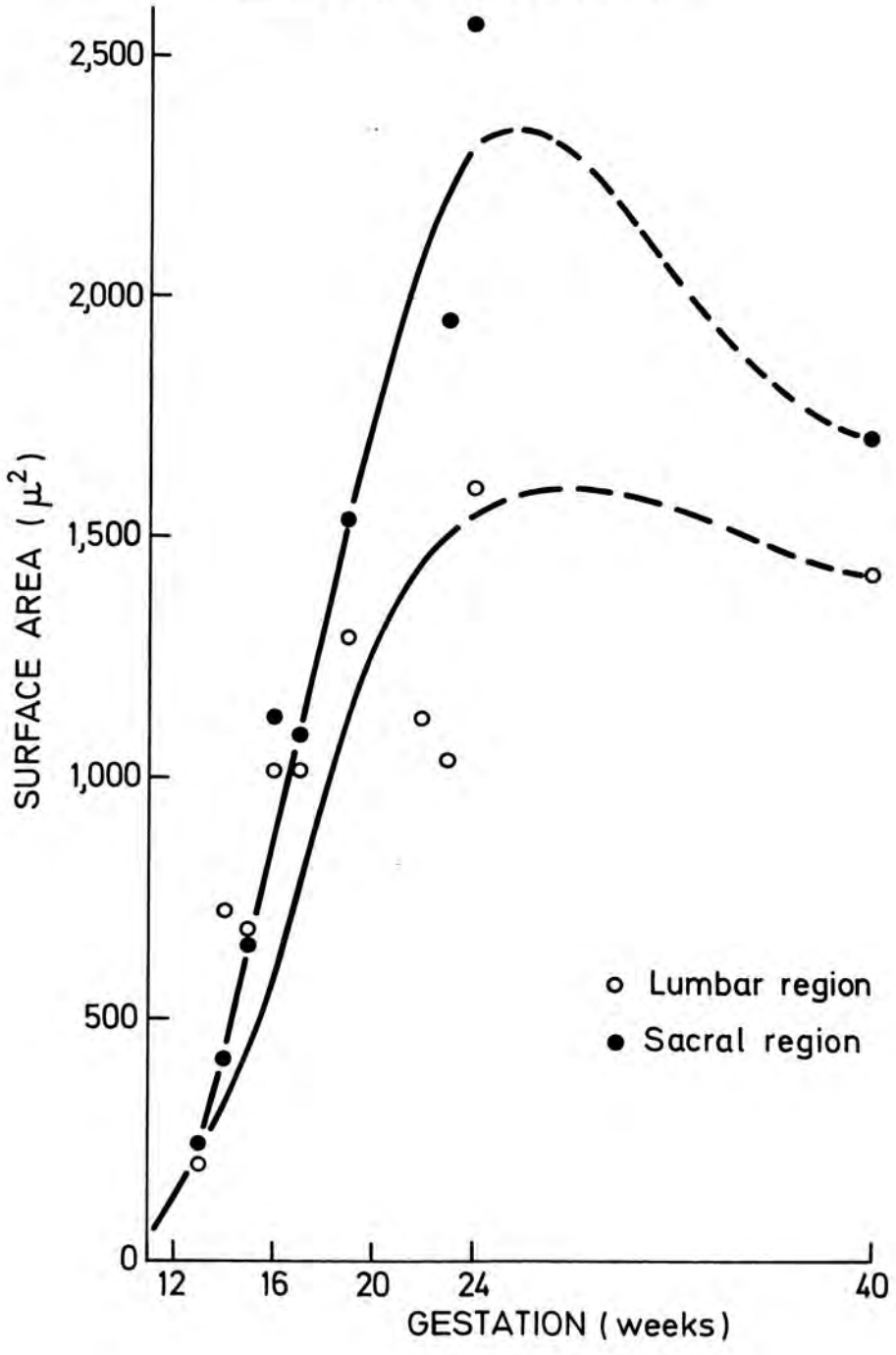
c



d

FIGURE 1

CHANGES IN SURFACE AREA OF HUMAN ANTERIOR HORN CELLS WITH GESTATION



sacral region is not nearly as marked. While the number of anterior horn cells in the fetus may increase by only fifty percent between 12 weeks gestation and birth, the number of muscle fibres increases far more. This is probably accounted for by an initially rapid growth in motor unit size, followed by a gradual decrease in this rate, which accompanies a gradual but much slower increase in the number of motor units.

The sizes of individual anterior horn cells may reflect one of two possibilities. The first is that when there are relatively few anterior horn cells present in any given area, there is less physical restriction on the growth of cells. A second and more likely possibility is that the size of each cell may reflect the number of skeletal muscle fibres it innervates. Sections taken from low in the sacral region, e.g. S2, may well contain more anterior horn cells which innervate large muscles with large, fast motor units e.g. gastrocnemius (FEINSTEIN et al., 1955) than at higher levels in the spinal cord.

The use of thick sections both in histological and histochemical studies was intended to make it possible to study the development of the group of anterior horn cells which innervates the triceps surae muscles, using Sharrard's data (SHARRARD, 1955) to localise the cell columns of interest. However it was eventually decided to sacrifice the precision of such a restricted study and involve all the anterior horn cells for the sake of a more accurate overall survey of their development. The use of thick sections also made it impossible to confirm the observations of ATSUMI (1971 c) and ROMANES (1941 - 42) that Nissl bodies first become visible at approximately the same time as the neurones become functional. However, it is perhaps significant that the first sign of acetylcholinesterase staining in the spinal cord is seen at 8 - 8½ weeks, while the primitive neuromuscular

connections appear in the muscles of the lower leg at 8 - 9 weeks (Chapter 3).

The results of the histochemical staining of anterior horn cells were difficult to interpret. By means of the acetylcholinesterase stain it was clearly possible to distinguish two differently staining cell types. Histochemical studies of enzyme levels in neurones may, however be misleading (EADIE et al., 1971). In Eadie's experiments various enzyme levels were measured in neurones of brain and spinal cord. In the conclusion it was pointed out that although histochemically there appeared to be two cell types, the enzyme concentration was in fact similar in all cells: but since neurones are of different sizes, total enzyme levels in different cells may be very different.

Studies on rat spinal cord motor neurones by GIACOBINI and HOLMSTEDT (1958) using histochemical and microchemical techniques led them to the conclusion that there were two significantly different groups of cells. Histochemically, the large cells were more darkly stained, while microchemical methods showed that one group of cells had a level of enzyme activity which was approximately four times higher than in the other group. In attempting to relate these findings to the muscle fibres innervated by these large motor neurones it was suggested either that the two types might represent flexor and extensor neurones, or, more likely, that they corresponded to the 'slow' and 'fast' muscle fibres.

If the anterior horn cell types in the present study were directly related to the two basic histochemical types of muscle fibres, whose properties they regulated neurotrophically, it would be expected that histochemical development of the anterior horn cells would parallel but precede the histochemical development of muscle. The fact that this simple relationship was not found means either that the AChE stain used to type the

anterior horn cells had no special relevance to this trophic aspect of cell function, or that there were other unknown variables involved. Staining for AChE might provide a meaningful indication of cellular activity, if acetylcholine were the synaptic transmitter of all cells whose processes impinge on anterior horn cells, but at present it is not known which synapses in the spinal cord are cholinergic (PHILLIS, 1970).

The numerical growth of muscle fibres in a motor unit may be gradual, but the rate of growth may also be different for 'fast' and 'slow' motor units. It has been shown in cat gastrocnemius muscle (McPHEDRAN et al., 1965) that fast motor units are larger than slow ones. Mixtures of fast and slow motor units (ATPase high and ATPase low) are found in all human muscles (JOHNSON et al., 1973), and the uncertainties with regard to the size and rate of growth of motor units during development thus make it impossible to know whether the histochemical development of anterior horn cells described bears any clear relationship to the histochemical events of myogenesis.

CHAPTER 5

STUDIES ON FETUSES AT HIGH RISK FOR DUCHENNE MUSCULAR DYSTROPHY.

Introduction.

Studies of the normal development of human skeletal muscle make it possible for comparison to be made between these findings and those made in cases when there is reason to suspect that there may be aberrations of muscle development. In the present investigation skeletal muscle was examined from five therapeutically aborted male fetuses at risk of developing Duchenne muscular dystrophy (DMD) and compared with skeletal muscle from normal controls.

The pathogenesis of Duchenne muscular dystrophy (which is the most severe of the muscular dystrophies) follows a well defined course. In neonatal males who later develop the disease a grossly elevated serum creatine kinase (CPK) level has been noted (BRADLEY et al., 1972), although at birth there are no clinical symptoms of the disease. These symptoms begin to appear at between 3 and 5 years of age, and consist of progressive muscle weakness which is seen first in the muscles of the pelvic girdle. With the progression of the disease there are distinctive changes in the skeletal muscle. The first signs are hyaline degeneration of fibres, and vigorous regeneration attempts in many fibres. As the disease progresses however, the amount of muscle tissue becomes greatly reduced; there is replacement of muscle by fat and connective tissue, and the regenerative effort of muscle fibres ceases.

Because muscle biopsies from boys suffering preclinical changes of Duchenne muscular dystrophy are so infrequent, there have to date been only two reports of such studies. The first was by PEARSON (1962) who reported the changes present in the skeletal muscle of a 10-month-old boy, who at the

time of study had three affected brothers. The abnormalities present in a biopsy of the quadriceps muscle included increased variability of fibre diameter, and the presence of hyaline fibres, but showed no fibre necrosis. In addition there was a moderate increase in the amount of interstitial connective tissue and some increase in basophilia in many fibres which were shown to contain significantly increased amounts of RNA.

In a second case, Bradley and his colleagues (BRADLEY et al., 1972) reported on the findings in a biopsy taken from a 2½-week-old boy, who in follow-up studies developed the full spectrum of histochemical and histological changes characteristic of Duchenne muscular dystrophy. The main observations here also were an increase in the variability of fibre diameter, the presence of hyaline fibres, as well as a slight increase in the amount of fibrous connective tissue.

The present investigation into skeletal muscle histology of male fetuses at risk for Duchenne muscular dystrophy was carried out to try and discover if there were any specific skeletal muscle abnormalities in such fetuses.

The preliminary findings of this study have been reported (TOOP and EMERY, 1974).

Materials and Methods.

In four of the fetuses studied in this investigation (599, 684, 734 & 73/375c) the mother was a possible carrier of DMD i.e. she had an affected son or brother. In the fifth case, (1018) the mother was a definite carrier having had two affected sons previously. Four of the five mothers had serum creatine kinase levels within the normal range (Table 2). In each case the sex of the fetus was established prior to therapeutic abortion, by sex chromatin and fluorescence studies on fixed and stained cultured amniotic

fluid cells. The controls were three male fetuses of comparable gestation, which had been similarly aborted. The gestations were checked by use of Streeter's heel-toe length table, (STREETER 1921), and by crown-rump measurements (see Chapter 2). Where possible samples both of quadriceps muscle and of cardiac blood were taken. In four of the five cases, the fetuses had been sent from up to two hundred miles away and in the fifth case, prostaglandin had been administered for 36 hours before abortion. While it was still possible to study muscle histology after the delay this involved, the blood had become very haemolysed.

Quadriceps muscle was chosen for this investigation since clinically it is one of the first muscles to become affected (WALTON and GARDNER-MEDWIN, 1969). Small blocks of muscle were frozen in isopentane chilled with liquid nitrogen, and transverse sections 10μ thick cut on a microtome (see Chapter 2). Sections were stained for H and E and with Gomori trichrome (ENGEL and CUNNINGHAM, 1963). In the case of 73/375c, 10μ sections were stained in addition for NADH-diaphorase and myosin ATPase at pH 4.3 and 9.4, and 20μ sections to demonstrate the peripheral motor innervation (see Chapter 2). Muscle fibre diameters of randomly selected fibres were measured in sections stained for H and E and Gomori trichrome using a calibrated eyepiece graticule at a magnification of x 400. In one case (684) 100 fibre diameters were measured, but in the other seven cases, 50 fibre diameters were measured. The percentage of hyaline fibres was detected by observing the incidence in 500 muscle fibres.

In addition to the histological studies, CPK estimations were carried out, where possible both on muscle and cardiac blood. The results from control muscle and from 73/375c were obtained fresh, but in the other fetuses at risk, estimations were made using muscle samples which had been stored at

-20°C for up to 32 months. As controls, estimations for numbers 878 and 1018 were made simultaneously with the others, and results compared with those obtained when these samples had been fresh, before up to 23 months storage at -20°C. For muscle CPK determinations, a 1/5 homogenate in distilled water was made using a Potter - Elvehjem homogeniser. Serum and muscle CPK levels were measured using Rosalki's method (ROSALKI, 1967). In addition muscle CPK isoenzymes were separated electrophoretically and stained by a modification of Rosalki's method (ROSALKI, 1965) on cellulose acetate strips. These were scanned in a Joyce, Loebl chromoscan, and the percentage of M and B subunits calculated.

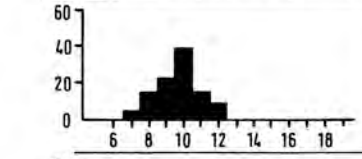
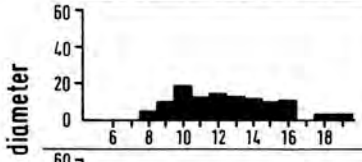
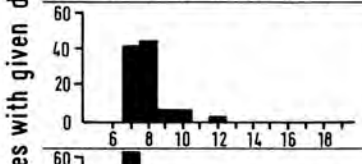
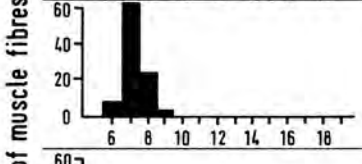
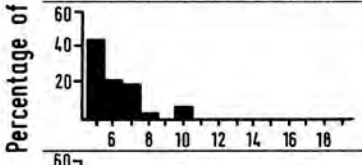
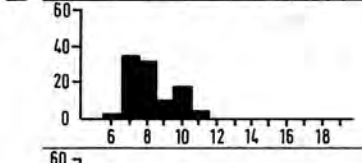
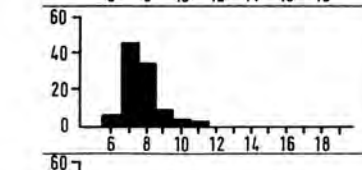
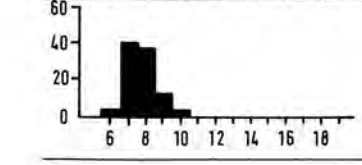
Results.

Muscle Histology.

There were no apparent histological abnormalities in two of the five fetuses at risk: the range of muscle fibre diameters was normal and there were no hyaline fibres (Table 1). One fetus at risk (73/375c) had a smaller mean muscle fibre diameter than expected by comparison with the normal controls and the variability of fibre diameter was slightly increased. Another fetus showed a slight increase in the mean and variability of muscle fibre diameter, but neither of these two specimens had any hyaline fibres. The fifth fetus (684) showed the most marked alterations in its muscle histology. The variability of muscle fibre diameter was much greater, and the mean fibre diameter was almost fifty percent larger than in any of the normal control specimens. There was also a significant number of hyaline fibres present which comprised between 2% (Gomori trichrome) and 3% (H and E) of the total number of fibres (Plate 1). In muscle from this fifth fetus, there also appeared to be more internal nuclei than expected in a 16 week fetus, but it was not possible to verify this quantitatively

TABLE 1.

MUSCLE FIBRE DIAMETERS AND INCIDENCE OF HYALINE FIBRES IN QUADRICEPS MUSCLES FROM FETUSES AT RISK AND CONTROLS

Muscle Fibre Diameter (μm) Distribution	Mean ± SD	Fetus Number	Gestation (weeks)	Hyaline Fibres (%)
	9·7 ± 1·2	599[r]	21	0·0
	12·4 ± 2·6	684[r]	16	2·0–3·0
	7·8 ± 1·0	734[r]	16	0·0
	7·2 ± 0·7	1018[r]	21	0·0
	5·8 ± 1·4	73/375[r]_c	19	0·0
	8·2 ± 1·3	73/258[c]	22	< 0·6
	7·6 ± 1·0	73/259[c]	17	0·0
	7·7 ± 0·9	73/271[c]	17	0·0

Percentage of muscle fibres with given diameter

[r] At risk [c] Control

Plate 1

a and b. Transverse sections of quadriceps muscle from control fetus, 73/271. H and E.

a. - x 135

b. - x 485

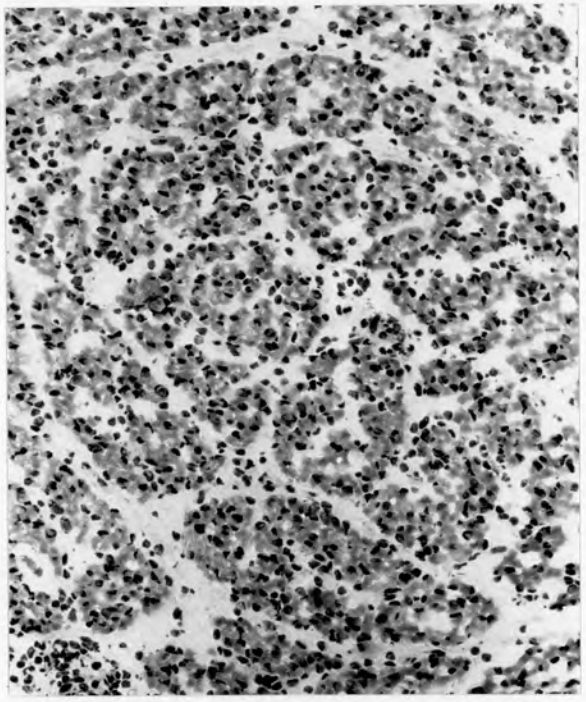
c and d. Transverse sections of quadriceps muscle from fetus at risk, 684. H and E.

c. - x 135

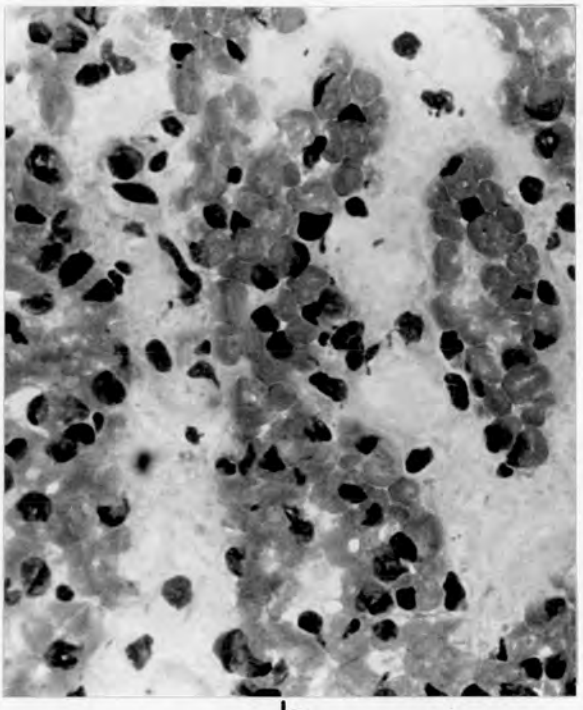
d. - x 485

Note the presence of hyaline fibres (arrowed).

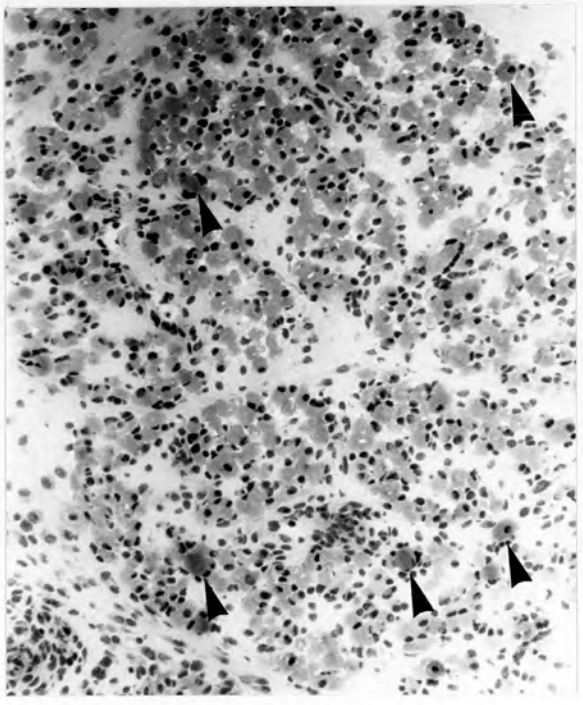
PLATE 1



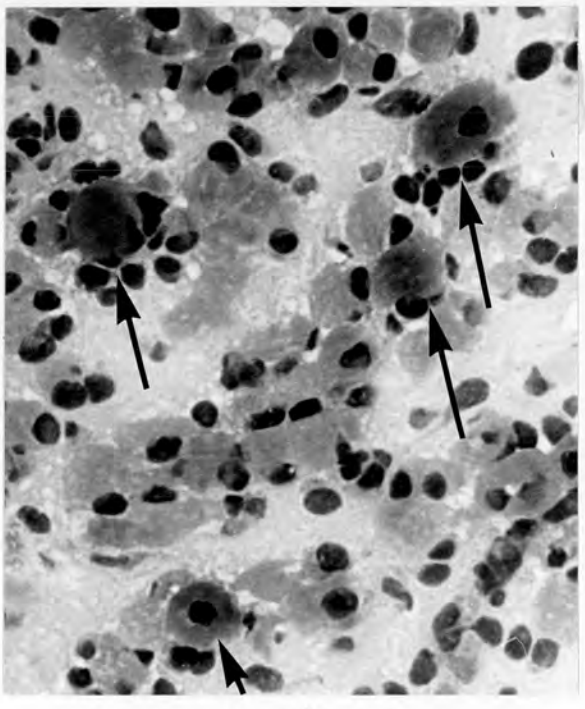
a



b



c



d

TABLE 2

Serum CPK levels and carrier status of mothers

Muscle and serum CPK levels and isoenzymes in fetuses at risk and controls.

Mother				Fetus							
							Muscle CPK (iu/mg protein)			Muscle CPK Isoenzymes	
Name	Age (yrs.)	Serum CPK (iu/l)	Carrier Status.	Number	Gestation (weeks)	Cardiac blood serum (iu/l)	Present level	Storage (months)	Original level (fresh)	% M	% B
M.I	23	32.8-42.8	Possible	599[r]	21	730	4.11	32		85.1	14.9
S.M.	23	22.5-28.0	Possible	684[r]	16	9,140●	2.12	29		too faint.	
S.D.	27	17.5	Possible	734[r]	16		1.65	27		88.4	11.6
I.A.	20	108	Definite	1018[r]	21	1,220●	0.57	18	0.39	too faint.	
M.C.	25	36.5	Possible	73/375c[r]	19	6,800●	17.1	fresh		92.0	8.0
				73/258[c]	22		5.9	fresh		85.2	14.8
				73/259[c]	17		5.7	fresh		76.4	23.6
				73/271[c]	17		4.7	fresh		76.3	23.7
				878	17		1.33	23	0.65		

[r] = at risk [c] = control ● = haemolysed

because of the variation in fetal gestations and the poor histological condition of the specimens. There was no evidence of fibre necrosis or regeneration in any of the specimens from fetuses at risk.

Muscle and Serum CPK.

The muscle CPK results from the five fetuses at risk were more variable than those from the three control specimens (Table 2). Repeat CPK estimations on the two stored samples (878, 1018) showed that enzyme activity increased relative to protein content during prolonged storage at -20°C, but during storage before freezing, as occurred in transit of 4 of the 5 fetuses at risk, muscle CPK levels tend to decrease at an unpredictable rate. Serum CPK results from the four fetuses at risk were even more variable than, and uncorrelated with, muscle CPK results. CPK isoenzyme determinations showed similar proportions of M and B subunits in control fetuses and those at risk.

Conclusion.

The findings in the two previous reports of muscle biopsies in preclinical cases of DMD were principally the presence of hyaline fibres and an increase in the variability of muscle fibre diameter, although there was also a slight increase in the amount of interstitial connective tissue. Four of the five fetuses at risk for DMD described in the present study showed none of these abnormalities in skeletal muscle. However, in the muscle of the fifth fetus (No. 684), there were both hyaline fibres and an increase in the variability of muscle fibre diameter. In addition, Morris (Dr. C.J. Morris, personal communication), has described some abnormally large fibres which stained very darkly for NADH-diaphorase in a biopsy of the deltoid muscle of an 18 week male fetus at risk for DMD. This fetus

also showed an apparently increased variability in muscle fibre diameter.

Serum CPK estimations have been successfully used to detect most carriers (PEARCE et al., 1964: EMERY, 1969) and even in very early stages of preclinical DMD, the serum CPK level may be grossly elevated (BRADLEY et al., 1972). The muscle and serum CPK estimations which were done in the present investigations were however inconclusive. In three of the present four cases, there was a raised level of CPK, but in each case there was marked haemolysis of the samples and the results were therefore unreliable. The results for muscle CPK estimations were more variable among fetuses at risk than among the normal controls, but the relative elevation or depression of each result was not correlated either with the serum CPK level of the fetus, or with the degree of pathological change in its muscle. In addition, the normal variation in CPK results for the quadriceps muscle is so wide that all of the results obtained may well be within the normal range (Dr. C. Foxall, personal communication).

The most interesting observation from any of the five fetuses at risk for DMD remains that of changes in muscle histology in fetus No. 684. These changes were not merely the results of autolysis (see footnote, page 17) which gives rise to very few, if any, hyaline fibres. Nor is it likely that these hyaline fibres were cells undergoing some form of autolytic degradation which Webb (WEBB, 1972) has shown to occur in the normal course of myogenesis. Rather, the changes described in the case of No. 684 are so similar to those described by Pearson and Bradley, in two cases of preclinical DMD that it seems likely that they are the earliest manifestations of DMD which has begun to express itself <u>in utero</u>.

CHAPTER 6

OBSERVATIONS ON FETUSES WITH CONGENITAL MALFORMATIONS OF THE CENTRAL NERVOUS SYSTEM

Introduction.

It has long been established (HARRISON, 1904) that the development of amphibian skeletal muscle is not totally dependent on an intact motor nerve supply, and Harrison demonstrated that even if the motor nerves to a limb muscle were removed, subsequent skeletal muscle development was apparently normal. In mammals, including man, diseases which result in degeneration of the motor nerve cells of the spinal cord, e.g. spinal muscular atrophy (EMERY, 1971), or diseases which cause axonal degeneration, invariably produce very marked alterations in muscle histology and histochemistry. Animal experiments have demonstrated the important role of the motor nerve in determining the properties of skeletal muscle. Cross-union of 'fast' and 'slow' motor nerves results in very marked alterations in the physiological and biochemical properties of the skeletal muscles whose innervation is altered (BULLER et al., 1960b). During the growth of chick skeletal muscle in tissue culture increasing SDH levels in the muscle cells have been shown to be associated with the development of multinucleated cells (COOPER and KONIGSBERG, 1961b). However, neither in this case nor during the growth of mammalian skeletal muscle in tissue culture has it been possible to demonstrate any histochemical differentiation in aneural cultures of skeletal muscle cells (ASKANAS et al., 1972: GALLUP et al., 1972). While the development of functional motor innervation thus appears to be essential to normal myogenesis in mammals, it is not known at which stage skeletal muscle in man becomes dependent on an effective motor nerve supply to maintain structural and biochemical integrity.

In the present study skeletal muscle and spinal cord from several anencephalic fetuses was studied to determine whether muscle development was histologically and histochemically normal, despite the fact that in the fetuses that had rachischisis, there was reason to suspect defective motor innervation.

A preliminary account of the findings reported here has already appeared (TOOP et al., 1973).

Materials and Methods.

Samples were taken from ten anencephalic fetuses at autopsy (Appendix 1). Gestational age was estimated both by the date of the last menstrual period and by use of Streeter's table of heel-toe measurements (STREETER, 1921), and varied between 23 and 31 weeks. Five of the ten had no visible lesion of the spinal cord at all, one had a lesion at the cranial end of the spinal cord which extended only slightly into the cervical region, while the remaining four fetuses had lesions which extended down to the lumbar region. Samples of soleus and gastrocnemius muscles were removed from nine of the ten fetuses, for comparison with normal fetal muscle, together with the lumbo-sacral spinal cord. No muscle sample could be obtained from one of the fetuses with rachischisis, but in the other three cases, as well as samples from the soleus and gastrocnemius muscles, additional samples were taken from the cervical spinal cord and from biceps brachii. This permitted study of several different skeletal muscles, as well as the region of the spinal cord from which their motor innervation normally arises.

Some of the muscle and spinal cord samples were frozen for histochemical and innervation studies, and others fixed and embedded in paraffin for histology. In four of the cases, because of inevitable delay involved in doing the post mortem examination, the time between abortion and obtaining the fetal material was up to three days. In the other six

fetuses, material was fixed or frozen on the same day. Before removing samples, and after removal before processing, all materials were stored at 4°C.

Samples for histochemical studies were frozen in isopentane chilled in liquid nitrogen. Sections were cut at 10μ (histochemistry) or at 20 - 30μ (innervation studies) and handled as described in Chapter 2. Sections of muscle tissue were stained with H and E and for NADH-diaphorase (SCARPELLI et al., 1958) and myosin ATPase at pH 9.4 (PADYKULA and HERMAN, 1955), or at pH 4.3. A combined AChE and silver impregnation stain (NAMBA et al., 1967) was used to stain the peripheral motor innervation. Frozen sections 10 to 50μ thick, taken from the lumbo-sacral region of six of the spinal cords, including three specimens from fetuses with rachischisis, were stained with H and E and toluidine blue. Muscle samples from three of the fetuses and sections of spinal cord from five were also fixed in 10% formol saline for at least one week before processing and double embedding in paraffin wax. Sections both of muscle and spinal cord were cut at 10μ and stained with H and E. In the spinal cord, sections were made at 50μ intervals.

In addition to the specimens from the ten anencephalic fetuses, a sample was taken as control from the lower cervical-upper thoracic portion of the spinal cord of the apparently normal non-identical twin of an anencephalic fetus of 27 weeks gestation. This specimen had been stored for three days at 4°C before fixation prior to paraffin embedding. 10μ sections were cut at intervals of 50μ and stained for H and E.

The method used for counting the large neurones of the spinal cord was that described by PAPAPETROPOULOS and BRADLEY (1972) by which, in order to exclude the possibility of counting one neurone twice, only those whose

nucleolus could be seen were counted. Non degenerate neurones were regarded as those with a well-defined area of cytoplasm and nucleus.

Results.

Muscle.

The histology and histochemistry of all skeletal muscles examined were apparently normal. Sections stained with H and E showed the presence only of normal mature muscle cells, as yet rounded in outline, as is the case in normal fetal muscle at this period of gestation. The fibres had cross-striations and peripheral nuclei, but no evidence of degeneration or atrophy (Plate 1 a, b).

Histochemical differentiation was clearly evident in sections from all of the fourteen skeletal muscles studied, but with some variations in the ratios of type II to type I fibres (Plates 1 c, d, 2). This variation was not always related to fetal gestation. One 26 week fetus (73/93), showed a pattern of histochemical differentiation which, in parts of the section from biceps muscle, was similar to that seen in the muscle of a neonate which has been studied (Chapter 3) (Plate 2 c, d). Other parts of the same section contained muscle fibres which were less well differentiated. Another fetus, of 31 weeks gestation (72/139), showed a considerably less mature development of its muscle: ATPase staining at pH 9.4 in this fetus showed only a few light muscle fibres and the difference in diameter between these type I fibres and the smaller type type II fibres was very marked (Plate 2 a, b), although it varied in different parts of the section. The percentages of different muscle fibre types also varied between three of the fetuses with rachischisis. The proportion of type I fibres was higher in fetus 956 (30 weeks) than in 965 (27 weeks), but neither was very different

Plate 1

a. Longitudinal section of biceps muscle from fetus 956.

H and E, x 330.

b. Longitudinal section of biceps muscle from fetus 965.

H and E, x 330.

c. Transverse section of biceps muscle from fetus 965.

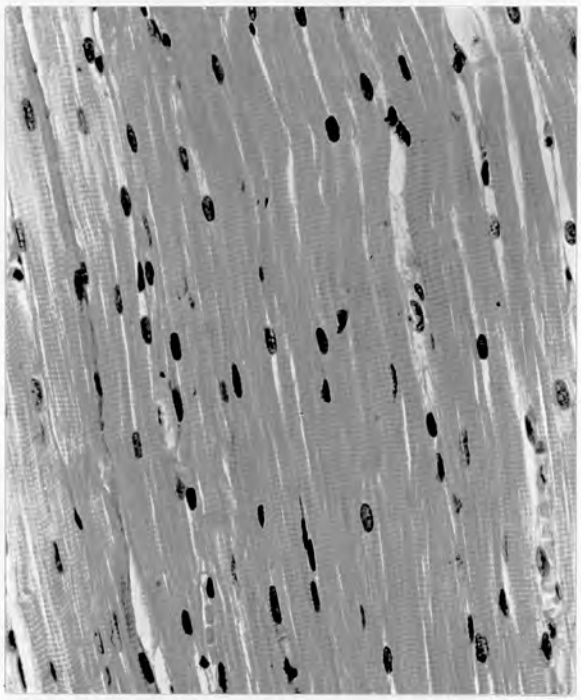
NADH-d, x 135.

d. Transverse section of gastrocnemius muscle from fetus 995.

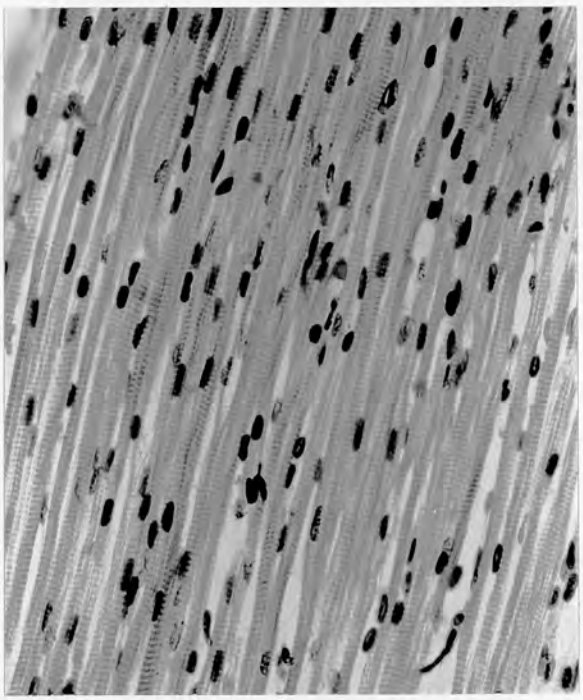
NADH-d, x 135.

Note the presence of scattered, larger, more darkly-staining fibres in c and d.

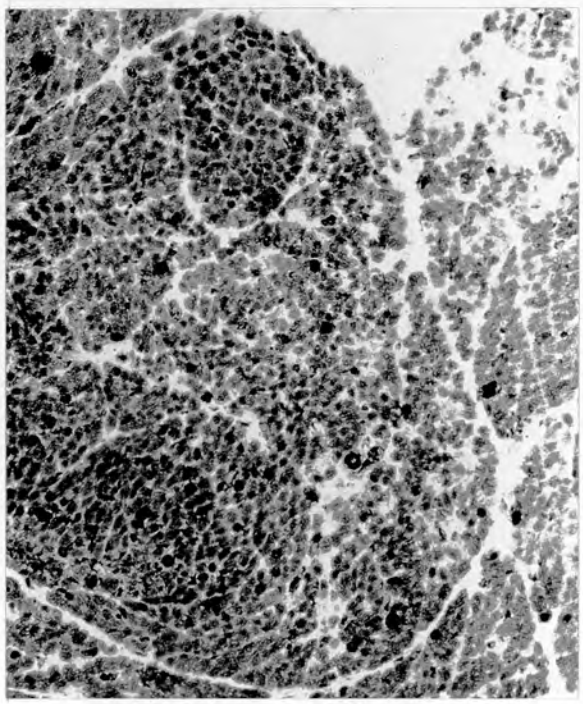
PLATE 1



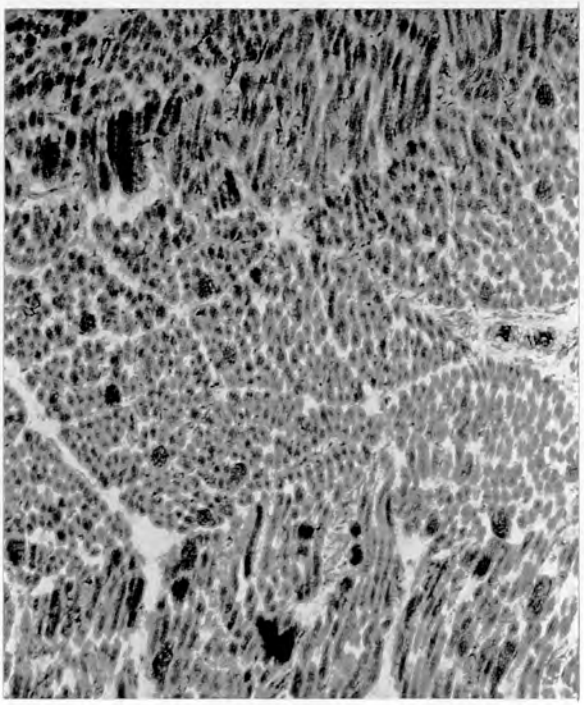
a



b



c



d

Plate 2.

a and b. Transverse serial sections of gastrocnemius muscle from fetus 72/139, x 135.

a. NADH-diaphorase.

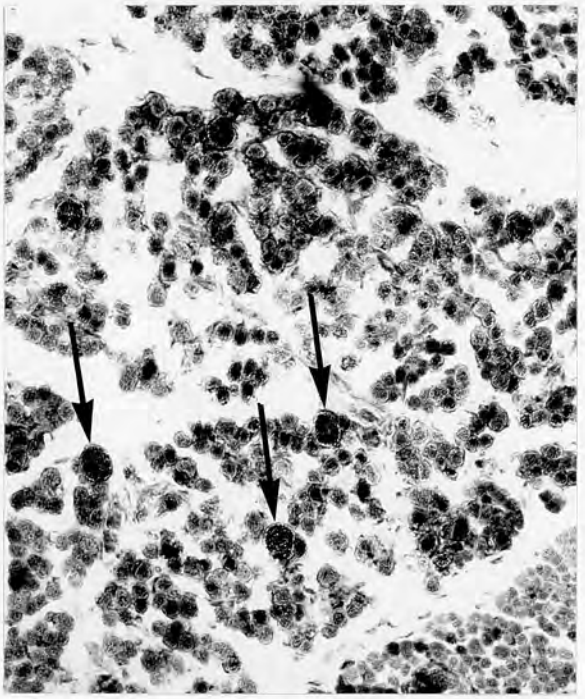
b. myosin ATPase pH 9.4.

Note the reciprocal staining pattern (arrows).

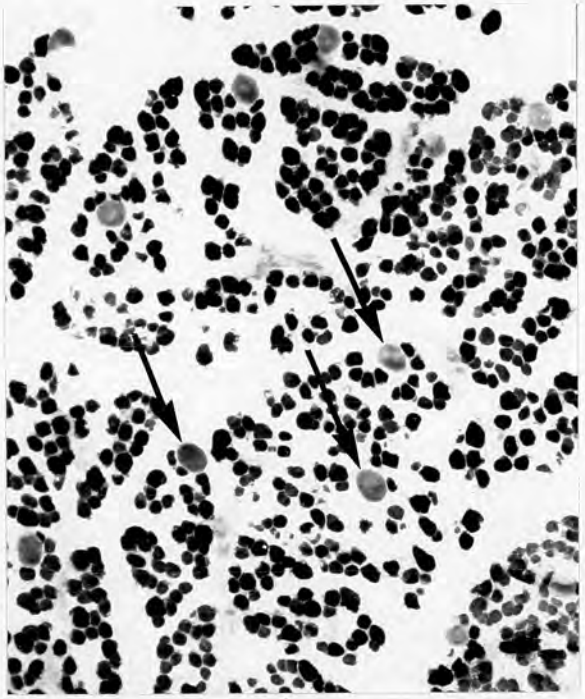
c. Transverse section of biceps muscle from fetus 73/93 NADH-d, x 330

d. Transverse section of biceps muscle from fetus 73/93. ATPase pH 9.4, x 330.

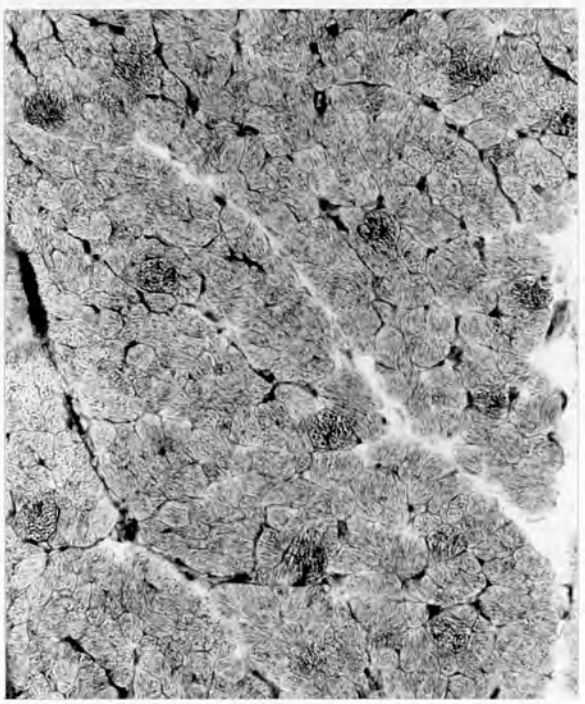
PLATE 2



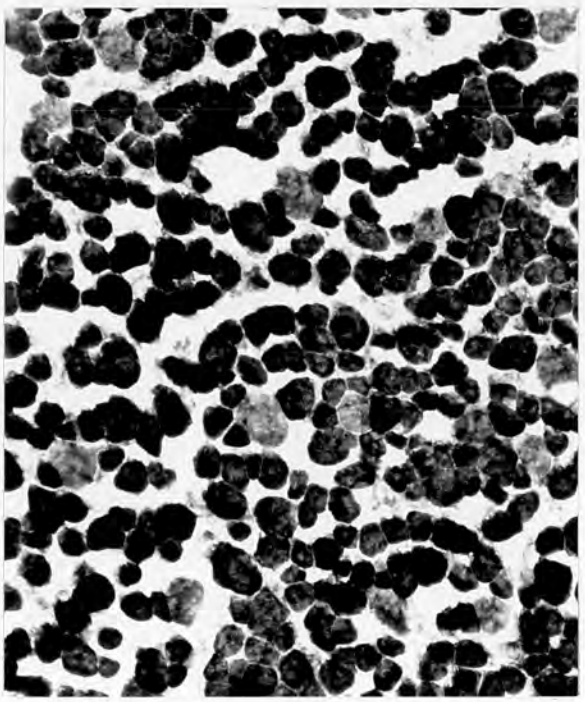
a



b



c



d

from the fibre type proportions seen in the six other anencephalic fetuses whose spinal cords were relatively normal. However, in fetus number 73/93 (26 weeks) the histochemical development of the muscle, as described above, was different, and more nearly complete than is normal in a fetus of this gestation.

The peripheral motor innervation was also apparently normal. The areas of AChE activity were of normal size and intensity and in most cases were restricted to one such area per muscle fibre, and never more than two (Plate 3 a, b). In some cases it was clear that not all terminal axons ended in only one motor end plate, or vice-versa. However, the limitations of the staining technique together with the extremely small diameter of the terminal axon made it impossible to quantitate accurately either of the above observations.

Spinal Cord.

The spinal cords of the fetuses with rachischisis were flat in cross-section, and in utero would have had only a thin layer of ependyma between the tissue of the spinal cord and the amniotic fluid (Plate 3 c). All these cords were extremely vascular and contained greatly reduced numbers of large neurones, which appeared to be degenerate (Plate 4 a, b: Table 1). In contrast to this, neurones in the dorsal root ganglia in two of these cords (956, 965), appeared normal. The findings in three spinal cords from fetuses without rachischisis were very different. One of these cords was more vascular than the other two, and contained large neurones whose cytoplasm was more diffuse than normal (Plate 4 c). The remaining two cords were very similar in all respects to the normal control (Plate 4 d).

Conclusion.

A simple conclusion to be drawn from these results is that anencephalic

Plate 3

a and b. Transverse section of soleus muscle from fetus 1009.

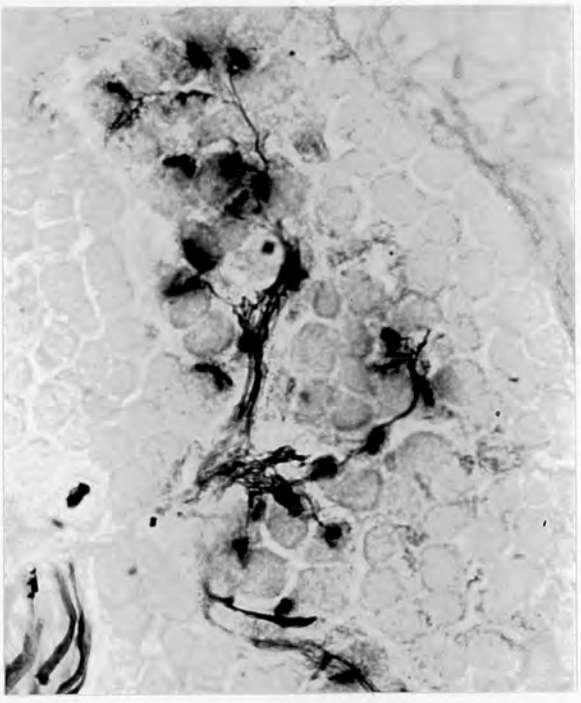
AChE and silver impregnation, x 520.

c. Transverse section of spinal cord from fetus 956.

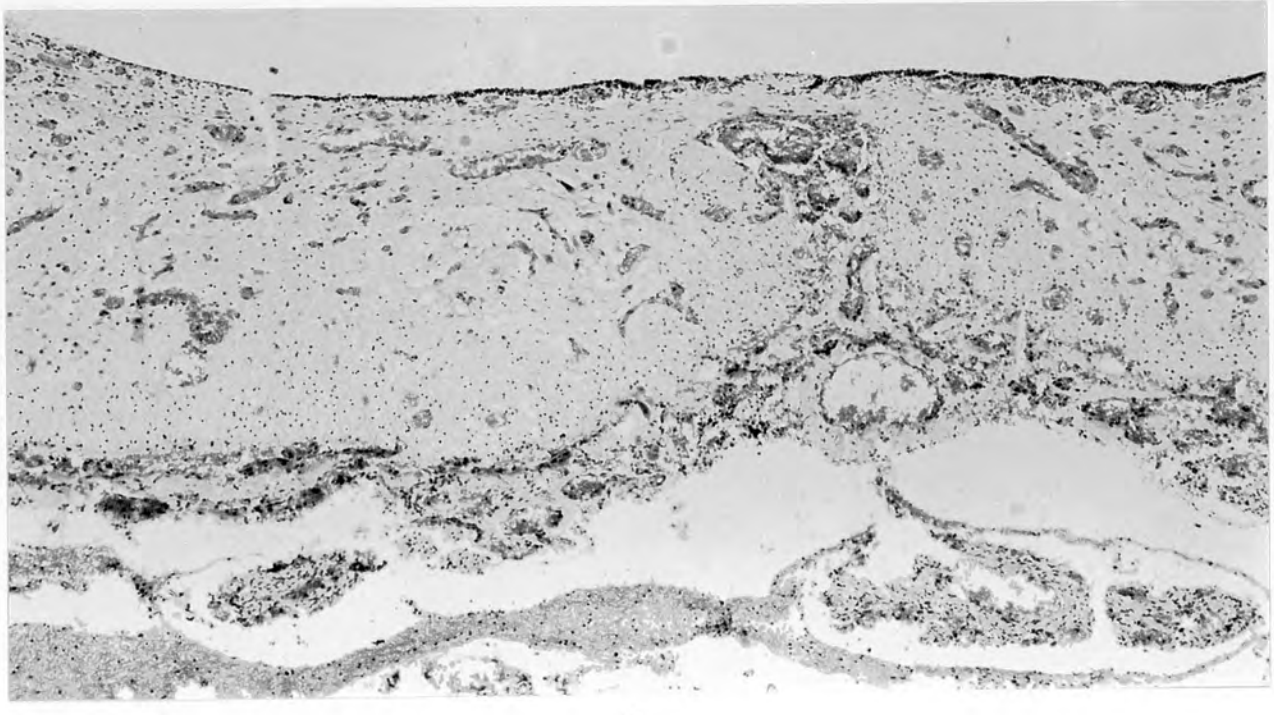
H and E, x 78.

PLATE 3

a



b



c

Plate 4

a and b. Transverse section of spinal cord from fetus 956.

H and E, x 155.

Note presence of many degenerate neurones.

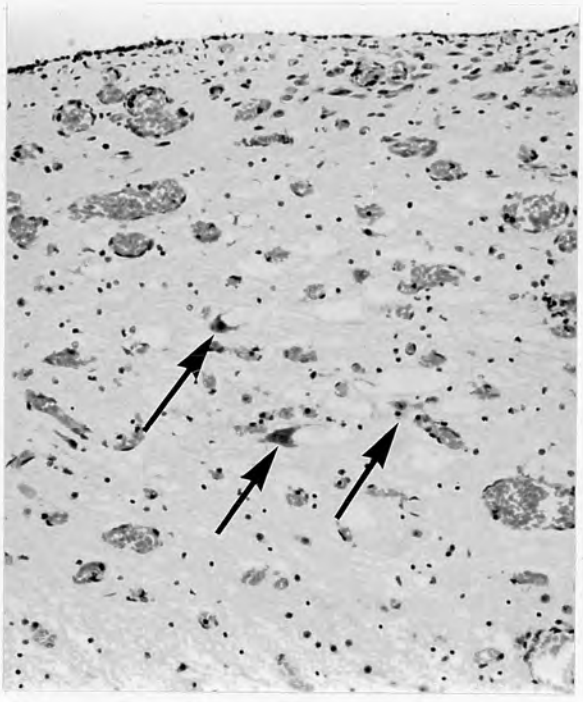
c. Transverse section of spinal cord from fetus 73/262R.

Toluidine blue, x 135.

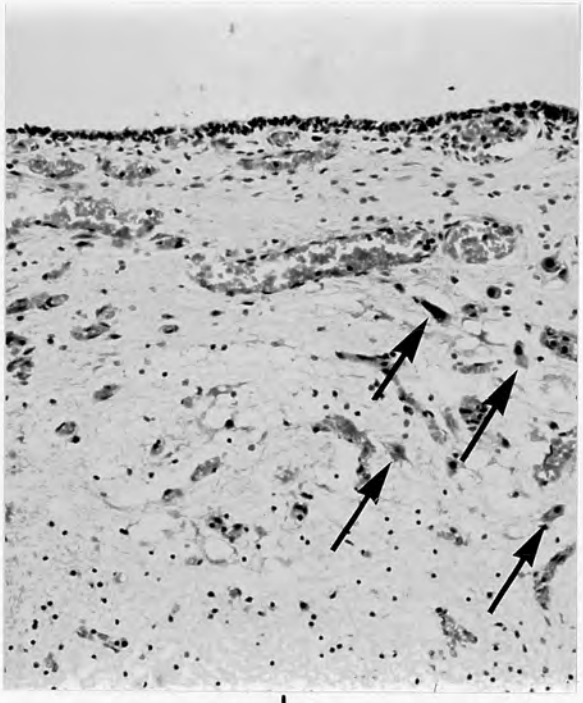
d. Transverse section of spinal cord from fetus 998.

H and E, x 135.

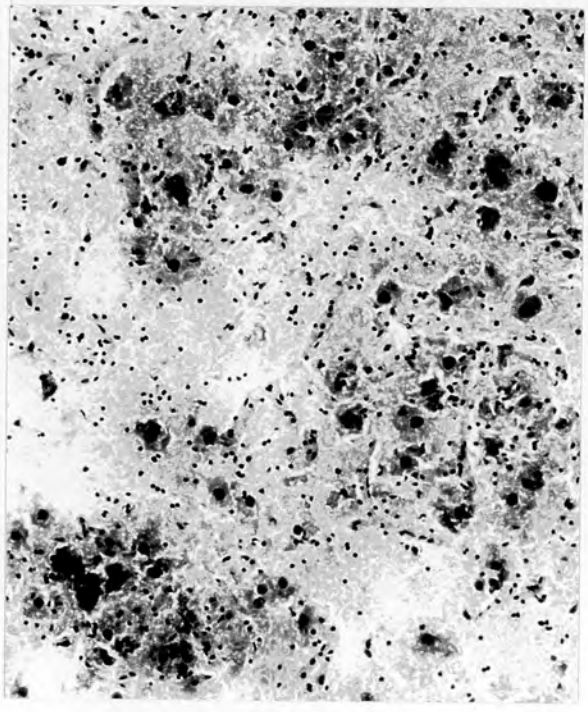
PLATE 4



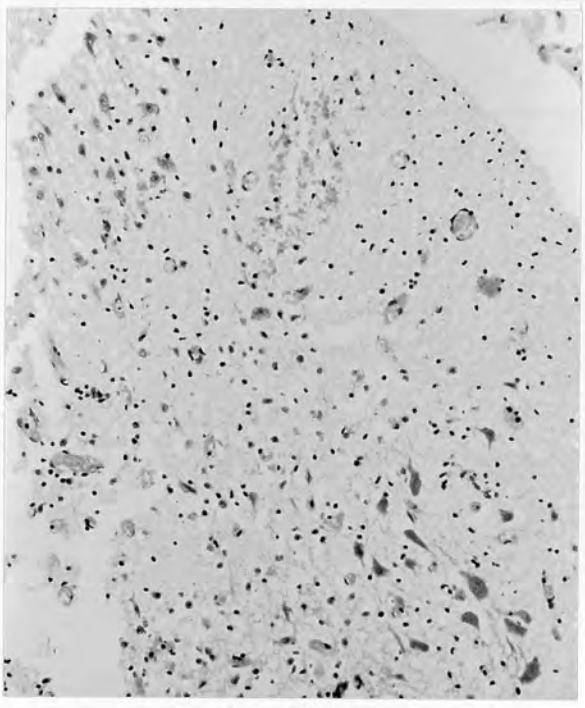
a



b



c



d

TABLE 1

Numbers of large neurones in spinal cord sections.

Fetus number	Status	Number of large neurones per section (No. of sections)		Section thickness (μ)
956	Anen-R	9.5	(8)	10
965	Anen-R	17	(7)	24
998	Anen.	55	(9)	10
72/124	Control	57	(10)	10
72/139	Anen.	44	(5)	10
72/186	Anen-R	8	(7)	10
72/262B	Anen.	167	(10)	50
73/93	Anen-R	10	(3)	20

Anen. = anencephalic fetus

Anen-R = anencephalic fetus with rachischisis.

fetuses with rachischisis have a deficient motor nerve supply to the skeletal muscles, as shown by the marked degeneration of the large neurones of the spinal cord. While no critical functional evaluation of these degenerate neurones was possible, their appearance suggested very strongly that their metabolism was far from normal. The anencephalic fetuses without rachischisis were different, in that the complement of anterior horn cells of the spinal cord was normal. However, in both rachischitic and non-rachischitic fetuses, the histological and histochemical development of muscle appeared normal.

Some studies on the spinal cords of children with myelomeningocele (LENDON, 1968, 1969) provide an interesting comparison with the present findings. These investigations showed that the reduction in number of anterior horn cells was greatest in the most severely affected segments of the spinal cords of these children, but was not as great in more normal segments. The present results paralleled this in that spinal cords from anencephalic fetuses with rachischisis appeared to be much more severely affected with regard to the motor neurone complement than those cords from anencephalic fetuses without rachischisis. Other studies on the spinal cord in myelomeningocele using clinical and electrophysiological criteria (STARK and DRUMMOND, 1971) have led to the tentative conclusion that the basic neurological disorder is of the upper motor neurone, while yet others (MORTIER and VON BERNUTH, 1971) have suggested that there may be early fetal denervation, since electrophysiology and histochemistry demonstrate that there may be impaired skeletal muscle maturation. Information obtained from studies on acephalic sheep fetuses (ROMANES, 1947) has shown that although the caudal half of the spinal cord can develop normally in the absence of the brain, there may again have been defective motor innervation

in these cases, since the skeletal muscles were poorly differentiated and small. Present results are somewhat at variance with the above findings, since all fetuses, with or without rachischisis, showed normal histological and histochemical development of skeletal muscle.

Nevertheless, if the motor nerve supply to the skeletal muscle of the fetuses with rachischisis was genuinely defective at the time when the studies reported here were carried out, there are several possible explanations for the present findings.

The first is that at no time during development was normal motor innervation present, and that the skeletal muscle developed largely or completely in its absence. This suggestion is reinforced by the finding in an even more extreme case than the present one (GILLASPIE and HEUSTON, 1917): an anencephalic fetus which had neither brain nor spinal cord showed "a complete and apparently normal development of the peripheral nervous system". In amphibia, development or regeneration of skeletal muscle may be possible in the absence of a motor nerve supply (HARRISON, 1904). However, studies on the effects of reciprocal cross-union of nerves to 'fast' and 'slow' muscles have led to the conclusion that in mammals the motor nerve plays a decisive role in muscle fibre type determination (BULLER et al., 1960b) probably through the mediation of a trophic substance. In situations where there was no motor nerve supply, as in aneural tissue culture of skeletal muscle, the normal pattern of histochemical differentiation has never been seen (ENGEL, 1961 ; ASKANAS et al., 1972: GALLUP et al., 1972). Many other experiments on animals also demonstrate the deterministic role of motor innervation in the initial differentiation of skeletal muscle fibres into different types (KARPATI and ENGEL, 1967) and probably also, through

some kind of repetitive stimulation to the muscle, the maintenance of its normal function (SALMONS and VRBOVÁ, 1969: AL-AMOOD et al., 1973). This first possibility, if true, would also cast doubt on previous studies of the histochemical development of human muscle (DUBOWITZ, 1968), in which it was suggested that the gradual increase in the percentage of type I fibres after 25 weeks gestation might be a neurally mediated alteration of some type II to type I fibres.

A second possible explanation is that there were only a few functional motor neurones at any time in the rachischitic spinal cords. In the anencephalic fetuses studied, the sizes both of muscles and of muscle fibres appeared normal, so that the number of muscle fibres was probably also close to normal. In the presence of a greatly reduced number of functional motor neurones, one might therefore expect the average size of a motor unit to be greatly increased. Although there are obvious technical difficulties in attempting to determine motor unit size in anencephalic fetuses, in principle it might be possible if specimens could be obtained freshly enough. The technique of prolonged single motor axon stimulation (EDSTRÖM and KUGELBERG, 1968: KUGELBERG, 1973) depletes the glycogen store of individual motor units, and histochemical staining for PAS and phosphorylase in transverse frozen sections may then be used to demonstrate the entire muscle fibre population of the motor unit. Alternatively, using either the techniques of McComas and his colleagues (McCOMAS et al., 1970), or by a study of the number of peripheral motor nerves in anencephalic fetuses with rachischisis compared with normal, it might be possible to estimate the actual number of motor units in a given muscle.

A third possibility is that normal motor innervation was present, and

necessary to produce the initial histochemical differentiation of muscle, but that the motor neurones then began to degenerate at some time before delivery. There are two points which must be remembered with respect to this suggestion. One is that in spite of the fact that histochemical differentiation is not normally present in human skeletal muscle before 20 weeks gestation, it was evident in all the muscles examined in the anencephalic fetuses studied here. The other is that the CNS defect in anencephaly must have developed at an early stage of gestation (PADGET, 1970), and Warren (WARREN, 1951) estimates that the critical period for the development of anencephaly is limited to the second month of gestation. Other evidence suggests that the defect occurs in the first month (GIROUD, 1960) and the Carnegie embryological collection contained one fetus 6 mm. long (equivalent to approximately 33 days gestation (HAMILTON, et al., 1952)), with anencephaly, and another only 2.1 mm. long with both anencephaly and spina bifida (MALL and MEYER, 1921). It is possible for adult (LEWIS, 1962: SYROVÝ et al., 1971) as well as neonatal skeletal muscle (LEWIS, 1973), to retain the different characteristics of 'fast' and 'slow' muscle fibres for some time after denervation, but in anencephaly it is not known when or why the degeneration of the large motor neurones begins. Irrespective of this, no satisfactory explanation of the present findings can as yet be given as to how apparently normal histochemical differentiation was able to take place in the anencephalic fetuses with rachischisis, despite the great neuronal deficit in the spinal cord which presumably had been present from an early stage of development.

CHAPTER 7

STUDIES ON THE MORPHOLOGY AND HISTOCHEMISTRY OF HUMAN FETAL MUSCLE CELLS IN VITRO

Introduction.

The growth of specific cell types *in vivo* is difficult to investigate, and the isolation and growth of specific cell lines in defined synthetic media has proved a satisfactory alternative in many instances. Under these circumstances the morphology and biochemical characteristics of particular cell types can be observed.

In 1885, ROUX (cited by PAUL, 1965) was apparently the first person to study an explant of biological tissue *in vitro*, and in 1906-07 HARRISON was able to maintain nervous tissue in a clot of frog lymph for some weeks. In subsequent years there were improvements in the techniques of tissue culture, which included the substitution of a plasma clot for the lymph clot (BURROWS, 1910), the discovery of the growth-promoting effects of chicken embryo extract (CARREL, 1913), and improvement in the nature of the vessels in which the different tissues were grown (CARREL, 1923: MAXIMOW, 1925). The transfer of cells from a primary culture to separate subcultures was first achieved by CARREL (1912), and in 1952, MOSCONA introduced trypsin into tissue culture to facilitate the removal of mononuclear precursor cells which could then be transferred to another vessel for subculture.

In 1915, LEWIS was the first person to study the growth of skeletal muscle *in vitro*. POGOGEFF and MURRAY investigated adult rat (1945) and human (1946) muscle grown *in vitro*, and indicated that multinucleated cells with cross-striations could develop from initial outgrowths of mononuclear cells. Subsequent experimentors obtained similar results (GEIGER and GARVIN, 1955, 1957). Much of the extensive work carried out on human skeletal

muscle *in vitro* has been reviewed by MURRAY (1965, 1972) and KONIGSBERG (1965).

The present study was concerned with human fetal skeletal muscle, a tissue whose growth *in vitro* has previously been reported only once (BATESON, 1968). The planning of the experiment therefore was largely based on experience gained by other workers during the culture of skeletal muscle from other species. The techniques which have been developed during the past 60 years for the study of vertebrate muscle *in vitro* form the basis of many of those still in common use today, as described by PAUL (1965).

The usefulness of tissue culture lies in the fact that specifically identifiable cell types can be studied, but a vital consideration when such a system is used is whether it is capable of maintaining *in vitro* the different morphological, biochemical and general behavioural properties which are its characteristics *in vivo*. Since this chapter is concerned only with human muscle in tissue culture, it is not proposed to describe in detail, experiments which have been carried out on skeletal muscle from animals. It is sufficient to say that in past experiments with both adult and fetal animal muscle it has been shown that individual myoblasts grow out from a muscle explant, and fuse to form multinucleated 'strap' cells. Later, either by means of fluorescent-labelled antimyosin antibody (HOLTZER et al., 1957: ENGEL and HORVATH, 1960), or by electron microscopy (MENDELL et al., 1972), myofibrils have been shown to develop, and finally cross-striations appear. These multinucleated cells have all the histological characteristics of mature muscle cells with peripherally displaced nuclei, and cultures of animal muscle cells may be seen to twitch spontaneously.

Such findings demonstrate that animal muscle cells in tissue culture behave in a manner similar to their behaviour *in vivo*; but perhaps the best evidence is that when portions of animal spinal cord are placed in contact

with growing animal muscle cells, axonal outgrowths to the muscle cells appear, and motor endplate formation can be demonstrated. The multinucleated muscle cells can then be made to contract by stimulation of the axons which innervate them (CRAIN, 1970: SHIMADA and KANO, 1971: PETERSON and CRAIN, 1972).

Studies of the growth of human muscle *in vitro* are not as numerous as those on animal muscle, although many similar features have been observed. Some of the earliest investigations on the growth of adult human muscle *in vitro* were conducted by POGOGEFF and MURRAY (1946), and since then the study of the growth of this tissue in culture has become generally accepted as a means of studying its properties in isolation, but the growth of human fetal muscle has remained unexploited.

The experiments undertaken in this section had several aims. One was to determine whether human fetal muscle *in vitro* develops morphologically in a manner similar to adult muscle in culture. A second aim was to discover whether, in long-term cultures, any histochemical differentiation could be detected in the muscle cell population. Each of these aims was considered in relation to the gestation of the fetus from which the initial muscle explant was made and the normal histological and histochemical state of the muscle *in vivo* at that gestation (see Chapter 3).

Materials and Methods.

(1) Setting up and maintenance of primary and subcultures.

Quadriceps muscle was taken from 63 fetuses all considered to be normal under the criteria laid down in Chapter 2. Muscle taken from a fetus removed by suction termination, or which for other reasons was considered unsterile, was washed before explanting, in a phosphate buffered saline solution (Dulbecco 'A') containing 500 u/ml Penicillin, 500 µg/ml Streptomycin, 200 µg/ml Cidomycin and 10 µg/ml Amphotericin B. This procedure prevented

infection occurring in primary cultures. Primary explants were made by dividing the muscle very finely with scalpels, then placing about 40 of these small muscle blocks (0.5 mm cube approximately) on the flat inside surface of each of 8-10 100 ml. glass bottles. The explants were clotted in place using a 1:1 mixture of chick embryo extract and cock plasma, and sufficient medium added just to cover them. The bottles were sealed with screw-on plastic caps and incubated for about 10 days at 37°C in an atmosphere of 5% CO_2: 95% air.

To remove the mononuclear cells from the bottles for subculture, the culture medium was removed from up to 5 bottles and a small amount of Trypsin (1:250, Difco, 0.25% in Dulbecco 'A') was added and immediately poured off. After 10 minute incubation the loose cells were removed as a suspension in fresh medium. A few drops of this suspension were retained to assess the concentration of suspended cells, while the rest was poured into the vessels chosen for subculturing. Simultaneously fresh medium was poured over the explants remaining in the primary culture to enable further growth to occur. The subculture vessels were initially 84 mm plastic petri dishes in which sterile coverslips had been placed, before coating the bottom of the dish with collagen film. The amount of medium necessary for maintaining these cultures was so great that later 50 mm petri dishes were substituted for the larger ones. Initial experiments appeared to show that the myoblasts grew as well on glass alone as on a collagen film, which was therefore omitted towards the end of the experiment. Also near the end of the experiment a few subcultures were grown in Leighton tubes with screw-on caps in order to try and eliminate infection.

The culture media used for these experiments were Minimal Essential Medium (Eagle) (Flow) initially, and later Hams F-10 (Flow) in either case

supplemented with fetal calf serum (Flow) to a final concentration of 10%, and containing 50 u/ml Penicillin, 50 μg/ml Streptomycin, 100 μg/ml Cidomycin and 2 μg/ml Amphotericin. Cell growth was assessed daily on an Olympus CK inverted phase contrast microscope, as was the state of the medium, which was changed when its pH reached approximately 6.8, or at weekly intervals. If a culture contained infection, which could not be suppressed by the antibiotics present in the culture medium, this was removed and the explants washed in several changes of the phosphate buffered saline containing antibiotics as described above, before fresh medium was added.

(2) Maintenance of sterility.

Sterility was achieved, insofar as was possible, either by virtue of the fact that the materials used had been sterilised during the production stage, or were sterilised in the laboratory. In the latter case, this was achieved either by passage through a bacterial filter, 0.22μ pore size, by steam (20 minutes at 15 lbs. per square inch) or by dry heat (at least 90 minutes at 160°C). The collagen film was denatured with .880 ammonia, washed with sterile water until neutral pH was achieved and sterilised by UV light for 24-48 hours. All handling of sterile materials was done in a laminar flow hood (Microflow) taking the usual precautions such as flaming pipettes and the open ends of bottles, and washing the hands with alcohol.

(3) Removal of coverslips and staining.

At the beginning of the experiment coverslips were sometimes removed from subculture after only a few days, but it soon became clear that they needed to be left considerably longer in culture. Coverslips were removed aseptically and rinsed for a few minutes in physiological saline

before staining. The stains used were H and E, NADH-d, and myosin ATPase at pH9.4. The methods used for histochemical stains were described in Chapter 2, as used to stain frozen sections of fetal muscle *in vivo*, but with H and E it was necessary to ensure that the coverslip remained in absolute alcohol no longer than one minute, since longer dehydration resulted in only very pale staining of the cells' cytoplasm.

The success of the growth in primary and secondary cultures was defined as the percentage of the culture vessels from which myoblasts could be removed, either for subculture or for staining on coverslips.

(4) Analysis of stained coverslips.

The entire area of each coverslip was scanned at low power for the presence of multinucleated muscle cells. In an attempt to quantify the growth, 5 areas of the diameter visible at a magnification of x400 on a Wild M40 inverted phase-contrast microscope were selected at random on each coverslip stained with H and E. Within these 5 areas, counts were made of the total numbers of nuclei, and the number of those which had become incorporated into multinucleated cells. From these counts, two figures were derived, each related proportionally to the actual counts, in order to try and quantify the degree of cell fusion. For each individual subculture, the differentiation index (DI) was defined as the number of multinucleated cells, and the nuclear index (NI) as the number of nuclei present in multinucleated cells, which would have been seen had 1,000 nuclei been counted. The slides stained for NADH-d and ATPase were scanned for the presence of stained cells.

(5) Analysis of nuclear counts.

Calculation of regression analyses or correlation coefficients were carried out on an ICL 4/75 computer.

Results.

The number of fetuses whose muscle grew sufficiently well in vitro for coverslips to be removed from subculture and stained, was 28, of which details can be found in Appendix 1. Infection either by yeast, bacteria, or latterly mycoplasma, was largely responsible for the loss of the other cultures, although for unknown reasons some primary cultures failed to grow at all, and a few others failed to grow after the first subculture was made. However, 6 primary cultures (from fetuses 274, 289, 296, 317, 319, 339), grew so well that four or more subcultures were made from each of them.

Morphological development.

All 28 successful primary cultures showed clearly the presence of mononuclear cell outgrowths from the initial muscle explants, and similar cells, identifiable as myoblasts, were seen in most of the subcultures.

The results of the growth and fusion analysis are presented in Table 1 and contain data on subcultures from 18 fetuses. Subcultures from 6 of the 28 fetuses contained very few cells, and none which were multinucleated, and in subcultures of 4 of the remaining 22 fetuses, the 5 microscope fields analysed contained no multinucleated cells, thus making estimation of the DI and NI impossible. The results of the analysis showed the very wide range of values which were found, with, for instance, the plating density varying between 0.4 and 6.7×10^4 cells/cm^2, the number of nuclei counted per 5 microscope fields between 9 and 651, and the number of myotubes/5 fields between 1 and 21.

In an attempt to extract some meaning from this great diversity of results, the data was put on to punched cards and analysed by computer to produce correlation coefficients or regressions for different paired or grouped

TABLE 1

GROWTH AND FUSION ANALYSIS

FETUS	Days in	SUBCULTURE							
Number	primary culture	Number	Secondary growth (%)	Plating density (cells/cm^2 X 10^4)	Collagen	Subculture vessel	Days in subculture	DI	NI
389	9	1	20	2.3	no	50 (a)	6	40	80
278	16	2	100	3.6	yes	84 (b)	26	26	52
	34	3	75	1.2	yes	50	14	11	28
							22	27	54
							30	17	34
	53	4	40	5.8	no	50	3	28	56
							13	25	50
382	6	1	20	3.3	no	50	11	54	108
	7	2	20	3.4	no	LT (c)	38	10	20
317	14	2	100	1.4	yes	50	7	14	28
							15	5	20
							25	31	77
274	8	1	50	0.7	yes	84	6	111	222
	21	2	100	1.8	yes	84	4	50	100
							7	125	250
							19	12	24
	32	3	100	2.0	yes	84	14	40	80
							21	31	70
							29	21	46
299	8	1	75	2.0	yes	84	13	7	14
							20	9	27
							28	15	34
	20	2	60	1.8	yes	50	14	17	34
303	12	1	100	1.6	yes	84	8	8	16
							15	19	38
							22	23	52
							29	18	49
	22	2	40	1.3	yes	50	15	31	62
	44	3	20	1.3	no	50	14	33	88
289	20	2	100	1.6	yes	84	8	19	38
							15	9	18
							22	28	60
	30	3	100	1.9	yes	50	25	12	24
	37	4	40	2.5	yes	50	8	51	130
339	8	1	80	1.5	yes	50	19	38	76
	12	2	60	2.3	yes	50	17	57	156
							33	51	102
	24	3	20	1.7	no	50	14	39	88
	35	4	80	4.5	no	LT	38	24	54
296	18	2	100	4.6	no	LT	14	15	30
						50	21	18	36
						LT	21	38	84
						50	28	21	42
						LT	28	21	42
						LT	62	32	77
	33	3		1.4	yes	50	29	21	42
352	16	1	60	1.8	no	50	7	27	54
							14	26	52
300	10	1	100	1.5	yes	84	11	29	88
							18	56	112
							25	40	86
							32	25	56
	20	2	80	0.8	yes	50	29	25	50
319	10	1	100	2.0	yes	50	7	26	58
							14	22	44
							22	22	47
	20	2	60	3.0	yes	50	8	29	58
							15	32	64
	34	4	20	3.2	no	50	14	24	48
	46	5	20	6.7	no	LT	38	33	71
345	10	2	40	1.0	no	50	15	35	70
341	37	3	20	0.5	no	50	10	29	58
316	7	1	40	1.1	yes	50	11	43	163
							22	15	69
	20	2	40	4.5	yes	50	7	22	61
318	13	2	60	4.7	yes	50	15	12	24
	46	3	20	1.4	no	LT	38	25	50
285	21	2	100	0.4	yes	84	15	22	44
							22	17	34
374	11	1	20	1.9	no	50	11	--	--
346	7	1	80	1.0	no	50	8	--	--

(a) - 50mm. petri dish (b) - 84mm. petri dish (c) - Leighton tube.

sets of observations respectively. Results of these analyses are given in Table 2. There is only one correlation coefficient with a probability value less than 0.05, but none of the other correlation coefficients or regression analyses were significant. In addition the mean and standard deviations of both the DI and NI were calculated to compare results in several contrasting classes: these results are also in Table 2. These showed that the means both of the DI's and NI's were higher in the presence of collagen than without it; higher in subcultures from female fetuses than from male; and highest when the subcultures were grown on 84 mm petri dishes. The standard deviations were so great however, that the differences were far from significant.

The results of this attempt at a more sophisticated analysis of the findings produced no clear evidence as to how the growth of a muscle culture could be quantified, or what were the best methods to use in trying to stimulate the growth and fusion of myoblasts. The comparative scarcity of large multinucleated cells in most of the subcultures contributed greatly to the difficulty of this analysis, but there were a few subcultures in which considerable myoblast fusion had occurred. Foremost of these was the second subculture from a 14 week fetus (339) in which many multinucleated cells, containing 5-60 nuclei each, could be seen on one coverslip, removed after 15 days in subculture (Plate 1). Results from this coverslip were not included in the analysis of cell fusion since the subculture had been grown for electrophysiological investigations, but in the first subculture of muscle from the same fetus there were also many large multinucleated cells present (Plate 2a). These subcultures were characterised not only by the presence of these very large multinucleated cells but also by the fact that

TABLE 2

STATISTICAL ANALYSIS OF GROWTH AND FUSION

(A)

Correlation of	with DI		with NI	
	r	probability	r	probability
Plating density	-0.08	0.51	-0.11	0.39
Nuclei/5 fields	-0.24	0.05	-0.17	0.16
Primary culture success	0.19	0.11	0.18	0.13
Secondary culture success	-0.15	0.22	-0.19	0.12
Gestation	-0.11	0.36	-0.03	0.82
Days in subculture	-0.22	0.08	+0.21	0.09

(B)

Multiple correlation of	with Nuclei/5 fields and plating density.		with Nuclei/5 fields and days in subculture.	
	r	probability	r	probability
DI	0.25	> 0.05	0.27	> 0.05
NI	0.19	> 0.05	0.23	> 0.05

(C)

Mean and standard deviation of DI and NI in different classes.

Class		DI	NI
Collagen		29.2 ± 22.7	65.2 ± 48.8
No collagen		27.9 ± 9.6	58.9 ± 22.00
Male		25.0 ± 10.2	54.5 ± 25.9
Female		27.5 ± 13.0	59.5 ± 31.1
Subculture number:	1	30.6 ± 22.5	70.2 ± 48.2
	2	28.4 ± 22.5	60.6 ± 47.4
	3	25.5 ± 9.5	55.2 ± 22.3
	4	30.4 ± 11.6	67.6 ± 35.0
	5	33.0 ± -	71.0 ± -
Subculture vessel:	84 mm petri	31.7 ± 29.5	67.1 ± 58.5
	50 " "	27.4 ± 12.6	62.0 ± 34.0
	Leighton tube	26.9 ± 7.9	58.3 ± 19.7
Termination method:	D & C	24.8 ± 13.8	50.3 ± 26.9
	Prostaglandin	30.7 ± 22.2	65.8 ± 46.4
	Spontaneous	25.7 ± 11.0	73.5 ± 46.1
	Hysterotomy	24.8 ± 15.6	55.7 ± 32.9

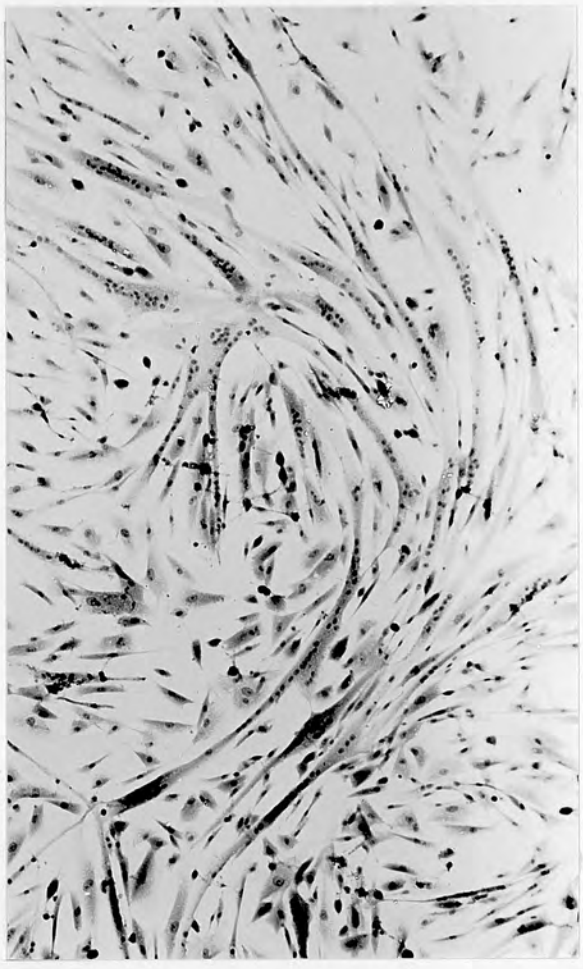
Plate 1

Multinucleated cells present in the second subculture from fetus 339, 15 days in culture. H and E.

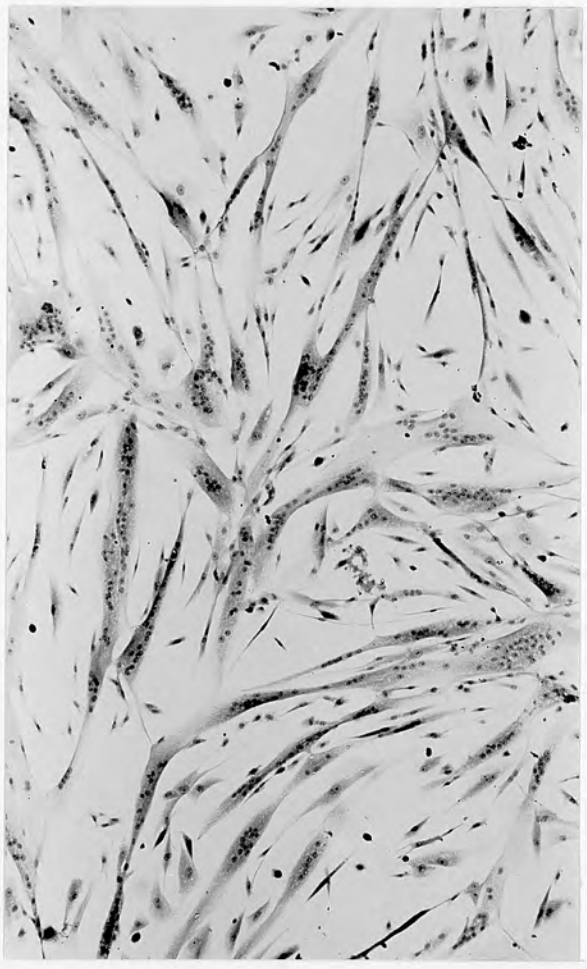
a and b - x 78

c - x 102.

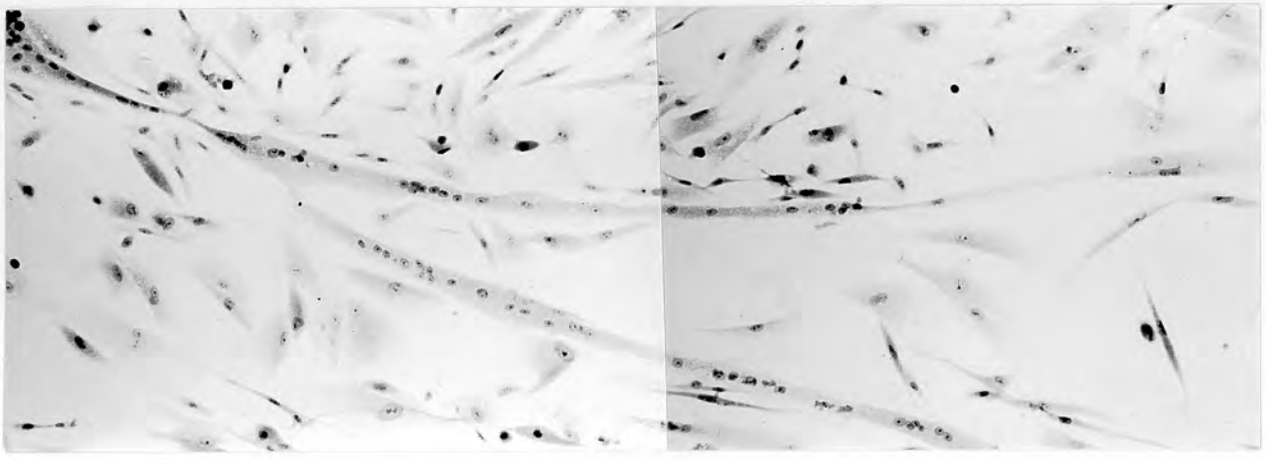
PLATE 1



a



b



c

the growing muscle cells were not as tightly packed together as in most of the other subcultures.

Similar but less striking observations were made in subcultures from other fetuses also. The second subculture of fetus 318 (22 weeks) showed some very large multinucleated cells, as well as smaller ones, while subcultures from fetuses numbers 316 (22 weeks), 319 (16 weeks), 346 (12 weeks) and 374 (11 weeks) contained more than the normal number of multinucleated cells, most of which were larger than average (Plates 2b - d, 3). While some of these larger-than-average multinucleated cells were found among almost confluent growths of uninucleated cells (316, 318, 319), the remainder, as in the subcultures of fetus 339, were found on coverslips on which the growth was not so nearly confluent. This less confluent pattern of growth was also seen in subcultures from four other fetuses (278, 10 weeks: 317, 11 weeks: 286, 26 weeks: 300, 16 weeks) (Plate 4a, b), but these subcultures contained only an average number of multinucleated cells. Three of these last four subcultures whose growth was less confluent were grown on a collagen film, as were five of the seven subcultures in which large multinucleated cells were seen.

Histochemical development.

The results using NADH-d stain were very different from those obtained using ATPase at pH9.4. With the latter, there were only three subcultures (from fetuses 278, 10 weeks: 319, 16 weeks: 346, 12 weeks) among all those which had been grown, for up to 37 days, whose cells had become at all stained, and even these cells were comparatively pale (Plate 4c, d). However, with NADH-d, many cells were stained, both uni- and multinucleated, in many subcultures from fetuses at different stages of

Plate 2

Multinucleated cells present in subcultures of human fetal muscle cells. H and E, x 135.

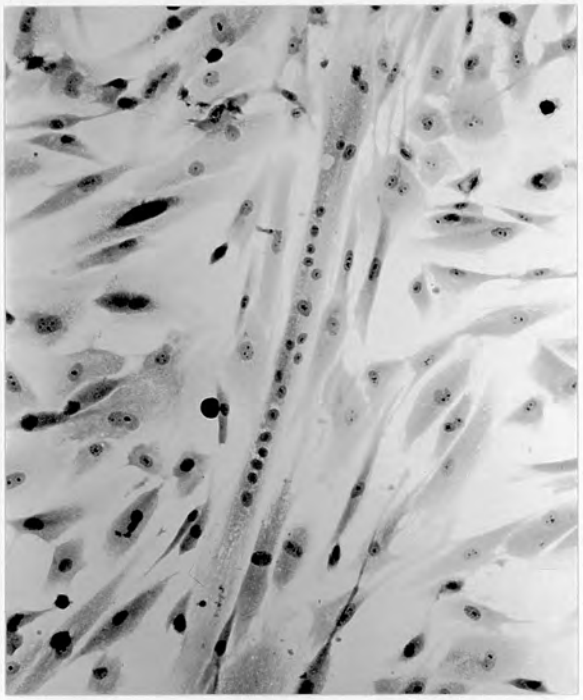
a. First subculture from fetus 339, 19 days in culture.

b. Second subculture from fetus 318, 7 days in culture.

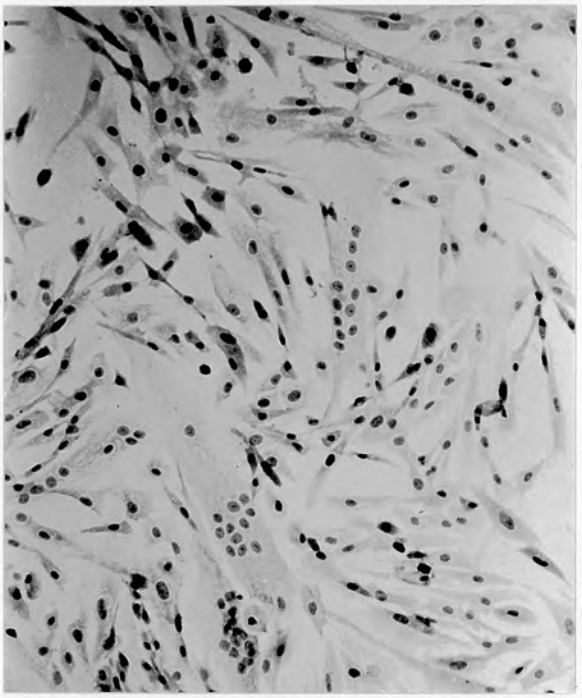
c. First subculture from fetus 346, 8 days in culture.

d. Second subculture from fetus 318, 15 days in culture.

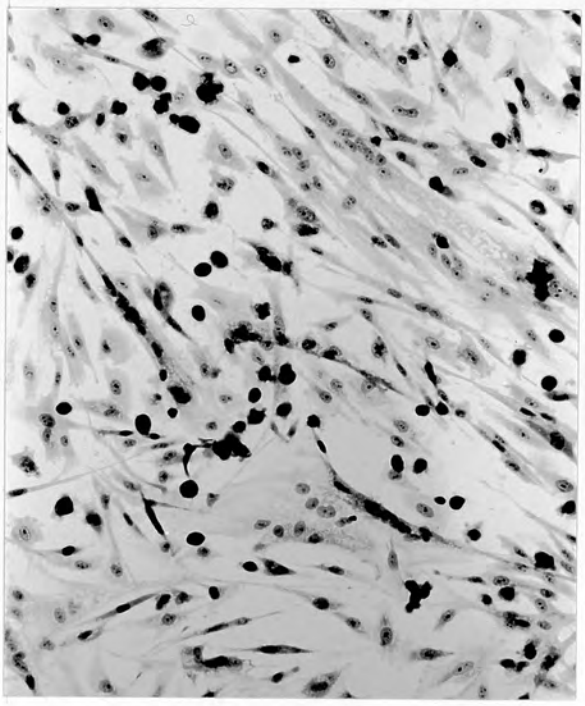
PLATE 2



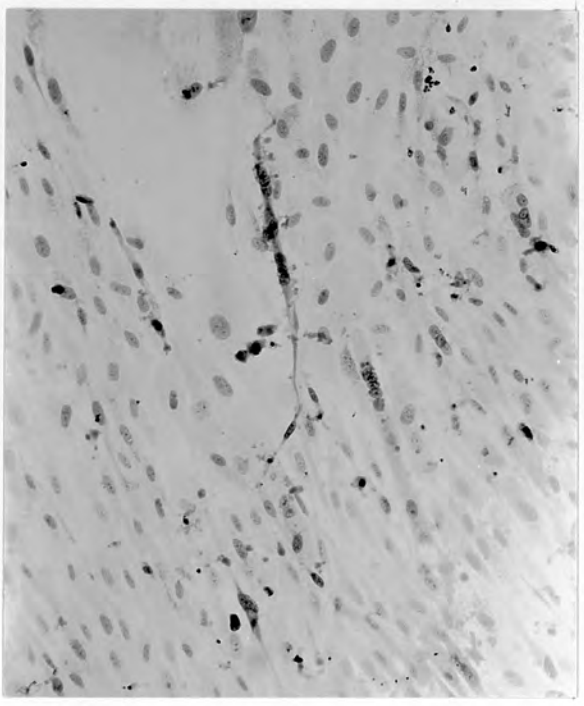
a



b



c



d

Plate 3

Multinucleated cells present in subcultures of human fetal muscle cells. H and E, x 135.

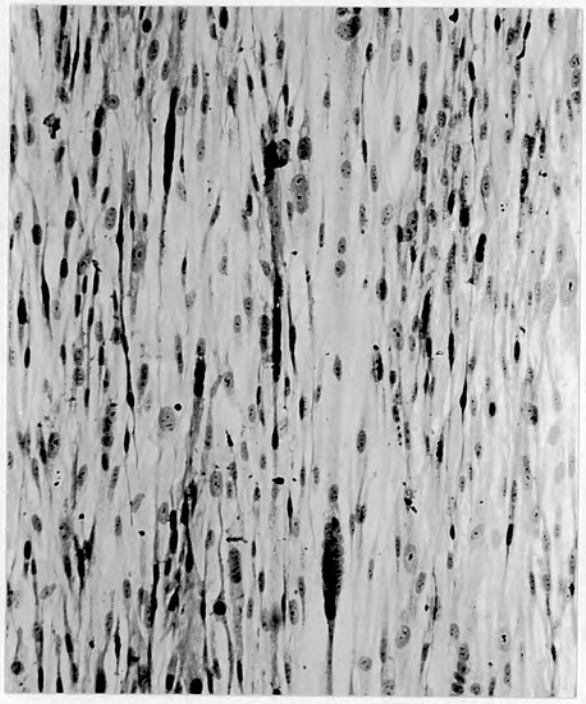
a. First subculture from fetus 316, 11 days in culture.

b. First subculture from fetus 316, 22 days in culture.

c. First subculture from fetus 319, 7 days in culture.

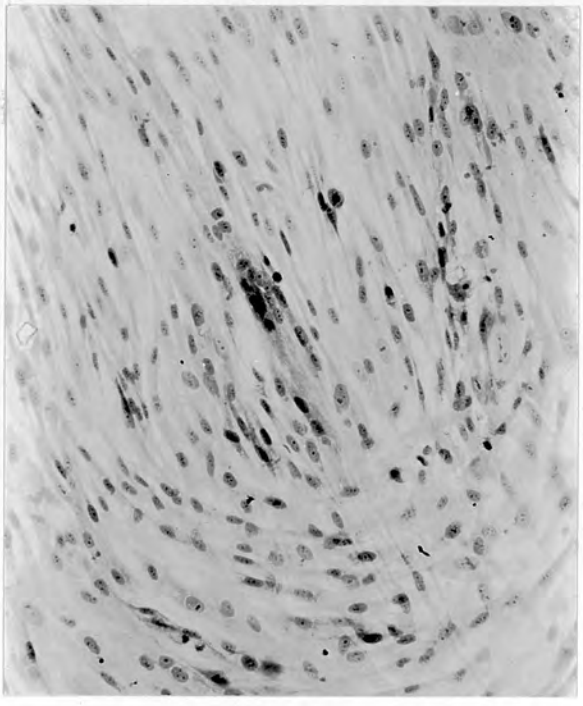
d. First subculture from fetus 374, 11 days in culture.

PLATE 3

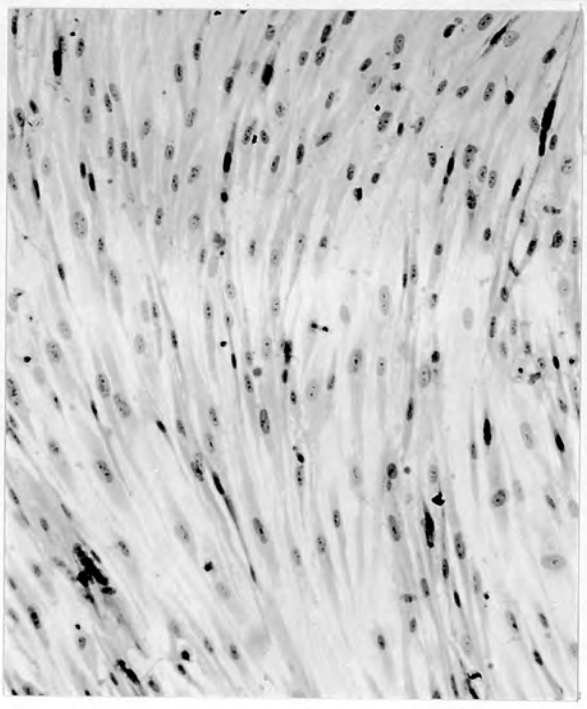


a

b



c



d

Plate 4

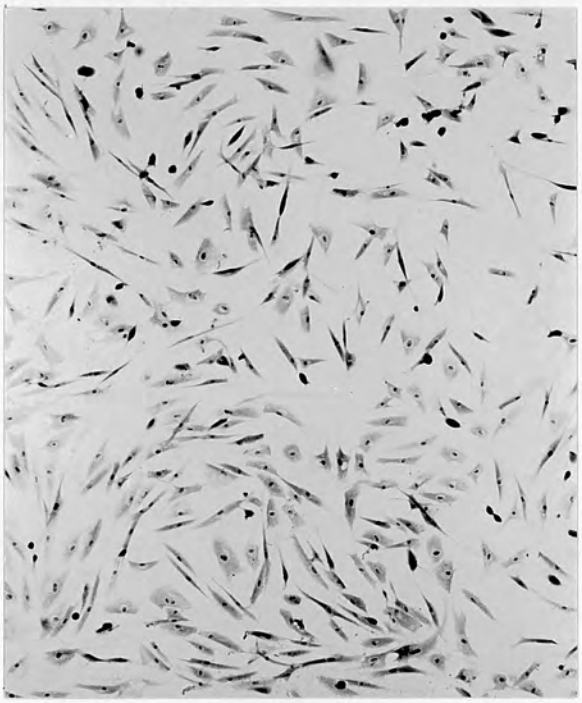
a. Second subculture from fetus 286, 16 days in culture.
H and E, x 53.

b. Second subculture from fetus 300, 38 days in culture.
H and E, x 53.

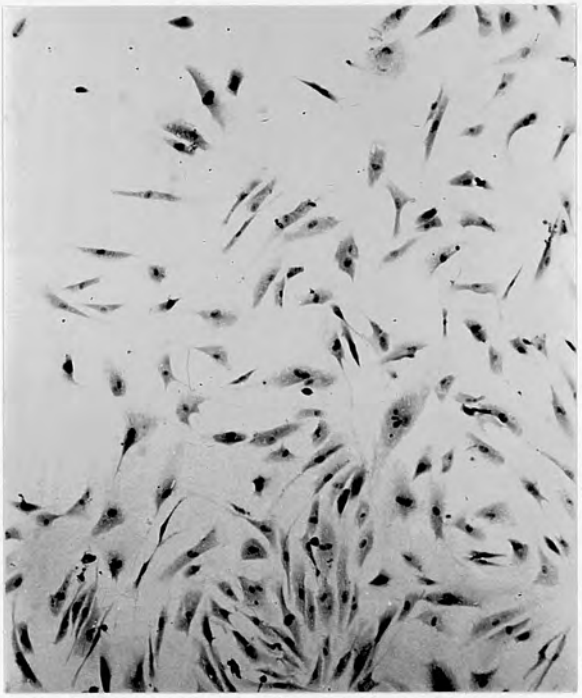
c. First subculture from fetus 346, 8 days in culture.
ATPase pH 9.4, x 135.

d. First subculture from fetus 319, 14 days in culture.
ATPase pH 9.4, x 135.

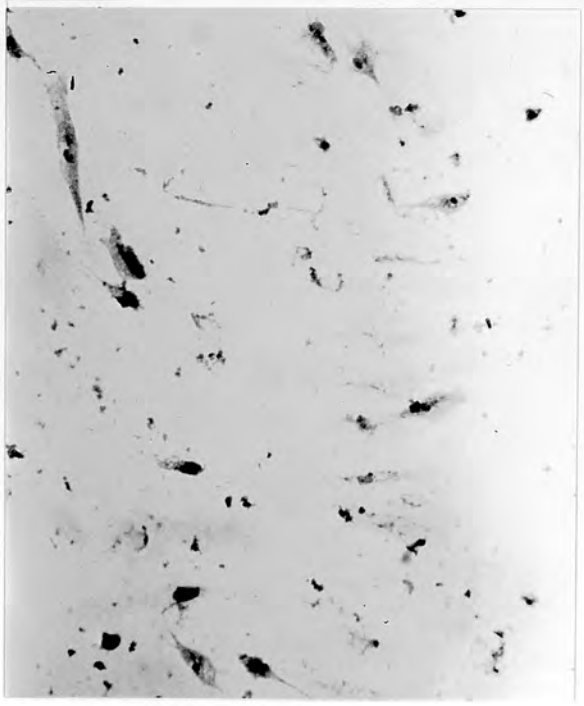
PLATE 4



a



b



c

d

Plate 5

Subcultures of human fetal muscle cells. NADH-d, x 135.

a. Second subculture from fetus 318, 7 days in culture.

b. Second subculture from fetus 316, 7 days in culture.

c. First subculture from fetus 300, 18 days in culture.

d. First subculture from fetus 339, 19 days in culture.

PLATE 5

a

b

c

d

gestation (Plate 5). There was some variation of staining intensity between the multinucleated cells, but this was also true for the uni-nucleated cells. Thus it was clear that no histochemical differentiation had occurred, at least in the sense in which that term is accepted for adult muscle, in this fetal muscle *in vitro*.

A totally unexpected, and inexplicable observation was that with NADH-d, the stained cells tended to occur in a narrow band at the periphery of the coverslip, although sometimes a few stained uni- or multinucleated cells could be seen among the large excess of unstained ones in the centre of the coverslip.

Coverslips removed for NADH-d staining from subcultures from which coverslips had been taken for H and E staining demonstrated that the rate of growth and fusion had not been the same in all vessels in which one subculture was being grown, although multinucleated cells were seen, with the exception of fetuses 339 and 346, in the same subcultures in which they were visible with H and E staining.

Discussion.

The results presented here appear at first sight not to follow any particular pattern, in that the degree of myoblast fusion seems not to be related to any specific conditions. It was found to occur to a considerable degree in fetuses of 14 and 22 weeks gestation, and to a lesser extent in fetuses of 11, 12 and 16 weeks gestation, but hardly at all in any of the fetuses of other gestations. The plating density of the subcultures in which most fusion is seen varies between 1.0 and 4.7 x 10^4 cells/cm^2, and successful growth in subculture from 20-100%.

The results of the growth and fusion analysis complicate rather than

clarify the situation. The fundamental reason for this is that in the analysis the number of variables was so great that it was almost impossible to make any meaningful statements. A great deal of variability was also introduced artificially as a result of the necessarily limited sampling methods which were employed in the growth and fusion analysis of the stained coverslips. The weakness of the sampling method in this respect is illustrated by the fact that even the results from the subcultures of fetuses 339 and 346, in which large multinucleated cells were found, appeared little different from those of any other subculture. Had the numbers of nuclei and multinucleated cells counted been an unbiased sample of the population, the DI's and NI's would have been expected to be a reliable indicator of the degree of myoblast fusion.

One variable over which there was little control was the success either of the primary or secondary cultures, although every attempt was made to standardise as far as possible the medium and handling techniques. This variability led in turn to variability in the subculture plating density, and since it was not known whether there was an optimum plating density, this was not artificially controlled. YAFFE (1973) has reported an optimum plating density for the fusion of rat myoblasts *in vitro*, but the present results appear to show that this is not true for human fetal muscle.

Other variables were deliberately introduced into the experiment in order to determine whether they produced significant differences in the results. Once again, statistically it was not possible to determine which conditions produced the highest degree of myoblast fusion, but when the subcultures in which most fusion actually occurred are considered, it is found in those grown on the smaller petri dishes, on coverslips coated with

collagen. Experiments have shown that with care it is possible to grow animal muscle *in vitro* in petri dishes (SHIMADA et al., 1967; SYTKOWSKI et al., 1973), and YAFFE (1973) has advocated the use of collagen or gelatin coated petri dishes to enhance myoblast growth and fusion in subcultures of rat muscle cells. The present results obtained from subcultures grown in Leighton tubes were few in number, but since the infection encountered using this method of culture was much reduced compared with growth on petri dishes, it is plausible to suggest that cultures grown on gelatin coated coverslips in Leighton tubes, would grow and fuse adequately.

There were also many possible variables in these experiments, whose influence on the growth of human muscle cells *in vitro* could not be investigated. One of these was the presence of calcium in the medium, which YAFFE (1973) has shown must be present in high concentrations to promote myoblast fusion in cultures of rat muscle cells.

Another possible variable was the medium itself and the type and concentration of serum included in it. Investigators who have grown animal muscle have used a variety of media, of which M.199 and Dulbecco's Modified Eagles Medium with 10% horse serum and 1% embryo extract (YAFFE, 1973), and F-14 medium with 10% fetal calf serum and 5% chick embryo extract (SYTKOWSKI et al., 1973) are but two examples. In the culture of human adult muscle also a variety of media have been employed (MENDELL et al., 1972: GALLUP et al., 1972), and growth in these media has resulted in the appearance of multinucleated muscle cells of similar morphology to those obtained from the culture of animal muscle cells, although ultrastructurally the human muscle cells did not always become fully differentiated (MENDELL, et al., 1972). In the one report to date of the growth of human fetal muscle *in vitro*, BATESON (1968) used a medium consisting of 35% Tyrode solution, 35% TC199,

20% embryo extract and 5% serum. Her results indicated that myoblast fusion had occurred, but that the number and size of the multinucleated cells was not very great. The medium selected for the present study was F-10, since although the concentration of calcium is low (0.3 mM) and is only slightly increased by the presence of the calcium in the added serum, there is a wide variety of amino acids, vitamins and inorganic salts present.

The possibility of variation in all the above factors helps to explain the difficulties involved in the use of any sampling method in the tissue culture situation. Estimation of the degree of fusion in a given culture clearly should be related to the number of cells in the culture which are available to fuse, but the final analysis must be made with a far larger sample than was used here. This might involve scanning 5-10% of the coverslip area, and estimating the number of myotubes formed per million cells originally plated out.

In the present results, as in Bateson's findings, there was no evidence of the appearance of adult muscle cell morphology in any of the cultures of human fetal muscle, although observation indicated that the proportion of myoblasts was high. The fact that 'strap' cells with cross-striations were not seen therefore probably indicates that, in the majority of samples, the myoblasts were not sufficiently active. However, if the second subculture from fetus 339 had been allowed to grow for more than 15 days, it seems very likely that muscle cells of a more mature form would have developed.

The observations of multinucleated cells which were made in the present cultures appeared at first sight to be haphazard, but may in fact be dependent on two interrelated factors. The first is the gestation of the fetus from which the muscle was taken, and the second is the part of the muscle from which

the explant was removed. In fetuses of about 9-13 weeks gestation, the number of myoblasts throughout a developing muscle is comparatively high (ISHIKAWA, 1966). Thus many parts of a muscle will contain a large number of myoblasts or myoblast precursors. In fetuses of 14 weeks gestation and over, the muscle cells are still growing in length. Thus a muscle explant taken from near the growing point may still contain sufficient active mononuclear cells to enable rapid muscle growth to occur *in vitro*.

Bateson did not stain her cultures histochemically, but had she done so, the result would probably have been similar to that obtained here, in that no histochemical differentiation of the cells would have been visible. The explanation for this assertion is simple if it is realised that in the development of human fetal muscle *in vivo* no consistent pattern of histochemical staining was ever seen before the majority of the muscle cells appeared morphologically to be adult, after 18-21 weeks gestation (see Chapter 3). Perhaps the most important factor of all is that the muscle cells *in vivo* have been innervated for a number of weeks by the time that histochemical differentiation becomes visible.

CHAPTER 8

GENERAL DISCUSSION

The studies which were undertaken during this project have concentrated on the development of motor innervation and its role in the histological and histochemical maturation of muscle. The muscles used in this main part of the study were soleus and gastrocnemius, but different muscles were investigated in other parts of the study. The histochemical stains throughout the study of fetal muscle development were unadapted from those normally used to stain adult muscle. The variability in selection of muscles to be studied, and the fact that unadapted histochemical stains were used are two of three factors which may have affected the results obtained, and therefore the validity of the interpretations placed on them.

The first factor concerns the presumed normality of the 75 fetuses from which samples were removed for study. These fetuses were presumed normal because there was no apparent abnormality on gross examination, no chromosomal abnormality (at least, in those few cases which were examined), and no family or obstetric history which might suggest the possibility of fetal abnormality. The differences in rate of development of different muscles and even in different fetuses of the same gestation made direct comparison between fetuses difficult, but the recorded observations indicated that the population of fetal samples was microscopically fairly homogeneous. Thus if there were muscular abnormalities present in any of the fetuses, they were not detectable as gross deviations from the findings in the majority of fetal samples. The original choice of muscles to be studied was made on the basis that in animals, e.g. cat (McPHEDRAN et al., 1965: WUERKER et al., 1965), the soleus contains a high proportion of type I fibres, and the gastrocnemius a high proportion of

type II fibres. A report which has appeared since the start of this study (JOHNSON et al., 1973) has shown a similar, although less distinct difference between the muscles in man, and thus to a degree justified the original choice. Since experiments have clearly shown that in adult animals muscle fibre typing is under neural influence, it is possible that the motor innervation to these two muscles might develop in different ways.

The second factor possibly affecting the results, was the stains which were used. Guth (GUTH and SAMAHA, 1972) has clearly pointed out the difficulty of applying the myosin ATPase stain at pH 9.4, as used for adult muscle, to the staining of fetal muscle. In the present research no attempt was made to use either the myosin ATPase or NADH-diaphorase reactions for typing fetal muscle fibres, until the stage at which there was histochemical differentiation. There should therefore be no uncertainty with regard to the usefulness of these stains. In adult muscle fibres, myosin ATPase staining has been very specifically associated with the myofibrils (SCHIAFFINO and BORMIOLI, 1973). In fetal muscle, the finding that there was originally most reaction for ATPase in the peripheral areas of the myotubes, agrees with both the light and electron microscope observations of myofibril formation occurring first in these areas.

The stain which was developed for the demonstration of the peripheral motor innervation gave satisfactory results but the nature of the desired observations created a problem. These observations of the motor innervation ideally required longitudinal sections, whereas transverse sections were necessary for histochemical studies. In practice, both longitudinal and transverse areas were nearly always visible in one section, so that alternate thin sections for histochemical reactions and thicker ones for innervation

studies was the best compromise which could be made. However, any studies of a tissue by means of sections inevitably face the twin problems of attempting to visualise the structure in three dimensions, as well as that of extrapolating from one small part of the tissue to an understanding of the whole.

The third factor which might have affected the results was the fact that, when studying either normal or possibly abnormal muscle development, several different muscles were subject to study. In the case of anencephalic fetuses a variety of skeletal muscles were studied, while in the cases of the fetuses at risk for DMD as well as in the tissue culture investigations, quadriceps muscle was investigated. The lack of any great differences between these control quadriceps muscles, and the samples of normal soleus and gastrocnemius muscles seems to indicate that valid comparisons can be made even if soleus, gastrocnemius and quadriceps muscles are used.

Histochemical observations in the present study showed in a few cases in the younger fetuses, the very close apposition of two cells, as though about to fuse. While this could represent myoblast precursor cell division, it is very unlikely in view of our present understanding of muscle fibre formation (COOPER and KONIGSBERG, 1961a: STOCKDALE and HOLTZER, 1961: KONIGSBERG, 1965: FISCHMAN, 1972) to have been, as CUAJUNCO suggested (1942), multinuclear cell division. Relatively few muscle cell fibre diameter measurements were made in the present research, but it is of interest to note that they were similar to those reported by DUBOWITZ (1968) and almost as variable. In the present study it was noted that after 13 weeks gestation, the mean muscle cell diameter appeared to increase very little compared with the increase in muscle bulk. There may therefore have been a considerable increase in the total number of cells present in the muscle, since the difference in size cannot be

accounted for by a marked change in the packing of the cells, or in the amount of connective tissue.

The relationship of the peripheral motor innervation to the developing histochemical pattern in normal fetal muscle has already been discussed in part in Chapter 3. The histochemistry of developing muscle has also been investigated in anencephalic fetuses, whose motor innervation with regard to large neurones of the spinal cord, was defective. However, the histochemistry of the developing muscle fibres in these anencephalic fetuses corresponds very closely to what is seen in normal skeletal muscle, as does their peripheral motor innervation. In the muscle from normal fetuses, scattered fibres, stained darkly for NADH-d, were visible at the stage of gestation equivalent to that of the anencephalic fetuses. These dark fibres in normal muscle were larger than average and this, together with their staining properties, placed them in the category of Wohlfart's 'B' fibres (WOHLFART, 1937: FENICHEL, 1963). However it seemed that the size and prevalence of the 'B' fibres was not as marked as either Fenichel's or Wohlfart's reports had suggested. Fibres with similar staining properties, but of a much larger diameter, were seen in one anencephalic fetus. This finding emphasises the normality of the skeletal muscle in anencephaly.

The present investigations into the histochemical staining properties of skeletal muscle cells *in vitro* were inconclusive because of the comparatively immature state of development reached by the cells. Many uni- and multinucleated cells did stain either lightly or darkly for NADH-d, and the variability of this staining was similar to that seen in the muscle of young fetuses *in vivo*. In contrast, the ATPase staining pattern was quite unlike that found *in vivo*. It seems likely that this reflects the immature state

of development of the myofibrils, even in the multinucleated cells which were present.

The evidence from the present studies concerning the histochemical differentiation of human muscle indicates clearly that it develops gradually over a long period, and that even in neonatal muscle the pattern of differentiation is not truly reciprocal. Dubowitz's results (DUBOWITZ, 1965, 1968) however, suggested that a reciprocal pattern of histochemical staining developed within a comparatively short space of time, and that when differentiation became evident, the muscle fibres could be divided into types I and II. Experiments on animals provide contradictory evidence on this point. Ashmore (ASHMORE et al., 1972) has suggested that muscle fibres in the fetal lamb have a "biphasic" development: the β fibres (red/slow) develop first, and are developmentally distinct from the α fibres (white/fast) which develop later. However, in this study ATPase at pH 9.4 was the only histochemical stain used, and the illustrations appear to show that in fact developing sheep muscle is histochemically very similar to human fetal muscle. Experiments on rats (BROOKE et al., 1971) indicate that in developing muscle the type I fibres are quite distinct, and that adult type II muscle fibres develop only from an embryonic type IIC. The present results have shown that the developmental sequence in the histochemistry of human muscle fibres is more similar to that found in the rat than that originally described in human fetal muscle by Dubowitz.

It is generally accepted that in mammalian fetal muscle motor innervation is essential for the normal development and survival of muscle, but the nature of the controls exerted by the motor innervation over muscle are unclear. Experiments on adult muscle have led to the conclusion that the controlling

influences of motor nerves are mediated by a trophic substance or substances (GUTH, 1968). These substances may be axonally transported macromolecules synthesised in the perikaryon. Experiments have been devised to test the trophic nature of acetylcholine (DRACHMAN, 1967) but the results have not been conclusive. Another possibility is that nervous activity itself controls the properties of the target muscle fibres (SALMONS and VRBOVÁ, 1969: PETTE et al., 1972: AL-AMOOD et al., 1973), a slow impulse rate giving rise to type I fibres,and a faster rate to type II. In human fetal muscle, functional motor endplates are present in the neck and upper limb-girdle region at 8 - 9 weeks gestation (HAMBURGER, 1963: HUMPHREY, 1968, 1969) shortly after the stage at which motor endplate formation can be seen in the intercostal muscles (JUNTUNEN and TERÄVÄINEN, 1972). The reflex actions which can be elicited at this stage of gestation are undoubtedly of a different quality from the spontaneous twitching which can sometimes be seen in uninnervated cultures of skeletal muscle cells. MAVRINSKAYA (1963) has also described how the first muscular contractions coincide with the localisation of cholinesterase on the muscle fibre at 8 weeks gestation. The present results have shown that very distinct motor endplates are visible in leg muscle at 9 - 10 weeks gestation. Thus functional neuromuscular transmission appears to be present long before histochemical differentiation becomes evident, and the present observations cannot further illuminate whether it is nerve impulses or acetylcholine, or neither of these, which exerts the trophic influence of nerve on muscle.

The function of the immature motor innervation in fetal muscle is similar to its function in the adult in that it initiates muscular contraction. However, the nature of the nerve impulses may be qualitatively different. Experiments on cats have shown that small diameter nerve fibres are slowly

conducting (HENNEMAN and OLSON, 1965) and generally innervate small, slowly contracting motor units (WUERKER et al., 1965). The axon diameters of peripheral motor nerves increase with gestation and it might therefore be expected that the first type of muscle fibre to develop would be that innervated by small diameter axons. These muscle fibres would therefore be type I. The finding that type I muscle fibres appear to be more resistant to atrophy than type II has already been discussed in Chapter 3. Other observations, on rats and mice (BAJUSZ, 1964), emphasise the relative independence of the 'red'fibres of neural control. These observations, together with Salmons and Vrbová's suggestion that type I motor units are innervated by axons which have a lower frequency of firing, would help to explain the consistent observation that type I muscle fibres appear to be the more fundamental.

One of the major concerns of the present study has been the early development of the neuromuscular junction. The localisation of acetylcholinesterase in the soleplate is an important indicator of the functional state of the motor endplate during development, as shown by the studies already referred to. Some studies have shown that there is a considerable amount of non-specific cholinesterase in the human motor endplate (CHOKROVERTY et al., 1971), but other studies that approximately 80% of the cholinesterase is acetylcholinesterase (NAMBA and GROB, 1970). Thus the use of a stain to demonstrate acetylcholinesterase in developing motor endplates appears to be justified.

Another method used to demonstrate the soleplate is one in which labelled α-bungarotoxin is allowed to become bound to the acetylcholine receptor sites. Experiments on rat diaphragm preparations have demonstrated the

non-identity of the acetylcholine receptor sites and the site of acetylcholinesterase activity (CHANG and SU, 1974), but for physiological efficiency the distribution of the two types of sites must be closely related in the soleplate. The specific binding of α-bungarotoxin to acetylcholine receptor sites (BERG et al., 1972) has been used in tissue culture experiments with interesting results. In aneural cultures of chick skeletal muscle cells these experiments showed that after 7-11 days there was a spontaneous concentration of acetylcholine receptor sites into several specific areas on the surface of a myotube. The concentration of acetylcholine receptor sites in these areas was ten times higher than on the rest of the muscle cell membrane (SYTKOWSKI et al., 1973). This spontaneous localisation of presumptive soleplate material has never been detected by means of acetylcholinesterase staining of skeletal muscle cells in vitro, and must be compared with the results of iontophoretic application of acetylcholine to muscle cells in culture. The development of acetylcholine sensitivity was found to be related to morphological differentiation (FAMBROUGH and RASH, 1971), but no uneven distribution of acetylcholine receptors was noted. Other experiments (FISCHBACH and COHEN, 1973) demonstrated that both innervated and uninnervated myotubes had several specific small areas of acetylcholine sensitivity which appeared very similar to the areas of acetylcholine receptor concentration described by Sytkowski.

The events described in the formation of neuromuscular junctions in vivo have many parallels in coupled cultures of mammalian skeletal muscle cells and spinal cord (SHIMADA and KANO, 1971: PETERSON and CRAIN, 1972). If Sytkowski's observations on the development of acetylcholine receptor sites were generally applicable, not only to muscle in vitro but also in vivo, they might help to explain the apparent specificity of the manner in which initial neuromuscular contact occurs.

In an ultrastructural study of rat motor endplate morphogenesis KELLY and ZACKS (1969) noted the apparently very brief close contact between the terminal motor axon and muscle prior to definitive motor endplate development, and suggested that this transient encounter could hardly provide the impetus for motor endplate formation. It was also noted that it was not possible to be certain that the region of the axon viewed in the electron microscope sections was in fact the area of presumptive motor endplate formation, since there were several exploratory terminal axon branches visible. These very fine branches would not be resolvable by the light microscope, and in the present project may therefore have been present but not recognised. The simultaneous presence of several possible motor endplate sites, as suggested by Sytkowski, and several fine terminal axon branches, would increase the chance of initial neuromuscular contact, which therefore need not be as specific as might be expected. In such a situation the presumptive soleplate sites would appear to be exerting an attractive influence on the terminal axon branches. Alternatively, if Sytkowski's findings were rare or an artifact of the tissue culture environment, terminal axons might induce soleplate formation at the points of the muscle cell membrane where they first made contact. At present it does not seem possible to distinguish between these two alternatives.

Whatever the precise mechanism of initial motor endplate formation, the results of the present studies indicate clearly that many if not all myotubes in human fetal muscle have, at least at some stage, double motor innervation, which persists for some months. Experiments on kittens (BAGUST et al., 1973) have also demonstrated that in the neonate there appears to be extensive multiple innervation of the soleus and flexor hallucis longus muscles, but that

during postnatal development there is a gradual change so that by 6 weeks of age the pattern of terminal motor innervation has reached a stable state. In adult muscle each fibre can normally be innervated at only one point by a single motor axon (BROWN and MATTHEWS, 1960: GUTH and ZALEWSKI, 1963: GWYN and AITKEN, 1970) although a recent experiment has shown that adult rat muscle may tolerate double innervation for some time (FRANK et al., 1974). Fetal muscle fibres may tolerate double innervation for some months simply because they are developmentally more "flexible" than adult fibres.

It has already been suggested that it may be the same myotubes which receive the first motor innervation that also show positive histochemical reactions first. A stain which enables simultaneous demonstration of NADH-d staining and peripheral motor innervation has been used to investigate reinnervation in patients with the Guillain-Barré syndrome (MORRIS and WOOLF, 1970) but its application to human fetal muscle has been unsuccessful. However, the present studies, as well as previous ones of mouse motor endplate morphogenesis (KELLY and ZACKS, 1969) have shown that groups of myotubes tend to become innervated together. The fact that the first myotubes which stain positively for histochemical reactions also occur in groups suggests that the histochemical properties of the myotubes may be directly related to their developing motor innervation.

It is difficult to trace the events which follow the establishment of definite neuromuscular junctions, because it is impossible to know precisely the nature of the changing relationships between nerve and muscle. Groups of myotubes may become innervated together, but studies of adult muscle both animal (EDSTRÖM and KUGELBERG, 1968, 1969: KUGELBERG, 1973) and human (BUCHTHAL et al., 1957, 1959) indicate that the muscle fibres of a motor unit

are dispersed within a muscle. Kelly and Zacks suggested that the muscle fibres which became innervated together later separate thus maintaining the integrity of each motor unit throughout development. The present observation of multiple terminal axon branching and the presence of more than one acetylcholinesterase positive site on many muscle fibres, suggests instead that the muscle fibres remain in their original location and receive different innervation. The phenomenon of terminal axon branching both in animal and human adult muscle is familiar enough (COËRS and WOOLF, 1959: HARRIMAN, 1961: MORRIS and RAYBOULD, 1971: COËRS et al., 1973) and occurs predominantly in disorders in which there is a deficiency of motor innervation. In such disorders, histochemical changes are visible in some of the muscle fibres which become reinnervated (MORRIS, 1970), and in developing fetal muscle it seems likely that the changes in fibre type are a result of direct neural influence.

It has been noted that human fetal muscle undergoes considerable changes during gestation and it is interesting to compare this with studies of the development of the longissimus muscle in the pig (DAVIES, 1972). The fibres of this muscle are arranged in very specific groups (JAMES, 1972) which in the neonatal pig bear striking resemblances to those found in human fetal muscle (see Chapter 3, Plate 10a), as well as in developing animal muscle (DUBOWITZ, 1965, 1968). However, when human skeletal muscle eventually matures, the distribution of fibre types appears almost random, whereas in the pig, although the proportion of different fibre types changes, the basic spatial organisation of the fibre-type groups does not. In rabbit and guinea-pig muscle (JAMES, 1971) there is also a non-random dispersion of histochemical fibre types, which it is suggested represents the arrangement necessary for maximum

physiological and mechanical efficiency of these fibre types. The different fibre types in pig muscle may be similarly arranged for the same reason. It seems unlikely that in developing fetal muscle this arrangement represents the need for maximum mechanical efficiency, but rather, as James suggested, it may be the arrangement in which maximum use is made of the available capillary oxygen.

It is suggested (DAVIES, 1972) that the histochemical changes in the developing pig longissimus muscle take place in response to the increasing load placed on the muscle as the animal grows. The histochemical composition of some human muscles may also reflect a degree of functional specialisation (JOHNSON et al., 1973), but it seems unlikely that developmental changes occur in either case purely in response to demand without some degree of neural mediation. The present study has been able to demonstrate the changes which are found in the histochemistry and motor innervation of developing human muscle, and in part the relationship between them. It has not, however, been possible to shed fresh light on the nature of the factors which initiate, or on the basic mechanisms which effect, the extensive changes which are seen.

Studies of human muscle development are necessarily limited because the work cannot be of an experimental nature. Although findings based on investigations of animal muscle are not always applicable when considering human muscle, such results could prove valuable. Firstly, it would be of interest to investigate, by means of a combined AChE and silver impregnation stain, the pattern of terminal motor innervation in the cat and rat, in which physiological evidence apparently shows double innervation in the neonate. Evidence from such investigations would help to further establish the occurrence of a pattern of development similar to what has been described here in human

muscle.

Some animal muscles are composed almost exclusively of fibres of one type, and investigations of the development of terminal innervation in these muscles, as well as a study of their histochemical development, would also be of interest. It may be that double fibre innervation and the pattern of scattered developing 'type I' fibres are present in all muscles at some stage of development. Such a finding would imply that the early distribution of fibres within a muscle occurred in response to the need to make maximum possible use of available energy resources and oxygen, but that later there is a neurally mediated change in the proportions of different fibres.

In the latter context, studies of developing spinal cord anterior horn cells in animals could be valuable. Such investigations would establish whether or not it were possible to discover a closer relationship between developing anterior horn cells and the muscle fibres they innervate than has been possible in human fetal muscle. It should be noted, however, that accurate sampling methods are more complex (TOMLINSON et al., 1973), and the distribution of anterior horn cells within the spinal cord more unequal (IRVING et al., 1974) than was anticipated when planning the studies of human anterior horn cell development.

The primary aim of this research as set out at the beginning was to define a baseline of development in normal human fetal muscle, so that it might then be possible to detect deviations from normal. Histological or histochemical studies may enable developmental abnormalities to be detected in skeletal muscle, since each area is relatively well defined. Part of this research has demonstrated the potential usefulness of such an application in determining whether or not fetuses at risk of developing Duchenne muscular

dystrophy begin to show the pathological changes of this disorder *in utero*. Further studies of other early-onset disorders, e.g. Werdnig-Hoffmann disease, would indicate whether abnormalities were evident antenatally in these disorders as well. In contrast to histological and histochemical studies of fetal muscle, investigations of the pattern of terminal motor innervation could be very difficult to interpret because of the complexity and variability of the normal pattern.

BIBLIOGRAPHY

AL-AMOOD, W.S., BULLER, A.J., POPE, R. (1973) 'Long-term stimulation of cat fast-twitch skeletal muscle.' Nature, 244, 225-227.

ALLBROOK, D.B., HAN, M.F., HELLMUTH, A.E. (1971) 'Population of muscle satellite cells in relation to age and mitotic activity.' Pathol., 3, 233-243.

ASHMORE, C.R., ROBINSON, D.W., RATTRAY, P., DOERR, L. (1972) 'Biphasic development of muscle fibres in the fetal lamb.' Exp. Neurol., 37, 241-255.

ASKANAS, V., SHAFIQ, S.A., MILHORAT, A.T. (1972) 'Histochemistry of cultured aneural chick muscle. Morphological maturation without differentiation of fibre types.' Exp. Neurol., 37, 218-230.

ATSUMI, S. (1971 a) 'The histogenesis of motor neurons with special reference to the correlation of their endplate formation.
I The development of end-plates in the intercostal muscle in the chick embryo.' Acta Anat. 80, 161-182.

ATSUMI, S. (1971 b) 'The histogenesis of motor neurons with special reference to the correlation of their endplate formation.
II The localisation of motor neurons innervating the intercostal muscle in the thoracic cord in the chicken.' Acta Anat. 80, 321-335.

ATSUMI, S. (1971 c) 'The histogenesis of motor neurons with special reference to the correlation of their endplate formation.
III The development of motor neurons innervating the intercostal muscle in the chick embryo.' Acta Anat. 80, 504-515.

BAGUST, J., LEWIS, D.M., WESTERMAN, R.A. (1973) 'Polyneuronal innervation of kitten skeletal muscle.' J. Physiol., 229, 241-255.

BAJUSZ, E. (1964) '"Red" skeletal muscle fibres: Relative independence of neural control.' Science, 145, 938-939.

BARDEEN, C.R. (1900) 'The development of the musculature of the body wall in the pig, including its histogenesis and its relations to the myotomes and to the skeletal and nervous apparatus.' John Hopk. Hosp. Rep., 9, 367-400.

BARDEEN, C.R., LEWIS, W.H. (1901-02) 'Development of the limbs, body-wall and back in man.' Amer. J. Anat., 1, 1-35.

BARKA, T., ANDERSON, P.J. (1963) 'Histochemistry: Theory, practice and bibliography.' Harper and Row, Publishers, Inc., New York, Evanston and London. pp.296-333.

BATESON, R.G. (1968) 'The origin and early development of skeletal muscle in the embryo, in tissue culture and, after injury, in vivo, in the chick, mouse and man.' Ph.D. Thesis, University of London.

BEATTY, C.H., BASINGER, G.M., BOCEK, R.M. (1967) 'Differentiation of red and white fibres in muscle from foetal, neonatal and infant rhesus monkeys.' J. Histoch. Cytoch., 15, 93-103.

BERG, D.K., KELLY, R.B., SARGENT, P.B., WILLIAMSON, P., HALL, Z.W. (1972) 'Binding of α-bungarotoxin to acetylcholine receptors in mammalian muscle.' Proc. Nat. Acad. Sci., 69, 147-151.

BERGMAN, R.A. (1962) 'Observations on the morphogenesis of rat skeletal muscle.' Bull. Johns Hopk. Hosp., 110, 187-201.

BOËTHIUS, J. (1969) 'Resting membrane potential in neck and leg muscles of young rats.' Acta Physiol. Scand., 75, 253-254.

BRADLEY, W.G. (1971) 'Nerve, muscle and muscular dystrophy.' Dev. Med. Child Neurol., 13, 528-531.

BRADLEY, W.G., HUDGSON, P., LARSON, P.F., PAPAPETROPOULOS, T.A., JENKISON, M. (1972) 'Structural changes in the early stages of Duchenne muscular dystrophy.' J. Neurol. Neurosurg. Psychiat., 35, 451-455.

BRADLEY, W.G., JENKISON, M. (1973) 'Abnormalities of peripheral nerves in murine muscular dystrophy.' J. Neurol. Sci., 18, 227-247.

BROOKE, M.H., KAISER, K.K. (1970) 'Muscle fibre types: how many and what kind?' Arch. Neurol., 23, 369-379.

BROOKE, M.H., WILLIAMSON, E., KAISER, K.K. (1971) 'The behaviour of four fibre types in developing and reinnervated muscle.' Arch. Neurol., 25, 360-366.

BROWN, M.C., MATTHEWS, P.B.C. (1960) 'An investigation into the possible existence of polyneuronal innervation in individual skeletal muscle fibres in certain hind-limb muscles of the cat.' J. Physiol., 151, 436-457.

BUCHTHAL, F., GULD, C., ROSENFALCK, P. (1957) 'Multielectrode study of the territory of a motor unit.' Acta Physiol. Scand., 39, 83-104.

BUCHTHAL, F., ERMINIO, F., ROSENFALCK, P. (1959) 'Motor unit territory in different human muscles.' Acta Physiol. Scand., 45, 72-87.

BULLARD, H.H. (1919) 'Histological as related to physiological and chemical differences in certain muscles of the cat.' Johns Hopk. Hosp. Rep., 18, 323-328.

BULLER, A.J., ECCLES, J.C., ECCLES, R.M. (1960 a) 'Differentiation of fast and slow muscles in the cat hind limb.' J. Physiol., 150, 399-416.

BULLER, A.J., ECCLES, J.C., ECCLES, R.M. (1960 b) 'Interactions between motoneurones and muscles in respect of the characteristic speed of their responses.' J. Physiol., 150, 417-439.

BULLER, A.J., MOMMAERTS, W.F.H.M., SERAYDARIAN, K. (1971) 'Neural control of myofibrillar ATPase activity in rat skeletal muscle.' Nature (New Biol.), 233, 31-32.

BURKE, R.E. (1967) 'Motor unit types of cat triceps surae muscle.' J. Physiol., 193, 141-160.

BURROWS, M.T. (1910) 'The cultivation of tissues of the chick-embryo outside the body.' J. Amer. Med. Assoc., LV, 2057-2058.

BURSTONE, M.S. (1957) 'Polyvinyl pyrrolidone as a mounting medium for stains for fat and for azo-dye procedures.' Amer. J. Clin. Path., 28, 429-430.

CAMPA, J.F., ENGEL, W.K. (1970 a) 'Histochemical differentiation of motor neurones and interneurones in the anterior horn of the cat spinal cord.' Nature, 225, 748-749.

CAMPA, J.F., ENGEL, W.K. (1970 b) 'Histochemistry of motor neurons and interneurons in the cat lumbar spinal cord.' Neurol., 20, 559-568.

CAMPA, J.F., ENGEL, W.K. (1971) 'Histochemical and functional correlations in horn neurons of the cat spinal cord.' Science, 171, 198-199.

CARLSON, B.M. (1973) 'The regeneration of skeletal muscle - A review.' Amer. J. Anat., 137, 119-150.

CARREL, A. (1912) 'On the permanent life of tissues outside of the organism.' J. Exp. Med., 15, 516-528.

CARREL, A. (1913) 'Artificial activation of the growth in vitro of connective tissue.' J. Exp. Med., 17, 14-19.

CARREL, A. (1923) 'A method for the physiological study of tissues in vitro.' J. Exp. Med., 38, 407-418.

CHANG, C.C., SU, M.J. (1974) 'Does α-bungarotoxin inhibit motor endplate acetylcholinesterase?' Nature, 247, 480.

CHOKROVERTY, S., PARAMESWAR, K.S., CO, C. (1971) 'Nonspecific esterases in the myoneural junction of human striated muscle.' J. Histoch. Cytoch., 19, 798-800.

CIACCIO, V. (1898) 'La découverte des muscles blancs et des muscles rouges, chez le lapin, revendiquée en faveur de S. Lorenzini.' Arch. Ital. Biol. 30, 287-288.

CLOSE, R. (1964) 'Dynamic properties of fast and slow skeletal muscles of the rat during development.' J. Physiol., 173, 74-95.

CLOSE, R.I. (1972) 'Dynamic properties of mammalian skeletal muscles.' Physiol. Rev., 52, 129-197.

COËRS, C. (1967) 'Structure and organisation of the myoneural junction.' Int. Rev. Cytol., 22, 239-267.

COËRS, C., TELERMAN-TOPPET, N., GERARD, J-M. (1973) 'Terminal innervation ratio in neuromuscular disease. II. Disorders of lower motor neuron, peripheral nerve, and muscle.' Arch. Neurol., 29, 215-222.

COËRS, C., WOOLF, A.L. (1959) 'The innervation of muscle. A biopsy study.' Blackwell Scientific Publications, Oxford.

COOPER, W.G., KONIGSBERG, I.R. (1961 a) 'Dynamics of myogenesis in vitro.' Anat. Rec., 140, 195-205.

COOPER, W.G., KONIGSBERG, I.R. (1961 b) 'Succinic dehydrogenase activity of muscle cells grown in vitro.' Exp. Cell Res., 23, 576-581.

COUTEAUX, R. (1941) 'Recherches sur l'histogénèse du muscle strié des mammifères et la formation des plaques motrices.' Bull. Biologique, 75, 101-239.

COUTEAUX, R. (1960) in 'The structure and function of muscle.' 1st. edition. Ed. Bourne, G.H. Academic Press, New York and London. Vol.1, pp. 337-380.

CRAIN, S.M. (1970) 'Bioelectric interactions between cultured fetal rodent spinal cord and skeletal muscle after innervation in vitro.' J. Exp. Zool., 173, 353-369.

CUAJUNCO, F. (1942) 'Development of the human motor end plate.' Contr. Embryol., XXX, 127-152.

DASTUR, D.K., RAZZAK, Z.A. (1973) 'Possible neurogenic factor in muscular dystrophy: its similarity to denervation atrophy.' J. Neurol. Neurosurg. Psychiat., 36, 399-410.

DAVIES, A.S. (1972) 'Postnatal changes in the histochemical fibre types of porcine skeletal muscle.' J. Anat., 113, 213-240.

DAVIES, A.S., GUNN, H.M. (1972) 'Histochemical fibre types in the mammalian diaphragm.' J. Anat., 112, 41-60.

DENNY-BROWN, D.E. (1929) 'The histological features of striped muscle in relation to its functional activity.' Proc. Roy. Soc. B., 104, 371-411.

DIAMOND, J., MILEDI, R. (1962) 'A study of fetal and new-born rat muscle fibres.' J. Physiol., 162, 393-408.

DRACHMAN, D.B. (1967) 'Is acetylcholine the trophic neuromuscular transmitter?' Arch. Neurol., 17, 206-218.

DUBALE, M.S., MURALIDHARAN, P. (1970) 'Histochemical studies on the fibre types in the developing jaw-muscles of the domestic fowl (Gallus domesticus).' Life Sciences, 9 Part II, 949-959.

DUBOWITZ, V. (1965) 'Enzyme histochemistry of skeletal muscle.' J. Neurol. Neurosurg. Psychiat., 28, 516-524.

DUBOWITZ, V. (1968) 'Developing and diseased muscle.' SIMP Research Monograph No.2. Spastics International Medical Publications with Heinemann, London.

DUBOWITZ, V., BROOKE, M.H. (1973) 'Muscle biopsy. A modern approach.' W.B. Saunders Co. Ltd., London, Philadelphia, Toronto.

DUBOWITZ, V., PEARSE, A.G.E. (1960) 'Reciprocal relationship of phosphorylase and oxidative enzymes in skeletal muscle.' Nature, 185, 701-702.

DUCHEN, L.W. (1970) 'Hereditary motor endplate disease in the mouse: light and electron microscope studies.' J. Neurol. Neurosurg. Psychiat., 33, 238-250.

DUCHEN, L.W. (1971) 'An electron microscopic study of the changes induced by botulinum toxin in motor endplates of slow and fast skeletal muscle fibres of the mouse.' J. Neurol. Sci., 14, 47-60.

EADIE, M.J., TYRER, J.H., KUKUMS, J.R. (1971) 'Selective vulnerability to ischaemia: studies in quantitative enzyme cytochemistry of single neurons and neuropil.' Brain, 94, 647-660.

EDSTRÖM, L., KUGELBERG, E. (1968) 'Histochemical composition, distribution of fibres and fatiguability of single motor units.' J. Neurol. Neurosurg. Psychiat., 31, 424-433.

EDSTRÖM, L., KUGELBERG, E. (1969) 'Histochemical mapping of motor units in experimentally reinnervated skeletal muscle.' Experientia, 25, 1044-1045.

ELLIOTT, H.C. (1942) 'Studies on the motor cells of the spinal cord. I. Distribution in the normal human cord.' Amer. J. Anat., 70, 95-117.

ELLIOTT, H.C. (1943) 'Studies on the motor cells of the spinal cord. II. Distribution in the normal human fetal cord.' Amer. J. Anat., 72, 29-38.

EMERY, A.E.H. (1969) 'Genetic counselling in X-linked muscular dystrophy.' J. Neurol. Sci., 8, 579-587.

EMERY, A.E.H. (1971) 'Review: the nosology of the spinal muscular atrophies.' J. Med. Genet., 8, 481-495.

ENGEL, W.K. (1961) 'Cytological localisation of glycogen in cultured skeletal muscle.' J. Histoch. Cytoch., 9, 38-43.

ENGEL, W.K., CUNNINGHAM, G.G. (1963) 'Rapid examination of muscle tissue. An improved trichrome method for fresh-frozen biopsy sections.' Neurol., 13, 919-923.

ENGEL, W.K., HORVATH, B. (1960) 'Myofibril formation in cultured skeletal muscle cells studied with antimyosin fluorescent antibody.' J. Exp. Zool., 144, 209-223.

FAMBROUGH, D., RASH, J.E. (1971) 'Development of acetylcholine sensitivity during myogenesis.' Dev. Biol., 26, 55-68.

FARKAS, E., TOMÉ, F.M.S., FARDEAU, M., ARSÉNIO-NUNES, M.L., DREYFUS, P., DIEBLER, M.F. (1974) 'Histochemical and ultrastructural study of muscle biopsies in 3 cases of Dystrophia myotonica in the newborn child.' J. Neurol. Sci., 21, 273-288.

FEINSTEIN, B., LINDEGÅRD, B., NYMAN, E., WOHLFART, G. (1955) 'Morphologic studies of motor units in normal human muscles.' Acta Anat., 23, 127-142.

FENICHEL, G.M. (1963) 'The B fibre of human fetal skeletal muscle. A study of fibre diameter size.' Neurol., 13, 219-226.

FIDZIAŃSKA, A. (1971) 'Electron microscopic study of the development of human foetal muscle, motor end-plate and nerve.' Acta Neuropath., 17, 234-247.

FIDZIAŃSKA, A. (1973) 'Electron microscopic study of the development of human fetal muscle and nerve.' Ann. Med. Sect. Pol. Acad. Sci., 18, 14-16.

FISCHBACH, G.D., COHEN, S.A. (1973) 'The distribution of acetylcholine sensitivity over uninnervated and innervated muscle fibers grown in cell culture.' Dev. Biol., 31, 147-162.

FISCHMAN, D.A. (1967) 'An electron microscope study of myofibril formation in embryonic chick skeletal muscle.' J. Cell Biol., 32, 557-575.

FISCHMAN, D.A. (1972) in 'The structure and function of muscle.' 2nd. edition. Ed. Bourne, G.H. Academic Press, New York and London. Vol. I, pp. 75-148.

FRANK, E., JANSEN, J.K.S., LØMO, T., WESTGAARD, R. (1974) 'Maintained function of foreign synapses on hyperinnervated skeletal muscle fibres of the rat. ' Nature, 247, 375-376.

GALLUP, B., DUBOWITZ, V. (1973) 'Failure of "dystrophic" neurones to support functional regeneration of normal or dystrophic muscle in culture.' Nature, 243, 287-289.

GALLUP, B., STRUGALSKA-CYNOWSKA, H., DUBOWITZ, V. (1972) 'Histochemical studies on normal and diseased human and chick muscle in tissue culture.' J. Neurol. Sci., 17, 109-125.

GARDNER-MEDWIN, D., WALTON, J.N. (1969) in 'Disorders of voluntary muscle.' 2nd. edition. Ed. Walton, J.N. J. and A. Churchill Ltd., London. pp. 411-453.

GEIGER, R.S., GARVIN, J.S. (1955) 'Growth pattern in vitro of skeletal muscle from pseudohypertrophic muscular dystrophy.' Fed. Proc., 14, 55.

GEIGER, R.S., GARVIN, J.S. (1957) 'Pattern of regeneration of muscle from progressive muscular dystrophy patients cultivated in vitro as compared to normal human skeletal muscle.' J. Neuropath. Exp. Neurol., 16, 532- 543.

HAMILTON, W.J., BOYD, J.D., MOSSMAN, H.W. (1952) 'Human embryology.' 2nd. edition. Heffer, Cambridge.

HANSON, J., HUXLEY, H.E. (1953) 'Structural basis of the cross-striations in muscle.' Nature, 172, 530-532.

HARRIMAN, D. (1961) in 'Electrodiagnosis and electromyography.' 2nd. edition. Ed. Licht, S., Licht, New Haven, Conn. pp.134-152.

HARRISON, R.G. (1904) 'An experimental study of the relation of the nervous system to the developing musculature in the embryo of the frog.' Amer. J. Anat., 3, 197-220.

HARRISON, R.G. (1906-7) 'Observations on the living developing nerve fiber.' Proc.Soc.Exp.Biol.Med., IV, 140-143.

HENDERSON, J.R. (1967) 'The use of silver for intensifying sulfide deposits in the cholinesterase technique.' Stain Technol., 42, 101-102.

HENNEMAN, E., OLSON, C.B. (1965) 'Relations between structure and function in the design of skeletal muscles.' J. Neurophysiol., 28, 581-598.

HEWER, E.E. (1927-28) 'The development of muscle in the human foetus.' J. Anat., 62, 72-78.

HEWER, E.E. (1934-35) 'The development of nerve endings in the human foetus.' J. Anat., 69, 369-379.

HOLTZER, H., MARSHALL, J.M., FINCK, H. (1957) 'An analysis of myogenesis by the use of fluorescent antimyosin. J. Biophys.Biochem.Cytol., 3, 705-724.

HUMPHREY, T. (1968) 'The development of mouth opening and related reflexes involving the oral area of human fetuses.' Alabama J. Med. Sci., 5, 126-157.

HUMPHREY, T. (1969) 'The prenatal development of mouth opening and mouth closure reflexes.' Pediatrics Digest, 11, 28-40.

GIACOBINI, E., HOLMSTEDT, B. (1958) 'Cholinesterase content of certain regions of the spinal cord as judged by histochemical and cartesian diver techniques.' Acta Physiol. Scand., 42, 12-27.

GILLASPIE, C., HEUSTON, H.H. (1917) 'Detailed study of a monster with craniorachischisis and other anomalies.' Anat. Rec., 13, 289-293.

GIROUD, A. (1960) 'Causes and morphogenesis of anencephaly', in CIBA Foundation Symposium on Congenital Malformations. Eds. Wolstenholme, G.E.W., O'Connor, C.M. Churchill, London. pp. 199-212.

GODLEWSKI, E. (1902) 'Die entwicklung des Skelet- und Herzmuskelgewebes des Säugethiere.' Arch. Mikr. Anat., 60, 111-156.

GOLDSPINK, G. (1972) in 'The structure and function of muscle.' 2nd. edition. Ed. Bourne, G.H. Acadamic Press, New York and London. Vol.I, pp. 179-236.

GOMORI, G. (1952) 'Microscopic histochemistry. Principles and practice.' Univ. Chicago Press. pp. 210-212.

GUTH, L. (1968) ' "Trophic" influences of nerve on muscle.' Physiol. Rev., 48, 645-687.

GUTH, L., SAMAHA, F.J. (1972) 'Erroneous interpretations which may result from application of the "myofibrillar ATPase" histochemical procedure to developing muscle.' Exp. Neurol., 34, 465-475.

GUTH, L., ZALEWSKI, A. (1963) 'Disposition of cholinesterase following implantation of nerve into innervated and denervated muscle.' Exp. Neurol., 7, 316-326.

GWYN, D.G., AITKEN, J.T. (1970) 'The formation of new motor endplates in mammalian skeletal muscle.' J. Anat., 100, 111-126.

HAMBURGER, V. (1963) 'Some aspects of the embryology of behaviour.' Quart. Rev. Biol., 38, 342-365.

HAMILTON, W.J., BOYD, J.D., MOSSMAN, H.W. (1952) 'Human embryology.' 2nd. edition. Heffer, Cambridge.

HANSON, J., HUXLEY, H.E. (1953) 'Structural basis of the cross-striations in muscle.' Nature, 172, 530-532.

HARRIMAN, D. (1961) in 'Electrodiagnosis and electromyography.' 2nd. edition. Ed. Licht, S., Licht, New Haven, Conn. pp.134-152.

HARRISON, R.G. (1904) 'An experimental study of the relation of the nervous system to the developing musculature in the embryo of the frog.' Amer. J. Anat., 3, 197-220.

HARRISON, R.G. (1906-7) 'Observations on the living developing nerve fiber.' Proc.Soc.Exp.Biol.Med., IV, 140-143.

HENDERSON, J.R. (1967) 'The use of silver for intensifying sulfide deposits in the cholinesterase technique.' Stain Technol., 42, 101-102.

HENNEMAN, E., OLSON, C.B. (1965) 'Relations between structure and function in the design of skeletal muscles.' J. Neurophysiol., 28, 581-598.

HEWER, E.E. (1927-28) 'The development of muscle in the human foetus.' J. Anat., 62, 72-78.

HEWER, E.E. (1934-35) 'The development of nerve endings in the human foetus.' J. Anat., 69, 369-379.

HOLTZER, H., MARSHALL, J.M., FINCK, H. (1957) 'An analysis of myogenesis by the use of fluorescent antimyosin. J. Biophys.Biochem.Cytol., 3, 705-724.

HUMPHREY, T. (1968) 'The development of mouth opening and related reflexes involving the oral area of human fetuses.' Alabama J. Med. Sci., 5, 126-157.

HUMPHREY, T. (1969) 'The prenatal development of mouth opening and mouth closure reflexes.' Pediatrics Digest, 11, 28-40.

HUXLEY, H.E. (1957) 'The double array of filaments in cross-striated muscle.' J. Biophys. Biochem. Cytol., 3, 631-648.

IRVING, D., REBEIZ, J.J., TOMLINSON, B.E. (1974) 'The numbers of limb motor neurones in the individual segments of the human lumbosacral spinal cord.' J. Neurol. Sci., 21, 203-212.

ISHIKAWA, H. (1966) 'Electron microscopic observations of satellite cells with special reference to the development of mammalian skeletal muscles.' Z. Anat. Entwicklungsgesch., 125, 43-63.

JAMES, N.T. (1971) 'A geometrical probability study of type I muscle fibres in the rabbit and guinea-pig.' J. Neurol. Sci., 14, 381-387.

JAMES, N.T. (1972) 'A quantitative study of the clumping of muscle fibre types in skeletal muscles.' J. Neurol. Sci., 17, 41-44.

JOHNSON, M., PEARSE, A.G.E. (1971) 'Differentiation of fibre types in normal and dystrophic hamster muscle.' J. Neurol. Sci., 12, 459-472.

JOHNSON, M.A., POLGAR, J., WEIGHTMAN, D., APPLETON, D. (1973) 'Data on the distribution of fibre types in thirty-six human muscles. An autopsy study.' J. Neurol. Sci., 18, 111-129.

JUNTUNEN, J., TERÄVÄINEN, H. (1972) 'Structural development of myoneural junctions in the human embryo.' Histochemie, 32, 107-112.

KARPATI, G., ENGEL, W.K. (1967) 'Neuronal trophic function. A new aspect demonstrated histochemically in developing soleus muscle.' Arch. Neurol., 17, 542-545.

KELLY, A.M., ZACKS, S.I. (1969) 'The fine structure of motor endplate morphogenesis.' J. Cell Biol., 42, 154-169.

KOELLE, G.B., FRIEDENWALD, J.S. (1949) 'A histochemical method for localising cholinesterase activity.' Proc.Soc.Exp.Biol.Med., 70, 617-622.

KONIGSBERG, I.R. (1965) in 'Organogenesis'. Eds. DeHaan, R.L., Ursprung, H. Holt, Rinehart and Winston, New York, Chicago, San Francisco, Toronto, London. pp. 337-358.

KUGELBERG, E. (1973) 'Histochemical composition, contraction speed and fatiguability of rat soleus motor units.' J. Neurol. Sci., 20, 177-198.

LARSON, P.F., HUDGSON, P., WALTON, J.N. (1969) 'Morphological relationship of polyribosomes and myosin filaments in developing and regenerating skeletal muscle.' Nature, 222, 1168-1169.

LARSON, P.F., JENKISON, M., HUDGSON, P. (1970) 'The morphological development of chick embryo skeletal muscle grown in tissue culture as studied by electron microscopy.' J. Neurol. Sci., 10, 385-405.

LENDON, R.G. (1968) 'Neuron population in the spinal cord of children with spina bifida and hydrocephalus.' Dev. Med. Child Neurol., Suppl. 15, 50-54.

LENDON, R.G. (1969) 'Neuron population in the lumbosacral cord of myelomeningocele children.' Dev. Med. Child Neurol., Suppl. 20, 82-85.

LEWIS, D.M. (1962) 'The effects of denervation on the speeds of contraction of striated muscle.' J. Physiol., 161, 24 P.

LEWIS, D.M. (1973) 'Effect of denervation on the differentiation of twitch muscles in the kitten hind limb.' Nature (New Biol.), 241, 285-286.

LEWIS, M.R. (1915) 'Rhythmical contraction of the skeletal muscle tissue observed in tissue cultures.' Amer. J. Physiol., 38, 153-161.

MAIR, W.G.P., TOMÉ, F.M.S. (1972) 'Atlas of the ultrastructure of diseased human muscle.' Churchill Livingstone, Edinburgh and London. pp. 160-167.

MALL, F.P., MEYER, A.W. (1921) 'Studies on abortuses: a survey of pathologic ova in the Carnegie embryological collection.' Contr. Embryol., XII, 193-202.

MALLORY, F.B. (1897) 'On certain improvements in histological technique.' J. Exp. Med., 2, 529-533.

MALLORY, F.B. (1900-01) 'A contribution to staining methods.' J. Exp. Med., 5, 15-20.

MASTAGLIA, F.L., WALTON, J.N. (1971) 'An ultrastructural study of skeletal muscle in polymyositis.' J. Neurol. Sci., 12, 473-504.

MAURO, A. (1961) 'Satellite cell of skeletal muscle fibers.' J. Biophys. Biochem. Cytol., 9, 493-495.

MAVRINSKAYA, L.F. (1963) 'Histochemical study of cholinesterase during development of somatic musculature in the human fetus.' Fed. Proc. (Transl. Suppl.) 22, T597-T604.

MAXIMOV, A. (1925) 'Tissue-cultures of young mammalian embryos.' Contr. Embryol., XVI, 47-113.

MENDELL, J.R., ROELOFS, R.I., ENGEL, W.K. (1972) 'Ultrastructural development of explanted human skeletal muscle in tissue culture.' J. Neuropath. Exp. Neurol., 31, 433-446.

MONTGOMERY, R.D. (1962) 'Growth of human striated muscle.' Nature, 195, 194-195.

MORRIS, C.J. (1970) 'The significance of intermediate fibres in reinnervated human skeletal muscle.' J. Neurol. Sci., 11, 129-136.

MORRIS, C.J., RAYBOULD, J.A. (1971) 'Fibre type grouping and endplate diameter in human skeletal muscle.' J. Neurol. Sci., 13, 181-187.

MORRIS, C.J., WOOLF, A.L. (1970) 'Mechanism of type I muscle fibre grouping.' Nature, 226, 1061-1062.

MORTIER, W., VON BERNUTH, H. (1971) 'The neural influence on muscle development in myelomeningocele: histochemical and electrodiagnostic studies.' Dev. Med. Child Neurol., Suppl. 25, 82-89.

MOSCONA, A. (1952) 'Cell suspensions from organ rudiments of chick embryos.' Exp. Cell Res., 3, 535-539.

MOSS, F.P., LEBLOND, C.P. (1971) 'Satellite cells as the source of nuclei in muscles of growing rats.' Anat. Rec., 170, 421-436.

MUIR, A.R., KANJI, A.H.M., ALLBROOK, D. (1965) 'The structure of the satellite cells in skeletal muscle.' J. Anat., 99, 435-444.

MURRAY, M.R. (1965) in 'Cells and tissues in culture.' Ed. Willmer, E.N. Academic Press, London, New York. Vol. 2, pp. 311-372.

MURRAY, M.R. (1972) in 'The structure and function of muscle.' 2nd. edition. Ed. Bourne, G.H. Academic Press, New York and London. Vol.I, pp. 237-299.

MacCALLUM, J.B. (1898) 'On the histogenesis of the striated muscle fibre, and the growth of the human sartorius muscle.' Johns Hopk. Hosp. Bull., 9, 208-215.

McCOMAS, A.J., MROZEK, K. (1967) 'Denervated muscle fibres in hereditary mouse dystrophy.' J. Neurol. Neurosurg. Psychiat., 30, 526-530.

McCOMAS, A.J., SICA, R.E.P., CURRIE, S. (1970) 'Muscular dystrophy: evidence for a neural factor.' Nature, 226, 1263-1264.

McPHEDRAN, A.M., WUERKER, R.B., HENNEMAN, E. (1965) 'Properties of motor units in a homogeneous red muscle (soleus) of the cat.' J. Neurophysiol., 28, 71-84.

NAMBA, T., GROB, D. (1970) 'Cholinesterase activity of motor endplate in human skeletal muscle.' J. Clin. Invest., 49, 936-942.

NAMBA, T., NAKAMURA, T., GROB, D. (1967) 'Staining for nerve fibre and cholinesterase activity in fresh frozen sections.' Amer. J. Clin. Path. 47, 74-77.

ODUTOLA, A.B. (1972) 'The organisation of cholinesterase-containing systems of the monkey spinal cord.' Brain Res., 39, 353-368.

OMMER, P.A. (1971) 'Histochemical differentiation of skeletal muscle fibres in the bovine fetus.' Experientia, 27, 173-174.

PADGET, D.H. (1970) 'Neuroschisis and human embryonic maldevelopment: new evidence of anencephaly, spina bifida and diverse mammalian defects.' J. Neuropath. Exp. Neurol., 29, 192-216.

PADYKULA, H.A. (1952) 'The localisation of succinic dehydrogenase in tissue sections of the rat.' Amer. J. Anat., 91, 107-145.

PADYKULA, H.A., GAUTHIER, G.F. (1970) 'The ultrastructure of the neuromuscular junctions of mammalian red, white and intermediate skeletal muscle fibres.' J. Cell Biol., 46, 27-41.

PADYKULA, H.A., HERMAN, E. (1955) 'Factors affecting the activity of ATPase and other phosphatases as measured by histochemical techniques.' J. Histoch. Cytoch., 3, 161-169.

PAGE, K. (1971) 'Histological methods for peripheral nerves - Part II.' Med. Lab. Technol., 28, 44-58.

PALAY, S.L., PALADE, G.E. (1955) 'The fine structure of neurons.' J. Biophys. Biochem. Cytol., 1, 69-88.

PALMGREN, A. (1960) 'Specific silver staining of nerve fibres. 1. Technique for vertebrates.' Acta Zoologica, 41, 239-265.

PANNESE, E. (1968) 'Developmental changes of the endoplasmic reticulum and ribosomes in nerve cells of the spinal ganglia of the domestic fowl.' J. Comp. Neurol., 132, 331-364.

PAPAPETROPOULOS, T.A., BRADLEY, W.G. (1972) 'Spinal motor neurones in murine muscular dystrophy and spinal muscular atrophy. A quantitative histological study.' J. Neurol. Neurosurg. Psychiat., 35, 60-65.

PAUL, J. (1965) 'Cell and tissue culture.' 3rd. edition. E. and S. Livingstone Ltd., Edinburgh.

PEARCE, J.M.S., PENNINGTON, R.J.T., WALTON, J.N. (1964) 'Some enzyme studies in muscle disease: Part III. Serum creatine kinase activity in relatives of patients with the Duchenne type of muscular dystrophy.' J. Neurol. Neurosurg. Psychiat., 27, 181-185.

PEARN, J.H. (1973) 'Fetal movements and Werdnig-Hoffmann disease.' J. Neurol. Sci., 18, 373-379.

PEARSON, C.K. (1963) 'A formalin-sucrose-ammonia fixative for cholinesterase.' J. Histoch. Cytoch., 11, 665-666.

PEARSON, C.M. (1962) 'Histopathological features of muscle in the preclinical stages of muscular dystrophy.' Brain, 85, 109-120.

PETERSON, E.R., CRAIN, S.M. (1972) 'Regeneration and innervation in cultures of adult mammalian skeletal muscle coupled with fetal rodent spinal cord.' Exp. Neurol., 36, 136-159.

PETTE, D., STAUDTE, H.W., VRBOVÁ, G. (1972) 'Physiological and biochemical changes induced by long-term stimulation of fast muscle.' Naturwissenschaften, 59, 469-470.

PHILLIS, J.W. (1970) 'The pharmacology of synapses.' 1st. edition. Pergamon Press. pp. 159-160.

POGOGEFF, I.A., MURRAY, M.R. (1945) 'Regeneration of adult mammalian skeletal muscle in vitro.' Science, 101, 174.

POGOGEFF, I.A., MURRAY, M.R. (1946) 'Form and behaviour of adult mammalian skeletal muscle in vitro.' Anat. Rec., 95, 321-335.

PYSH, J.J., KHAN, T. (1972) 'Variations in mitochondrial structure and content of neurons and neuroglia in rat brain: an electron microscopic study.' Brain Res., 36, 1-18.

RAGAB, A.H.M.F. (1971) 'Motor-end-plate changes in mouse muscular dystrophy.' Lancet II, 815-816.

RANVIER, L. (1874) 'De quelques faits relatifs à l'histologie et à la physiologie des muscles striés.' Arch. Phys. 2e série, 1, 1 - 15.

REDFERN, P.A. (1970) 'Neuromuscular transmission in new-born rats.' J. Physiol., 209, 701-709.

REIER, P.J., HUGHES, A. (1972) 'Evidence for spontaneous axon degeneration during peripheral nerve maturation.' Amer. J. Anat., 135, 147-152.

ROMANES, G.J. (1940-41) 'Cell columns in the spinal cord of a human foetus of fourteen weeks.' J. Anat., 75, 145-152.

ROMANES, G.J. (1941-42) 'The development and significance of the cell columns in the ventral horn of the cervical and upper thoracic spinal cord of the rabbit.' J. Anat., 76, 112-130.

ROMANES, G.J. (1947) 'Notes on the spinal cord in acephalic sheep foetuses.' J. Anat., 81, 391-392.

ROMANES, G.J. (1951) 'The motor cell columns of the lumbo-sacral spinal cord of the cat.' J. Comp. Neurol., 94, 313-363.

ROMANUL, F.C.A. (1964) 'Enzymes in muscle. I Histochemical studies of enzymes in individual muscle fibers.' Arch. Neurol., 11, 355-368.

ROSALKI, S.B. (1965) 'Creatine phosphokinase isoenzymes.' Nature, 207, 414.

ROSALKI, S.B. (1967) 'An improved procedure for serum creatine kinase determination.' J. Lab. Clin. Med., 69, 696-705.

SALMONS, S., VRBOVÁ, G. (1969) 'The influence of activity on some contractile characteristics of mammalian fast and slow muscles.' J. Physiol., 201, 535-549.

SAMAHA, F.J., GUTH, L., ALBERS, R.W. (1970) 'The neural regulation of gene expression in the muscle cell.' Exp. Neurol., 27, 276-282.

SCARPELLI, D.G., HESS, R., PEARSE, A.G.E. (1958) 'The cytochemical localisation of oxidative enzymes. I Diphosphopyridine nucleotide diaphorase and triphosphopyridine nucleotide diaphorase.' J. Biophys. Biochem. Cytol., 4, 747-752.

SCHIAFFINO, S., BORMIOLI, S.P. (1973) 'Histochemical characterisation of adenosine triphosphatases in skeletal muscle fibres by selective extraction procedures.' J. Histoch. Cytoch., 21, 142-145.

SHAFIQ, S.A., ASIEDU, S.A., MILHORAT, A.T. (1972) 'Effect of neonatal neurectomy on differentiation of fibre types in rat skeletal muscle.' Exp. Neurol., 35, 529-540.

SHARRARD, W.J.W. (1953) 'Correlation between changes in the spinal cord and muscle paralysis in poliomyelitis - A preliminary report.' Proc. Roy. Soc. Med., 46, 346-349.

SHARRARD, W.J.W. (1955) 'The distribution of the permanent paralysis in the lower limb in poliomyelitis.' J. Bone Joint Surg., 37B, 540-558.

SHIMADA, Y., FISCHMAN, D.A., MOSCONA, A.A. (1967) 'The fine structure of embryonic chick skeletal muscle cells differentiated in vitro.' J. Cell Biol., 35, 445-453.

SHIMADA, Y., KANO, M. (1971) 'Formations of neuromuscular junctions in embryonic cultures.' Arch. Histol. Jap., 33, 95-114.

STARK, G.D., DRUMMOND, M. (1971) 'The spinal cord lesion in myelomeningocoele.' Dev. Med. Child Neurol., Suppl. 25, 1-14.

STOCKDALE, F.E., HOLTZER, H. (1961) 'DNA synthesis and myogenesis.' Exp. Cell Res., 24, 508-520.

STRAUS, W.L., RAWLES, M.E. (1953) 'An experimental study of the origin of the trunk musculature and ribs in the chick.' Amer. J. Anat., 92, 471-509.

STREETER, G.L. (1921) 'Weight, sitting height, head size, foot length and menstrual age of the human embryo.' Contr. Embryol., XI, 143-170.

SYROVÝ, I., GUTMANN, E., MELICHNA, J. (1971) 'Differential response of myosin-ATPase activity and contraction properties of fast and slow rabbit muscles following denervation.' Experientia, 27, 1426-1427.

SYTKOWSKI, A.J., VOGEL, Z., NIRENBERG, M.W. (1973) 'Development of acetylcholine receptor clusters on cultured muscle cells.' Proc. Nat. Acad. Sci., 70, 270-274.

TELLO, J.F. (1917) 'Génesis de las terminaciones nerviosas motrices y sensitivas. I En el sistema locomotor de los vertebrados superiores. Histogenesis muscular.' Trab. Lab. Invest. Biol. Madrid, 15, 101-199.

TELLO, J.F. (1922) 'Die entstehung des motorischen und sensiblen Nervendigungen. 1. In dem lokomotorischen Systeme der höheren Wirbeltiere Muskuläre Histogenese.' Z. Anat. Entwicklungsgesch., 64, 348-440.

TERÄVÄINEN, H. (1968) 'Development of the myoneural junction in the rat.' Z.Zell. Mikr. Anat., 87, 249-265.

TIEGS, O.W. (1953) 'Innervation of voluntary muscle.' Physiol. Rev., 33 90-144.

TOMLINSON, B.E., IRVING, D., REBEIZ, J.J. (1973) 'Total numbers of limb motor neurones in the human lumbosacral cord and an analysis of the accuracy of various sampling procedures.' J. Neurol. Sci., 20, 313-327.

TOOP, J., JACKSON, P.D. (1973) 'Histochemical development of anterior horn cells in the human fetus.' Int. Res. Com. Syst. (Med.Sci.) (73-10) 1-0-11.

TOOP, J., WEBB, J.N., EMERY, A.E.H. (1973) 'Muscle differentiation in anencephaly.' Dev. Med. Child Neurol., 15, 164-170.

TOOP, J., EMERY, A.E.H. (1974) 'Muscle histology in fetuses at risk for Duchenne muscular dystrophy.' Clin. Genet. (in press).

WALTON, J.N., GARDNER-MEDWIN, D. (1969) in 'Disorders of voluntary muscle.' 2nd. edition. Ed. Walton, J.N. J. and A. Churchill Ltd., London. pp.455-500.

WARMOLTS, J.R., ENGEL, W.K. (1972) 'Open-biopsy electromyography. I. Correlation of motor unit behaviour with histochemical muscle fibre type in human limb muscle.' Arch. Neurol., 27, 512-517.

WARREN, H.S. (1951) 'Acrania induced by anencephaly.' Anat. Rec., 111, 653-668.

WEBB, J.N. (1972) 'The development of human skeletal muscle with particular reference to muscle cell death.' J. Path., 106, 221-228.

WIRSÉN, C., LARSSON, K.S. (1964) 'Histochemical differentiation of skeletal muscle in foetal and newborn mice.' J. Embr. Exp. Morphol., 12, 759-767.

WOHLFART, G. (1937) 'Über das Vorkommen verschiedener arten von Muskelfasern in der Skelettmuskulatur des Menschen und einiger Säugethiere.' Acta Psychiat. Neurol., Suppl. XII.

WUERKER, R.B., McPHEDRAN, A.M., HENNEMAN, E. (1965) 'Properties of motor units in a heterogeneous pale muscle (M. gastrocnemius) of the cat.' J. Neurophysiol., 28, 85-99.

YAFFE, D. (1973) in 'Tissue culture. Methods and applications.' 1st. edition. Eds. Kruse, P.F., Jr., Patterson, M.K., Jr. Academic Press, New York and London. pp. 106-114.

ZACKS, S.I. (1964) 'The motor endplate.' W.B. Saunders Company, Philadelphia, London.

ZACKS, S.I., BLUMBERG, J.M. (1961) 'Observations on the fine structure and cytochemistry of mouse and human intercostal neuromuscular junctions.' J. Biophys.Biochem.Cytol., 10, 517-528.

APPENDIX 1

FETUSES FROM WHICH SAMPLES WERE OBTAINED FOR STUDY.

Abbreviations.

LMP = last menstrual period

D + C = dilation and curettage

Hyst. = hysterotomy

Spont. = spontaneous abortion

PG = Prostaglandin

(A) Fetuses for the study of muscle development in vivo.

Fetus number	Gestation (wks) by Crown-rump length	Gestation (wks) by Heel-toe length	Gestation (wks) by First day of LMP	Gestation taken as (wks.)	Method of termination
761	16	17		17	Hyst.
803		10	10	10	D + C
806		13	13	13	Hyst.
825		16	14	16	Hyst.
827	28		18	28	Still birth
837	14		16	15	Hyst.
839		15	14	15	Hyst.
938		16	14	16	
955		20	21	20	Hyst.
980		15	15	15	
981	20	22	23	22	
987		11	13	11	D + C
988		10	10	10	D + C

Appendix 1(A) (Contd.)

Fetus number	Gestation (wks) by Crown-rump length	Gestation (wks) by Heel-toe length	Gestation (wks) by First day of LMP	Gestation taken as (wks.)	Method of termination
994		23	22	23	Spont.
1006		8	11	8	D + C
1007	5-6			5-6	Hyst.
1015	26	28		28	Still birth
72/12		11		11	PG
72/20		12		12	
72/22		22		22	
72/23		14		14	
72/37		9		9	D + C
72/48		7	9	7	D + C
72/55		9	9	9	D + C
72/56		12	11	12	D + C
72/61		10	10	10	D + C
72/62	19	18		18	PG
72/76		8	10	8	D + C
72/77		10	11	10	D + C
72/80	25	25	24	25	Spont.
72/99	18	19		19	PG
72/126	22	23		23	Spont.
72/198	18	24		21	Hyst.
72/200	13	14	16	14	PG.
72/202	14	14		14	Hyst.
72/205		25		25	Spont.
72/207		11		11	D + C

Appendix 1(A) (Contd.)

Fetus number	Gestation (wks.) by Crown-rump length	Gestation (wks.) by Heel-toe length	Gestation (wks.) by First day of LMP	Gestation taken as (wks.)	Method of termination
72/210	20	19		19	
72/211	11	12	11	12	Hyst.
72/230	14	14	12	14	Hyst.
72/289	14	14	14	14	PG
72/291		8		8	
72/293	15	14	15	14	PG
72/303	12		12	12	PG
72/345	16	17		17	Spont.
72/380	13	13		13	
73/16	14	14	21	14	Hyst.
73/31		24		24	PG
73/32	16	15	14	15	PG
73/55	16	16	14	16	PG
73/68	12	12	15	12	PG
73/69		13	13	13	PG
73/76		24	26	24	Spont. (lived 3 days)
73/81	17	18	18	18	PG
73/85	13	13	14	13	Spont.
73/88	12	12	13	12	PG
73/89		39	42	Neonate	Died after 54 hrs. of Pulmonary hypoplasia
73/90	28	28		28	Still birth

Appendix 1(A) (Contd.)

Fetus number	Gestation (wks.) by Crown-rump length	Gestation (wks.) by Heel-toe length	Gestation (wks.) by First day of LMP	Gestation taken as (wks.)	Method of termination
73/101	20	21		21	PG
73/105	14	14	14	14	PG
73/124		9		9	D + C
73/125	19	20		20	Hyst.
73/127		22		22	Spont.
73/130		18	19	18	PG
73/131		12	13	12	PG
73/132	14	13	17	13	PG
73/134	8			8	D + C
73/138	11	10		10	Hyst.
73/181		11		11	D + C
73/186A	8	8		8	D + C
73/210	13	13		13	PG
73/211	11	12	13	12	PG
73/221	11	12		12	Hyst.
73/259	18	17		17	Hyst.
73/384	27	29		29	Spont.

Appendix 1 (B) Fetuses for the study of Anterior Horn Cell development.

Fetus number	Gestation (wks.) by Crown-rump length	Heel-toe length	First day of LMP	Gestation taken as (wks.)	Method of termination	Delay before freezing/ fixation (days)
72/198	18	24		21	Hyst.	0
72/210	20	19		19		0
72/226		17		17	PG	1
72/252		23	23	23	Spont.	0
72/264	13			13		0
72/300	15	16		16	Hyst.	0
72/318	20	22		22		0
73/9		23	24	23	Spont.	3
73/10	19	19	19	19		3
73/12	24	24		24		2
73/16	14	14	21	14	Hyst.	0
73/24		40		Neonate	Died of anoxia	1
73/25		33		33	Spont. Difficulty breathing.	1
73/32	16	15	14	15	PG	1
73/34	7			7	D + C	1
73/49	13	12		12	PG	1
73/56	16	17		17	PG	1
73/58	15	16		16	Hyst.	0
73/59		8	9	8	D + C	1
73/77		14	14	14	PG	1
73/83		11		11	D + C	0
73/90	28	28		28	Still birth.	1

Appendix 1 (B) (Contd.)

Fetus number	Gestation (wks.) by Crown-rump length	Heel-toe length	First day of LMP	Gestation taken as (wks.)	Method of termination	Delay before freezing/ fixation (days)
73/91		13		13	PG	1
73/101	20	21		21	PG	1
73/103		18		18		0
73/108	7			7	D + C	0
73/124		9		9	D + C	0
73/125	19	20		20	Hyst.	0
73/131		12	13	12	PG	2
73/134	8			8	D + C	1
73/138	11	10		10	Hyst.	0
73/188		15	15	15	PG	0
73/207		10	19	10	D + C	0
73/210	13	13		13	PG	2
73/255		8		8	D + C	1

Appendix 1 (C). Anencephalic fetuses studied.

Fetus number	Gestation (wks.) by Heel-toe length	Gestation (wks.) by First day of LMP	Gestation taken as (wks.)	Rachischisis
956	30	34	30	Yes
965	27	29	27	Yes
995		29	29	No
998	26	34	26	No
1008	27		27	No
1009	31	31	31	No
72/139	31	32	31	No
72/186		31	31	Yes
73/93	26	27	26	Yes
73/262B	23	23	23	No

Appendix 1 (D) Fetuses for the study of human skeletal muscle in vitro.

Fetus number	Gestation (wks.) by Crown-rump length	Heel-toe length	First day of LMP	Gestation taken as (wks.)	Method of termination	Subcultures which grew.
72/268		15		15	D + C	1
72/270		10		10	D + C	1
72/271		11		11	D + C	2
72/274		12		12	PG	3
72/278	10	10		10	D + C	4
72/285		26		26	Spont.	2
72/286	15	16		16	PG	3
72/289	14	14	14	14	PG	4
72/296	16	16	15	16	PG	4
72/297	15	16	21	16	PG	2
72/299	12	13		13	Hyst.	2
72/300	15	16		16	Hyst.	3
72/303	12		12	12	PG	3
72/316	21	22		22	Spont.	3
72/317	12	11		11	D + C	4
72/318	20	22		22		2
72/319	16	16	18	16	PG	5
72/336	12		13	12	PG	1
72/339	14	14		14	PG	4
72/341	16	18	18	18	PG	2
72/345	16	17		17	Spont.	1
72/346		12		12	D + C	2
72/352	15	16	16	16	PG	1

Appendix 1 (D) (Contd.)

Fetus number	Gestation (wks.) by Crown-rump length	Heel-toe length	First day of LMP	Gestation taken as (wks.)	Method of termination	Subcultures which grew.
72/372	15	15	14	15	PG	1
72/374		11		11	D + C	1
72/382	10		10	10	D + C	2
72/383	11	11	12	11	D + C	1
72/389	10	9	11	9	PG	1

APPENDIX 2

STAINING METHODS USED TO DEMONSTRATE THE PERIPHERAL MOTOR INNERVATION IN HUMAN FETAL MUSCLE.

(A) Staining for acetylcholinesterase activity.

1. Make frozen sections 10 - 20μ thick.
2. Allow to dry at room temperature for about 30 minutes.
3. Fix in formalin - sucrose - ammonia fixative for 10 minutes at room temperature.

Formalin	10 ml.
Sucrose	15 gm.
.880 NH_3	1 ml.

Make up with distilled water to 100 ml.

4. Wash for 2 minutes in distilled water.
5. Incubate in acetylcholinesterase medium at 37°C for different periods of time (next page)

Stock solution:-	$CuSO_4$	0.3	gm.
	Maleic acid	1.75	gm.
	Glycine	0.375	gm.
	$MgCl_2$	1.0	gm.
	N NaOH	30	ml.
	20 - 25% solution of Na_2SO_4	170	ml.

Stock solution keeps indefinitely at room temperature.

For use:- dissolve about 20 mg substrate (acetylthiocholine iodide) in distilled water, and add 10 ml. stock.

Incubation times:-

up to 9 weeks	gestation,	incubate	for	9	minutes.
10 or 11	"	"	"	7	"
12 or 13	"	"	"	$4\frac{1}{2}$	"
14 or 15	"	"	"	$3\frac{1}{2}$	"
16 - 18	"	"	"	3	"
19 - 24	"	"	"	$2\frac{1}{2}$	"
25 +	"	"	"	2	"

6. Rinse three times in distilled water.
7. Place in 0.5% $K_3Fe(CN)_6$ for 10 minutes at room temperature.
8. Wash in distilled water for 1 minute.
9. Place in 5% aqueous silver nitrate for about $\frac{1}{2}$ minute.
10. Rinse briefly in distilled water.
11. Place in 5% sodium thiosulphate for about $\frac{1}{2}$ minute.
12. Rinse in three changes of distilled water.
13. Stain in 1% aqueous light green for 20 seconds.
14. Rinse briefly in two changes of distilled water.
15. Dehydrate in graded alcohols, clear for no more than 5 minutes in xylene, and mount in DPX.

(B) <u>Combined acetylcholinesterase incubation and silver impregnation for the simultaneous demonstration of motor endplates and axons</u>.

1. Make frozen sections 20 - 50μ thick.
2. Allow to dry thoroughly (overnight) at room temperature.
3. Fix in formalin - sucrose - ammonia fixative for 5 minutes at room temperature (Method A)
4. Wash for 2 minutes in distilled water.

5. Incubate in acetylcholinesterase medium at 37°C (Method A) for periods of time as follows:-

up to 9	weeks	gestation,	incubate for	8	minutes.
10 or 11	"	"	"	6	"
12 or 13	"	"	"	4	"
14 or 15	"	"	"	3	"
16 - 18	"	"	"	$2\frac{1}{2}$	"
19 - 24	"	"	"	2	"
25 +	"	"	"	$1\frac{1}{2}$	"

6. Rinse three times in distilled water.

7. Place in 0.5% $K_3Fe(CN)_6$ for 10 minutes at room temperature. (Solution must be fresh.)

8. Rinse three times in distilled water.

9. Fix for 1 hour in Bouin's fixative at 4°C.

Saturated aqueous picric acid	75 ml.
Formalin	25 ml.
Acetic acid	5 ml.

10. Wash in distilled water for 1 - 2 minutes.

11. Treat with 1% .880 NH_3 in 70% alcohol to remove yellow colour from sections.

12. Wash in changes of distilled water for 5 minutes.

13. Impregnate in 10% silver nitrate in 0.02% aqueous pyridine at 37°C for between 1 minute (for muscle from older fetuses) and 2 minutes(for very early muscle). (Keep pyridine as stock solution. Make up silver solution fresh at least twice a week.)

14. Wash in changes of distilled water for 3 minutes.

15. Place in 2% borax in distilled water for $\frac{1}{2}$ - 1 minute.

16. Develop in (1 gm. quinol + 5gm. Na_2SO_3 in 100 ml. 2% borax in distilled water) at 37°C for 2 - 3 minutes. (This lasts approximately 1 week in the incubator.)

17. Wash in 50% alcohol for 5 minutes.

At this stage the sections should be inspected to decide whether (i) re-impregnation is necessary, in which case go back to step 13; or (ii) the fine detail of the axons would be demonstrated more clearly by gold intensification.

If neither is necessary, proceed to step 21. If (ii) is necessary, proceed:-

18. Tone section for 15 - 30 seconds in a few drops of a 0.5% solution of yellow gold chloride ($NaAuCl_4.2H_2O$) which contains 3 drops glacial acetic acid per 100 ml. Section turns grey - blue. (This solution keeps well on the shelf.)

19. Rinse briefly in distilled water.

20. Intensify for 2 - 3 minutes at 37°C in 0.5% oxalic acid in 50% alcohol. (This solution may be used for 1 week in the incubator.)

21. Rinse briefly in distilled water.

22. Fix in 5% sodium thiosulphate for ½ - 1 minute.

23. Rinse in distilled water.

24. Dehydrate, clear in xylene for no longer than 5 minutes and mount.

If addition intensification is required after step 23, repeat steps 18 - 23.

References.

GOMORI, 1952 HENDERSON, 1967. PAGE, 1971.

PALMGREN, 1960. PEARSON, 1963.

APPENDIX 3

PUBLICATIONS

TOOP, J., JACKSON, P.D. (1973) 'Histochemical development of anterior horn cells in the human fetus.' Int. Res. Com. Syst. (Med. Sci.) (73-10) 1 - 0 - 11.

TOOP, J., WEBB, J.N., EMERY, A.E.H. (1973) 'Muscle differentiation in anencephaly.' Dev. Med. Child Neurol., 15, 164 - 170.

TOOP, J., EMERY, A.E.H. (1974) 'Muscle histology in fetuses at risk for Duchenne muscular dystrophy.' Clin. Genet. (in press).

HISTOCHEMICAL DEVELOPMENT OF ANTERIOR HORN CELLS IN THE HUMAN FETUS

J. Toop and P.D. Jackson,
University Department of Human Genetics, Western General Hospital, Edinburgh, EH4 2XU, Scotland.

Paper received: 7th September, 1973

For reprints please quote:
(73-10) 1-0-11

Human Biology
Growth and Development
(73-10) 5-7-8

Neurology
Nerve and Muscle Anatomy
(73-10) 16-1-7

Cell and Membrane Studies
General Aspects
(73-10) 1-0-11

The results of nerve cross-union experiments in animals suggest that biochemical aspects of muscle are under direct neuronal control; other experiments suggest that each motor unit is biochemically and physiologically homogeneous. The motoneurones in the anterior horn of the spinal cord (AHC's) of the adult cat, can be divided into 'fast' and 'slow' on the basis of the twitch properties of their respective motor units, and more recently (1) several types of AHC's have been histochemically distinguished. In the adult monkey Odutola (2) has used acetylcholinesterase (AChE) staining to distinguish different types of AHC's. Adult human AHC's,and fetal (3), have been studied histologically, but histochemically different types of AHC's have not yet been differentiated. We have investigated whether there are different histochemical types of AHC's in the human fetus, and if so, whether the changing histochemical pattern of developing muscle (4) can be related to changes in the proportions of the different types of AHC's supplying these muscles.

The caudal end of the spinal cord was removed, following therapeutic or spontaneous abortion, from 16 fetuses of between 7 and 28 weeks gestation. The cord was frozen in isopentane chilled in liquid nitrogen, and transverse frozen sections cut at 30 - 50 μ, depending on gestational age. Sections were stained for AChE and succinic dehydrogenase (SDH), and with haematoxylin and eosin. Staining times for each stain were kept constant for all sections.

Percentages of anterior horn cells staining darkly or lightly for acetylcholinesterase at different gestational ages.

Age (weeks)	Light cells (%)	Dark cells (%)
12	65	35
13	61	39
14	66	34
15	69	31
17	66	34
18	69	31
20	61	39
22	70	30
28	72	28

No AChE or SDH activity could be detected in spinal cords of less than 8½ weeks gestation, at which time there was only a narrow band of AChE activity visible at the periphery of the anterior horn. This band was visible in fetuses of up to 10 weeks gestation; but at 11-12 weeks gestation the AHC's could definitely be divided into light and dark types, and were grouped in columns as in the adult spinal cord. At this age there were about 65% light cells and 35% dark, and these proportions did not alter greatly with increasing gestational age (table). Throughout the gestational age range, the light cells were smaller than the dark ones, seemed to occur more at the peripheries of the columns, and appeared to correlate in number, size and distribution with those cells which stained intensely for SDH.

Previous workers (1) have suggested that two of the types of AHC's differentiated histochemically may correspond to two types of motoneurones each of which supplies either 'fast' or 'slow' muscle fibres. Fast and slow motor units do not necessarily contain equal numbers of muscle fibres (5), but if the above suggestion is correct, one would still expect the ratio of 'fast' to 'slow' muscle fibres to be similar to the ratio of 'fast' to 'slow' AHC's even though, allowing for axonal transport of a trophic factor, there might be a time lag.

Our results therefore appear not to support this suggestion, since the AHC's seem to reach their histochemically adult status by 11-12 weeks, and thereafter alter very little, in contrast to the great histochemical changes which take place in skeletal muscle between 11 and 30 weeks gestation (4).

1. Campa, J.F. and Engel, W.K. (1971) Science, 171, 198
2. Odutola, A.B. (1972) Brain Res., 39, 353
3. Romanes, G.J. (1940-41) J. Anat., 75, 145
4. Dubowitz, V. (1968) 'Developing and Diseased Muscle'. SIMP Research Monograph No. 2. London: Spastics International Medical Publications with Heinemann. p.47
5. McPhedran, A.M., Wuerker, R.B. and Henneman, E. (1965) J. Neurophysiol., 28, 71

Acknowledgement

This work was supported by a grant from the Medical Research Council.

Reprinted from Developmental Medicine and Child Neurology Vol. 15. No. 2. April 1973, pp. 164–170

Muscle Differentiation in Anencephaly

James Toop **John N. Webb**† **Alan E. H. Emery**‡*

Introduction

Myogenesis in tissue culture and the classical experiments of Harrison (1904) are clear demonstrations of the ability of skeletal muscle to grow and differentiate morphologically in the absence of a neural stimulus. Harrison demonstrated that the skeletal muscle of amphibian limbs continued to differentiate even after the spinal cord tissue and complete nervous supply had been excised. On the other hand, adult human muscle fibres which are deprived of their nerve supply atrophy and die, for example in diseases which primarily destroy the anterior horn cells of the spinal cord or the motor nerve fibres. In man, it is not known at what stage in development the motor nerve supply becomes essential for the continued integrity of skeletal muscle, nor is it known how the nerve supply exerts its trophic influence on muscle. It has been suggested that innervation is essential for the initial differentiation of muscle into histochemical fibre types, and in tissue culture the development of histochemical differentiation cannot be demonstrated in cultures of initially undifferentiated fetal myoblasts (Engel 1961) or adult muscle (Gallup *et al.* 1972) grown in the absence of nerve. Cross-innervation experiments in animals (Engel 1961, Buller *et al.* 1960) lend support to the idea that the neural stimulus controls the development of different fibre types.

In an attempt to further elucidate these problems, we have examined the central and peripheral nervous systems and skeletal muscles of two anencephalic fetuses, one at 30 weeks and the other at 27 weeks gestation. We were interested to know whether the abnormality in the spinal cord would be reflected by a disturbance in muscle development, histologically or histochemically, or both.

Material and Method

Both rachischitic anencephalic fetuses were still-born, and examination was carried out in Case 1 one day later and in Case 2 three days later, during which time the intact fetuses were kept at 4°C. At the time of examination, the approximate post-conceptional age was determined by means of heel-toe measurements (Streeter 1921). Muscle tissue (biceps brachii, gastrocnemius, deltoid, vastus lateralis) and the whole spinal cord were removed. Parts of the biceps and gastrocnemius muscles were taken from near the motor point and frozen in liquid nitrogen before being sectioned at 10μ in a cryostat. The remainder of these muscles, as well as the deltoid and vastus lateralis muscles, were processed for paraffin embedding and, after sectioning, were stained with haema-

* Ph.D. Student; † Consultant Pathologist; ‡ Professor of Human Genetics; University Department of Human Genetics, Western General Hospital, Edinburgh EH4 2XU.

toxylin and eosin. Frozen sections of muscle were stained for NADH-diaphorase (Scarpelli *et al.* 1958) and myosin ATPase, pH 9·4 (Padykula and Herman 1955).

In order to study the peripheral motor innervation, a combined cholinesterase and silver impregnation technique was used (Namba *et al.* 1967). Only the most caudal part of the spinal cord was used for frozen sections, which were stained with toluidine blue (1 per cent aqueous solution, 1 to 6 hours at room temperature) and studied alongside sections of gastrocnemius muscle. The lower cervical and upper thoracic regions were blocked for paraffin sectioning, and sections stained with haematoxylin and eosin were studied together with sections of the biceps muscle.

Two other spinal cords were used for controls, one from an anencephalic fetus without rachischisis of 34 weeks gestation, and the other from the apparently normal non-identical twin fetus of an anencephalic of 27 weeks gestation. Before fixation and embedding for paraffin sectioning, each had been stored for 3 days at 4°C. The spinal cords were processed in the same way as those from the rachischitic anencephalic fetuses; 10μ sections were cut from the lower cervical and upper thoracic regions at intervals of 50μ, and stained with haematoxylin and eosin.

The counting of neurones was done in the manner described by Papapetropoulos and Bradley (1972). Non-degenerate neurones were regarded as those with a well-defined area of cytoplasm and nucleus, and only those with visible nucleoli were counted.

Results

Case 1

Female anencephalic fetus at 30 weeks gestation with complete craniorachischisis.

Central nervous system. A knob of cerebellar tissue was present, but with no identifiable brain stem. Cervical and thoracic cord consisted of a thin vascular plate of atrophic neural tissue; there were only a few neurones present, and most of these showed advanced degenerative changes when stained either with toluidine blue or haematoxylin and eosin. The nerve roots were well developed and dorsal root ganglia were normal. In the lumbar region, the cord consisted of a thin core of neural tissue with a double canal.

Peripheral nervous system. The peripheral nerves and their motor end-plates on the muscle fibres appeared normal in distribution and size. The acetylcholinesterase reaction was restricted to the end-plate region and was of normal intensity (Fig. 1).

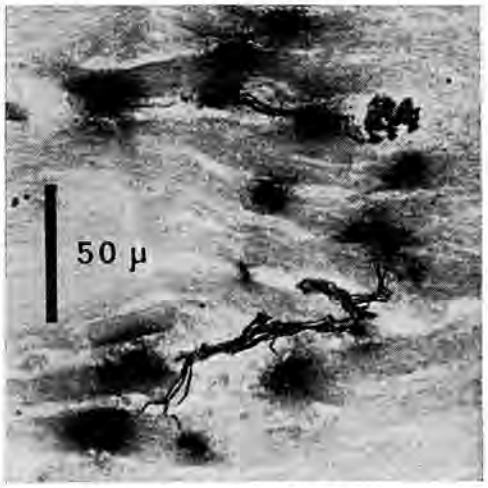


Fig. 1. Biceps brachii muscle, 30 week anencephalic fetus. Combined cholinesterase and silver impregnation preparation (Namba *et al.* 1967) showing motor nerves and endplates. × 360.

Muscle histology and histochemistry. The histology of all muscles was normal; the fibres showed cross-striations and subsarcolemmal nuclei, with no evidence of degeneration or atrophy (Fig. 2). There was a normal pattern of fibre type differentiation in the biceps and gastrocnemius muscles (Dubowitz 1968) (Fig. 3).

Case 2

Female anencephalic fetus at 27 weeks

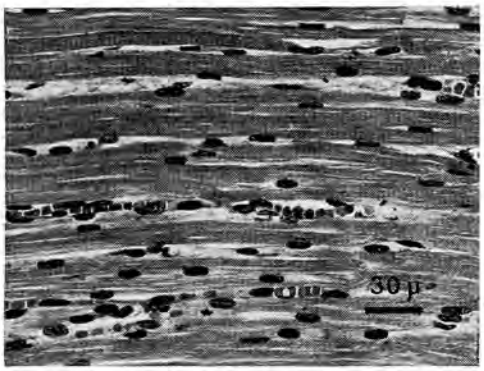


Fig. 2. Biceps brachii muscle, 30 week anencephalic fetus. Longitudinal section showing normal muscle histology. (H. & E. × 230.)

gestation with complete craniorachischisis.

Central nervous system. No cerebral or cerebellar tissue was identified. The spinal cord was represented by a thin, flat vascular membrane, in which were fairly numerous neurones, most of which were degenerate.

Peripheral nervous system. This appeared similar in every respect to Case 1.

Muscle histology and histochemistry. No abnormality could be seen in the morphology of the fibres of any muscles or in the end-plate or in the pattern of fibre type histochemical differentiation (biceps and gastrocnemius muscles).

Neurone Counts

The spinal cords from the two control fetuses (anencephalic without rachischisis and normal) gave almost identical results. Although the total neurone count varied somewhat on successive recounts of the same section, the results for the normal and non-rachischitic anencephalic spinal cords could not be confused with those from the rachischitic anencephalic spinal cord, due to the great difference between their means. The mean number of neurones/whole spinal cord section were: normal = 241 (10 sections); non-rachischitic anencephalic = 212 (5 sections); rachischitic anencephalic = 53 (8 sections).

Since the larger neurones in the anterior

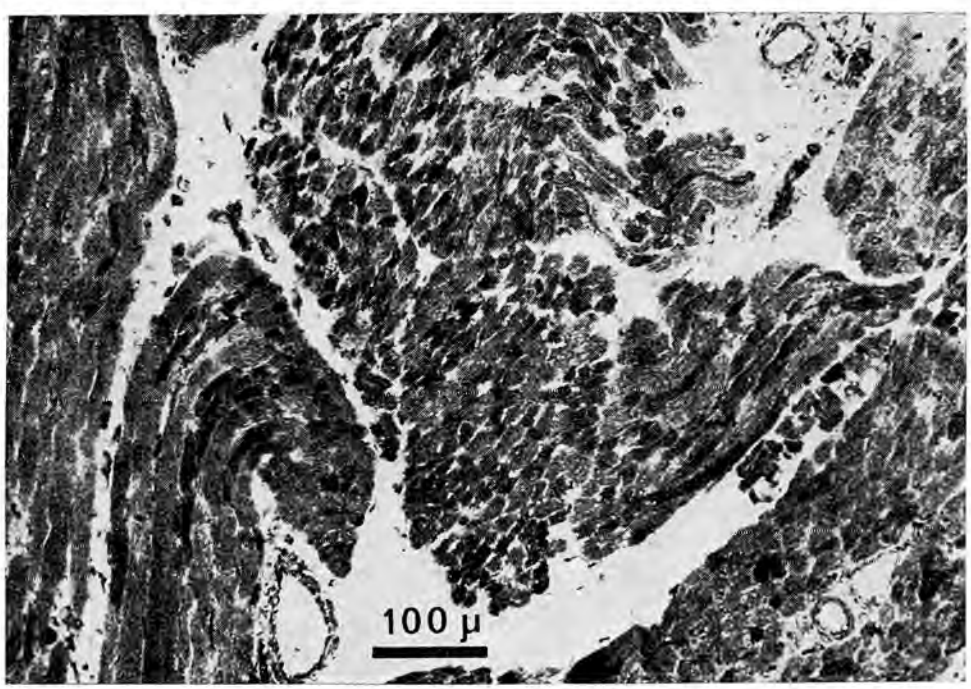


Fig. 3. Biceps brachii muscle, 30 week anencephalic fetus. NADH-diaphorase stain showing differentiation into fibre types I and II. × 150.

horns determine the development of the muscle motor units, these were counted separately in another series in which 'large' was defined as greater than 20μ in diameter. These results were less variable, but also gave a clear separation into normal and affected (mean number of neurones/whole spinal cord section: normal = 57 (10 sections); non-rachischitic anencephalic = 55 (9 sections); rachischitic anencephalic = 9·5 (8 sections)).

The majority of the large neurones in the rachischitic anencephalic cord sections were, in pathological terms, grossly degenerate. While it was not possible to determine whether or not these cells were functional, their advanced state of degeneration suggested very strongly that their metabolism was far from normal.

Discussion

Previous work on anencephaly has not included quantitative estimates of the numbers of large neurones in the spinal cord, and the present finding that their numbers are reduced is paralleled by quantitative studies by Lendon (1968, 1969). He showed that in children with myelomeningocele those cord segments which are most severely affected, at any level in the spinal cord, contain the lowest number of neurones. There is a further parallel between these two studies and our own, in that not only are the numbers of non-degenerate large neurones (as well as the total numbers) lowest in the most severely affected spinal cords, but also the numbers are closer to normal in other cords which are less affected.

There are three possible explanations for the present findings.

The first is that the motor neurones were never functionally normal and that, contrary to present concepts, the muscle had differentiated both morphologically and histochemically in the total absence of functional motor innervation. This explanation would appear to fit in with Gillaspie and Heuston's finding (1917) of an anencephalic fetus in which 'there was a complete and apparently normal development of the peripheral nervous system', despite the fact that there was neither brain nor spinal cord. But if this explanation is correct, skeletal muscle may not become dependent on its nerve supply to maintain its normal structural and biochemical integrity until much later in development than is generally supposed. It would also cast doubt on a great number of the studies which have been carried out on the histochemical development of human muscle. It has been shown that there is no well-differentiated pattern of histochemical staining in human fetal muscle until after 20 weeks gestation (Dubowitz 1968), yet the motor innervation is firmly established for some weeks before this. At 25 weeks gestation there are only about 5 per cent of type I fibres, but this increases to about 50 per cent at birth. Following the suggestion of Buller *et al.* (1960), Dubowitz postulates that this increase in the proportion of type I fibres may be brought about by a neurally mediated change of some type II fibres to type I (Dubowitz 1968). The results of animal cross-innervation experiments would also have to be questioned, since it is widely accepted that nerve exerts a powerful effect in reversing the histochemical pattern in a fast muscle cross-innervated by a nerve which normally innervates a slow muscle, and *vice versa* (Buller *et al.* 1960, Engel 1961).

The second possible explanation is that there may have been only a few functional neurones at any time. Since in anencephaly the sizes of both muscle and muscle fibre are normal, the number of muscle fibres is probably also close to normal. In the presence of only a few functional neurones, the size of the motor units in the corresponding muscles should therefore be greatly increased. Whether this is so could

possibly be determined by using the electrophysiological technique developed by McComas *et al.* (1970)

The third possibility is that motor innervation may be necessary for histochemical differentiation but that in these cases the initially normal motor neurones, having brought about normal morphological and histochemical differentiation in the muscle, degenerated at some time before delivery.

Padget (1970) has recently suggested that the spinal defect in anencephaly arises as a result of the re-opening of a closed neural tube, rather than initial failure to close. The defect in CNS development must thus have been determined at an early stage. Other earlier reports on anencephaly have also stressed that the disturbance of development in the brain and spinal cord occurs at an early stage. Warren (1951) estimates that the critical period for the development of anencephaly is limited to the second month of gestation, but other evidence suggests that the defect occurs in the first month (Giroud 1960). However, it is not yet clear when the large neurones of the rachischitic spinal cord begin to decrease in number, or even what is the primary cause of this decrease. A study of both rachischitic and non-rachischitic spinal cords at stages of gestation before 25 weeks would help in elucidating this problem.

If it is correct to assume that any changes in muscle are neurally mediated through a trophic substance, any account of the present findings must explain how histochemical differentiation of the muscle was able to develop in the presence of such a great neuronal deficit in the spinal cord.

Acknowledgements: We are indebted to Mr. J. Paul for producing the photographs, and to Mr. Robert Green for preparing the slide for Fig. 2. This work was supported by grants from the Medical Research Council and the Muscular Dystrophy Group of Great Britain.

SUMMARY

When adult human muscle is deprived of its motor nerve supply the fibres atrophy and die. In anencephaly, there is a disturbance in the development of the central nervous system at an early stage, yet the limb muscles are well-formed. This study reports an investigation on two anencephalic fetuses at 27 and 30 weeks gestation. Despite atrophy of the spinal cord and marked degeneration of neurones, muscle histology was normal, peripheral motor innervation was normal, and there was normal histochemical differentiation into two fibre types. It would appear, therefore, that there was normal muscle development despite the disturbance to the development of motor innervation.

These findings lend support to the theory that the spinal cord defect in anencephaly is the result of a re-opening and degeneration of a closed neural tube, rather than of an initial failure to close.

RÉSUMÉ

La différenciation musculaire dans l'anencéphalie

Quand un muscle humain d'adulte est privé de son nerf moteur, il y a une atrophie des fibres, qui disparaissent. Dans l'anencéphalie, il y a une perturbation très précoce du système nerveux central et néanmoins les muscles des membres sont bien formés. Cette étude rapporte des examens de deux fétus anencéphales à 27 et 32 semaines de gestation. En dépit de l'atrophie de la moelle épinière et d'une dégénération accentuée des neurones, l'histologie musculaire fut trouvée normale ainsi que l'innervation motrice périphérique et

la différenciation histo-chimique en deux types de fibres. Il semblerait donc y avoir eu un développement musculaire normal en dépit des perturbations dans le développement de l'innervation motrice.

Ces découvertes semblent en faveur d'une théorie qui ferait de l'altération de la moelle épinière dans l'anencéphalie une conséquence d'une réouverture et dégénération d'un tube neural fermé plutôt que d'un défaut initial de fermeture.

ZUSAMMENFASSUNG

Muskeldifferenzierung bei Anencephalie

Wenn beim erwachsenen Menschen ein Muskel von seiner motorischen Nervenversorgung abgeschnitten wird, atrophieren die Fasern und sterben ab. Bei Anencephalie liegt eine Störung in der Entwicklung des Zentralnervensystems in einem frühen Stadium vor, aber dennoch ist die Extremitätenmuskulatur gut ausgebildet. In dieser Studie wird über die Untersuchung von zwei anencephalen Feten der 27. und 32. Schwangerschaftswoche berichtet. Trotz Atrophie des Rückenmarkes und deutlicher Degeneration der Neurone, fand man eine normale Histologie der Muskeln, eine normale periphere motorische Innervation und eine normale histochemische Differenzierung in zwei Fasertypen. Daraus könnte man schließen, daß die Muskelentwicklung normal war trotz der Störung in der Ausbildung der motorischen Innervation.

Diese Befunde sprechen dafür, daß der Rückenmarksdefekt bei Anencephalie wahrscheinlicher durch eine Wiederöffunug und Degeneration eines bereits geschlossenen Neuralrohres als durch einen primären Schließungsdefekt bedingt ist.

RESUMEN

Diferenciación muscular en la anencefalia

Cuando un músculo humano adulto es desprovisto de su aporte nervioso motor, las fibras se atrofian y mueren. En la anencefalia, hay una alteración en el desarrollo del sistema nervioso central a una edad precoz, y a pesar de ello los músculos de las extremidades están bien formados. Este estudio aporta una investigación en dos fetos anencefálicos de 27 y 32 semanas de gestación. A pesar de la atrofia medular y de la marcada degeneración de las neuronas, la histología muscular era normal, así como la inervación motora periférica y la diferenciación histoquímica en dos tipos de fibras. Parece pues que había un desarrollo muscular normal a pesar de la alteración en el desarrollo de la invervación motora. Estos hallazgos llevan a apoyar la teoría de que el defecto espinal en la anencefalia es el resultado de una reapertura y degeneración de un tubo neural cerrado, más bien que un fallo inicial de cierre.

REFERENCES

Buller, A. J., Eccles, J. C., Eccles, R. M. (1960) 'Interactions between motoneurones and muscles in respect of the characteristic speeds of their responses.' *Journal of Physiology*, **150,** 417.

Dubowitz, V. (1968) *Developing and Diseased Muscle.* SIMP Research Monograph No. 2. London: Spastics International Medical Publications with Heinemann. p. 47.

Engel, W. K. (1961) 'Cytological localisation of glycogen in cultured skeletal muscle.' *Journal of Histochemistry and Cytochemistry*, **9,** 38.

Gallup, B., Strugalska-Cynowska, H., Dubowitz, V. (1972) 'Histochemical studies on normal and diseased human and chick muscle in tissue culture.' *Journal of the Neurological Sciences*, **17,** 109.

Gillaspie, C., Heuston, H. H. (1917) 'Detailed study of a monster with craniorachischisis and other anomalies.' *Anatomical Record*, **13,** 289.

Giroud, A. (1960) 'Causes and morphogenesis of anencephaly.' *In* Wolstenholme, G. E. W., O'Connor, M. (Eds.) *Ciba Foundation Symposium on Congenital Malformations.* London: J. & A. Churchill. p. 199.

Harrison, R. G. (1904) 'An experimental study of the relation of the nervous system to the developing musculature in the embryo of the frog.' *American Journal of Anatomy*, **3**, 197.
Lendon, R. G. (1968) 'Neurone population in the spinal cord of children with spina bifida and hydrocephalus.' *Developmental Medicine and Child Neurology*, Suppl. 15, 50.
— (1969) 'Neurone population in the lumbosacral cord of myelomeningocele children.' *Developmental Medicine and Child Neurology*, Suppl. 20, 82.
McComas, A. J., Sica, R. E. P., Currie, S. (1970) 'Muscular dystrophy: evidence for a neural factor.' *Nature*, **226**, 1263.
Namba, T., Nakamura, T., Grob, D. (1967) 'Staining for nerve fibre and cholinesterase activity in fresh frozen sections.' *American Journal of Clinical Pathology*, **47**, 74.
Padget, D. H. (1970) 'Neuroschisis and human embryonic maldevelopment: new evidence on anencephaly, spina bifida and diverse mammalian defects.' *Journal of Neuropathology and Experimental Neurology*, **29**, 192.
Padykula, H. A., Herman, E. (1955) 'Factors affecting the activity of adenosine triphosphatase and other phosphatases as measured by histochemical techniques.' *Journal of Histochemistry and Cytochemistry*, **3**, 161.
Papapetropoulos, T. A., Bradley, W. G. (1972) 'Spinal motor neurones in murine muscular dystrophy and spinal muscular atrophy.' *Journal of Neurology, Neurosurgery and Psychiatry*, **35**, 60.
Scarpelli, D. G., Hess, R., Pearse, A. G. E. (1958) 'The cytochemical localisation of oxidative enzymes. I. Diphosphopyridine nucleotide diaphorase and triphosphopyridine nucleotide diaphorase.' *Journal of Biophysical and Biochemical Cytology*, **4**, 747.
Streeter, G. L. (1921) 'Weight, sitting height, head size, foot length and menstrual age of the human embryo.' *Contributions to Embryology*, **XI**, 143.
Warren, H. S. (1951) 'Acrania induced by anencephaly.' *Anatomical Record*, **111**, 653.